ADDENDUM

The Forensic Toxicology of Antipsychotic Drugs: Method Development, Stability and Redistribution Studies

A Thesis submitted to the Faculty of Medicine in candidacy for the Degree of DOCTOR OF PHILOSOPHY

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August 2012

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"We are at the very beginning of time for the human race. It is not unreasonable that we grapple with problems. But there are tens of thousands of years in the future. Our responsibility is to do what we can, learn what we can, improve the solutions, and pass them on."

Richard Feynman

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GENERAL DECLARATION

Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17/ Doctor of Philosophy and Master of Philosophy (MPhil) regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes five original papers published in peer reviewed journals and no unpublished publications. The core theme of the thesis is the forensic toxicology of antipsychotic drugs with a focus on stability and redistribution studies. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Forensic Medicine under the supervision of Professor Olaf Drummer, Doctor Dimitri Gerostamoulos and Doctor Jochen Beyer.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 1-5, my contribution to the work involved the following:

Thesis chapter	**Publication title**	**Publication status**	**Nature and extent of candidate's contribution**
2.1	Review: The Analysis of Antipsychotic Drugs in Human Matrices using LC-MS(/MS)	Published	Located references, reviewed articles and wrote the paper.
2.2	Identification and Quantification of 30 Antipsychotics in Blood using LC-MS/MS	Published	Conducted literature review, performed method validation experiments. Wrote article.
3.1	Assessment of the Stability of 30 Antipsychotic Drugs in Stored Blood Specimens	Published	Conducted literature review, performed stability experiments and interpreted data. Wrote article.
4.1	Identification of 2-Hydroxymethyl-olanzapine as a Novel Degradation Product of Olanzapine	Published	Conducted literature review, performed degradation studies and interpreted data. Wrote article.
5.2	The Time-dependent Post-mortem Redistribution of Antipsychotic Drugs	ePub	Conducted case searches, performed case analyses and interpreted the data. Wrote article.

Signed .....[redacted].....

Date 27/07/2012

ACKNOWLEDGEMENTS

I would like to express my gratitude to several individuals that have significantly contributed to the completion of my Ph.D thesis from 2009 till 2012 at the Victorian Institute of Forensic Medicine and the Department of Forensic Medicine, Monash University.

It is with immense gratitude that I acknowledge the support of my supervisors Professor Olaf Drummer, Dr Dimitri Gerostamoulos and Dr Jochen Beyer. Thank you for giving me the opportunity to test and prove myself by doing a Ph.D in Forensic Toxicology. Your expertise and feedback during my candidature proved invaluable.

A big thanks to all the staff at VIFM who have assisted me in many ways during my candidature, especially to Natalia George, Melynda Hargreaves, Katherine Wong, Penny Tayler and Matthew Di Rago, thank you for helping me to remember to laugh!

Thanks to fellow Ph.D student Luke Rodda for his technical and moral support – turns out there is light at the end of the tunnel, so keep going!

A special thanks also to librarian Kerry Johannes for the sourcing of uncountable references as well as Cereil Pereira for her amazing organisational skills.

My thanks goes to my aunt Ruth Eisenbrand as well as my uncle Dieter Saar and close family friends, especially Ruth Biegel, Uli Müller, Ilse and Peter Rech, Christine and Wolfgang Thomas. Your ongoing support has been greatly appreciated.

A big thanks to all my friends 'back home', especially Tine Lang, Nicole Klatt, Lisa Müller, Barbara Schulz, Jan Mertens and Michael Ungers - the last three years would not have been possible without your enduring support and encouragement.

A special thanks to my former Ph.D buddy Dr. Jennifer Pilgrim: Apart from your endless proof reading and uncountable cups of coffee alongside 'yet another pep talk' preventing me from throwing in the towel when the frustrations just seemed to be overwhelming, our trip to the TIAFT conference in San Francisco 2011 will always be one of my greatest memories: I consider myself privileged to have a friend like you!

I would like to reserve my most special thanks to my parents Margret and Dr. Günther Saar: Thank you for giving me the tools to become the person I am and teaching me the importance of standing up for what you believe in. The values you have taught me form the basis of everything I do, and I will be forever grateful.

To Mark: Thanks for being my positivity when I was most frustrated. Thanks for being my patience when I had none left. Thanks for being my focus, when everything was a blur. Thanks for being my strength when I felt the weakest - thanks for making my life complete.

This thesis is dedicated to my grandfather, Dr. Erwin Saar (14.10.1920 - 10.08.2001), who valued education above all.

ABBREVIATIONS

5-HT	serotonin
9OH RIS	risperidone
AP	antipsychotic drug
APCI	atmospheric-pressure chemical ionisation
BSTFA	N,O-Bis(trimethylsilyl)trifluoracetamide
CAN	acetonitrile
DA	dopamine
ESI	electrospray ionisation
GC	gas chromatography
I.D	inner diameter
LC	liquid chromatography
LLE	liquid-liquid extraction
OLZ	olanzapine
p	significance level
PDD	pervasive developmental disorders
PFAA/PFA	pentafluoropropionic anhydride / pentafluoro-1 - propanol
PMR	post-mortem redistribution
RIS	9OH-risperidone
SPE	solid-phase extraction
TFAA	trifluoracetic anhydride
TIC	total ion chromatogram
TMCS	trimethylchlorsilane
VIFM	Victorian Institute of Forensic Medicine
α	adrenergic

LIST OF ORIGINAL PUBLICATIONS RELATING TO DISSERTATION

1. Saar, E., Gerostamoulos, D., Beyer, J & Drummer, O.H. (2012) The analysis of antipsychotic drugs in human matrices using LC-MS(/MS). Drug Testing and Analysis **6** (2012) 376-394.

2. Saar, E., Gerostamoulos, D., Drummer, O.H. & Beyer, J. (2010) Identification and quantification of 30 antipsychotics in blood using LC-MS/MS. J Mass Spectrom, 2010. **45**(8): p. 915-25.

3. Saar, E., Gerostamoulos, D., Drummer, O.H. & Beyer, J. (2012) Assessment of the stability of 30 antipsychotic drugs in stored blood specimens. Forensic Sci Int, 2012. **215**(1-3): p. 152-8.

4. Saar, E., Gerostamoulos, D., Drummer, O.H. & Beyer, J. (2012) Identification of 2-hydroxymethyl-olanzapine as a novel degradation product of olanzapine. Forensic Sci Int, 2012. **220** (1-3): p. 74-79.

5. Saar, E., Beyer, J, Gerostamoulos, D., & Drummer, O.H (2012) The time-dependent post-mortem redistribution of antipsychotic drugs, Forensic Sci Int, 2012. (ePub)

ABSTRACT

Antipsychotic drugs are commonly prescribed for the treatment of a number of metal illnesses. However, people suffering from mental illness are at a higher risk of suicide than healthy individuals and these drugs are associated with the onset and exacerbation of particular comorbidities. Accordingly, antipsychotic drugs are commonly detected in cases reported to the coroner. The detection and quantification of antipsychotic drugs in post-mortem specimens and the ensuing interpretation of these results therefore play an important role determining the cause of death. A study is presented which describes the development of a comprehensive detection method for the analysis of antipsychotic drugs in whole blood, as well as research into several factors that can potentially alter blood concentrations after death, with a focus on post-mortem redistribution and stability of blood samples containing antipsychotic drugs.

The research indicated that several antipsychotic drugs are prone to significant losses if stored at temperatures above 0 ºC, potentially compromising post-mortem drug results. Furthermore, the atypical antipsychotic drug olanzapine showed significant losses even if stored at temperatures below 0 ºC. Further analysis of the degradation of this commonly prescribed drug revealed 2-hydroxymethyl olanzapine as a new degradation product of olanzapine in aqueous solutions. The investigation of the post-mortem redistribution of antipsychotic drugs in peripheral blood specimens revealed that the majority of targeted drugs underwent a time-dependent bi-phasic process with an initial increase followed by a decrease in concentration. However, different patterns of change were also present. The results highlighted the need for immediate sample collection post-admission of a deceased person to the mortuary, in addition to appropriate sample storage; pre- and post-analysis.

When interpreting post-mortem blood results of antipsychotic drugs, the factors described in this thesis must be considered as sources of potential variation.

Chapter 1

Introduction

Chapter 1.1 and 1.3-1.5 have been extracted from the book “LC-MS in Drug Bioanalysis” (Springer Book, *2012*, ISBN 1461438276, *in press*), chapter “The Analysis of Antipsychotic Drugs in Human Biosamples by LC-MS” (Saar, E., Gerostamoulos, D., Drummer, O.H., Beyer, J.)

1.1 HISTORY OF THE TREATMENT OF PSYCHOTIC ILLNESSES

Prior to the 1950s, electroconvulsive therapy and psychosurgery were considered suitable treatments for patients suffering from mental illnesses. Due to a lack of knowledge surrounding the pathophysiology of psychotic disorders such as schizophrenia, the only pharmacological "treatment" at this time consisted of sedation with barbiturates and drug-induced epileptic seizures [1]. The first notable development in drug-therapy for people suffering from bipolar disorder – a disorder characterised by alternating manic and depressive episodes, was the discovery of lithium as a suitable treating agent in the late-1940s [2]. While the mechanism of action of lithium still remains unclear, its effectiveness in the treatment of bipolar disorders has been accepted worldwide and it is still considered the leading prophylactic treatment for this condition, even 60 years after its discovery [1].

In the 1950s, a more evidence-based approach to antipsychotic drug (AP) therapy was undertaken when structural variations of antihistamines were produced by a French scientist (Paul Charpentier), in order to make use of the "unwanted" sedative side effect produced by these drugs. Initially used to lower body temperature in patients undergoing cardiac surgery, chlorpromazine (Figure 1I) was the first drug with antipsychotic properties successfully used in clinical trials [3].

1.1.1 TYPICAL ANTIPSYCHOTIC DRUGS

Since its official release in 1952, chlorpromazine has been considered the prototype of so-called "typical" APs. Chlorpromazine has a characteristic phenothiazine structure, which formed the basis of other APs synthesised in subsequent years, such as fluphenazine, perphenazine, promazine, promethazine, thioridazine, trifluoperazine, and triflupromazine (Figure 1II - 1VIII). These APs formed the largest subgroup of all drugs commonly referred to as "Typical APs". They are also known as "First Generation APs". Other subgroups within the typical APs are also characterised by their chemical structures, such as the butyrophenones (e.g. droperidol, haloperidol, trifluperidol, melperone, and pipamperone (Figure 1IX - 1XIII)), the thioxanthenes (e.g. flupentixol, zuclopenthixol, chlorprothixene, and thiothixene (Figure 1XIV - 1XVII)), diphenylbutylpiperidines (e.g. pimozide, fluspirilene, penfluridol (Figure 1XVIII - 1XX)); indoles (e.g. molindone (Figure 1XXI), and others (e.g. loxapine (Figure 1XXII)).

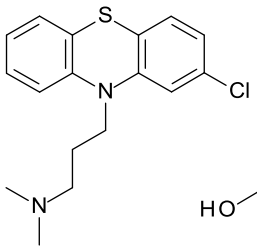
Despite having varying chemical structures, all typical APs have a significant affinity to dopamine (DA) receptors, mainly the D_2-type, while also showing minor antagonism at muscarinic, 5-HT, adrenergic (α), and histaminergic (H1) receptors. By blocking receptors in the prefrontal cortex and the limbic area of the brain, both areas which are linked with mood and emotional behaviour, an improvement in positive symptoms is achieved. However, the same action of APs in other cerebral areas such as the striatum, which is associated with motor control, has shown to lead to serious side effects.

Patients treated with typical APs are likely to suffer parkinsonian symptoms such as mobility difficulties (tremor, bradykinesia, postural instability). The blockage of DA receptors leads to a decrease in DA ultimately causing symptoms consistent with patients suffering from Parkinson's disease.

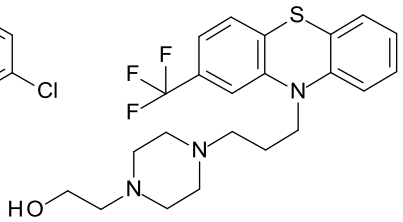
The use of typical APs is associated with a number of side-effects which can outweigh their positive outcomes at times. Neuroleptic Malignant Syndrome (NMS), although difficult to distinguish from other disorders [4], is characterised by elevated temperature, changed mental status and severe muscle rigidity. While recent studies suggest that the prevalence of NMS has decreased from around 2.4 % [5] to 0.01 % - 0.02 % [6], this is most likely due to more conservative prescription patterns of typical APs and a greater awareness of the illness [7]. The mortality rate associated with NMS was reported to be as high as 20 % at one stage [5, 8]. However, in the last two decades, mortality rates have fallen below 10 % due to early recognition and improved management [6].

Additional problems associated with typical APs include cardiotoxicity [9], seizures, and an increased risk of sudden cardiac death [10, 11].

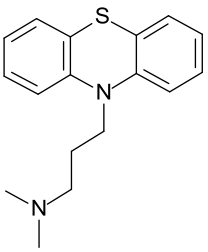
I) Chlorpromazine (318.1)
$C_{17}H_{19}ClN_2S$



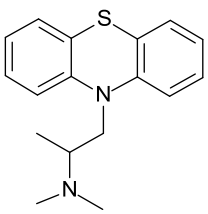
II) Fluphenazine (437.2)
$C_{22}H_{26}F_3N_3OS$



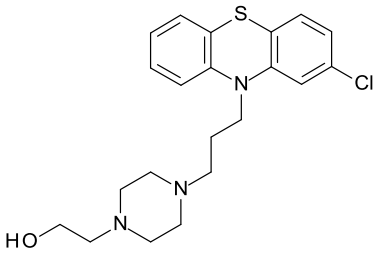
III) Promazine (284.1)
$C_{17}H_{20}N_2S$



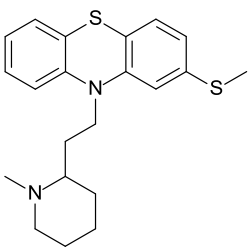
IV) Promethazine (284.1)
$C_{17}H_{20}N_2S$



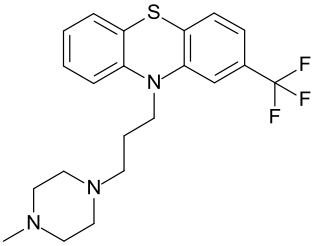
V) Perphenazine (403.1)
$C_{21}H_{26}ClN_3OS$



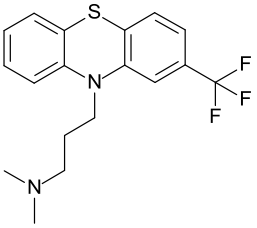
VI) Thioridazine (370.2)
$C_{21}H_{26}N_2S_2$



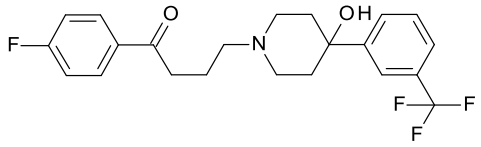
VII) Trifluoperazine (407.2)
$C_{21}H_{24}F_3N_3S$



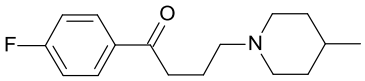
VIII) Triflupromazine (352.1)
$C_{18}H_{19}F_3N_2S$



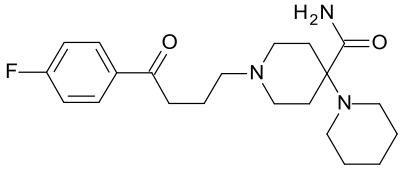
XI) Trifluperidol (409.2)
$C_{22}H_{23}F_4NO_2$



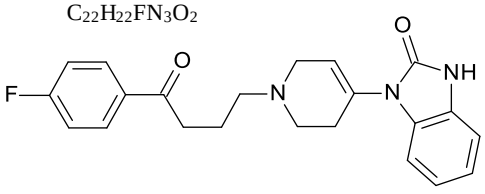
XII) Melperone (263.2)
$C_{16}H_{22}FNO$



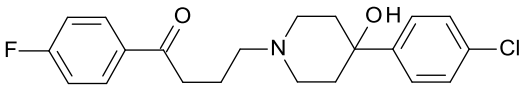
XIII) Pipamperone (375.2)
$C_{21}H_{30}FN_3O_2$



IX) Droperidol (379.2)
$C_{22}H_{22}FN_3O_2$



X) Haloperidol (375.1)
$C_{21}H_{23}ClFNO_2$



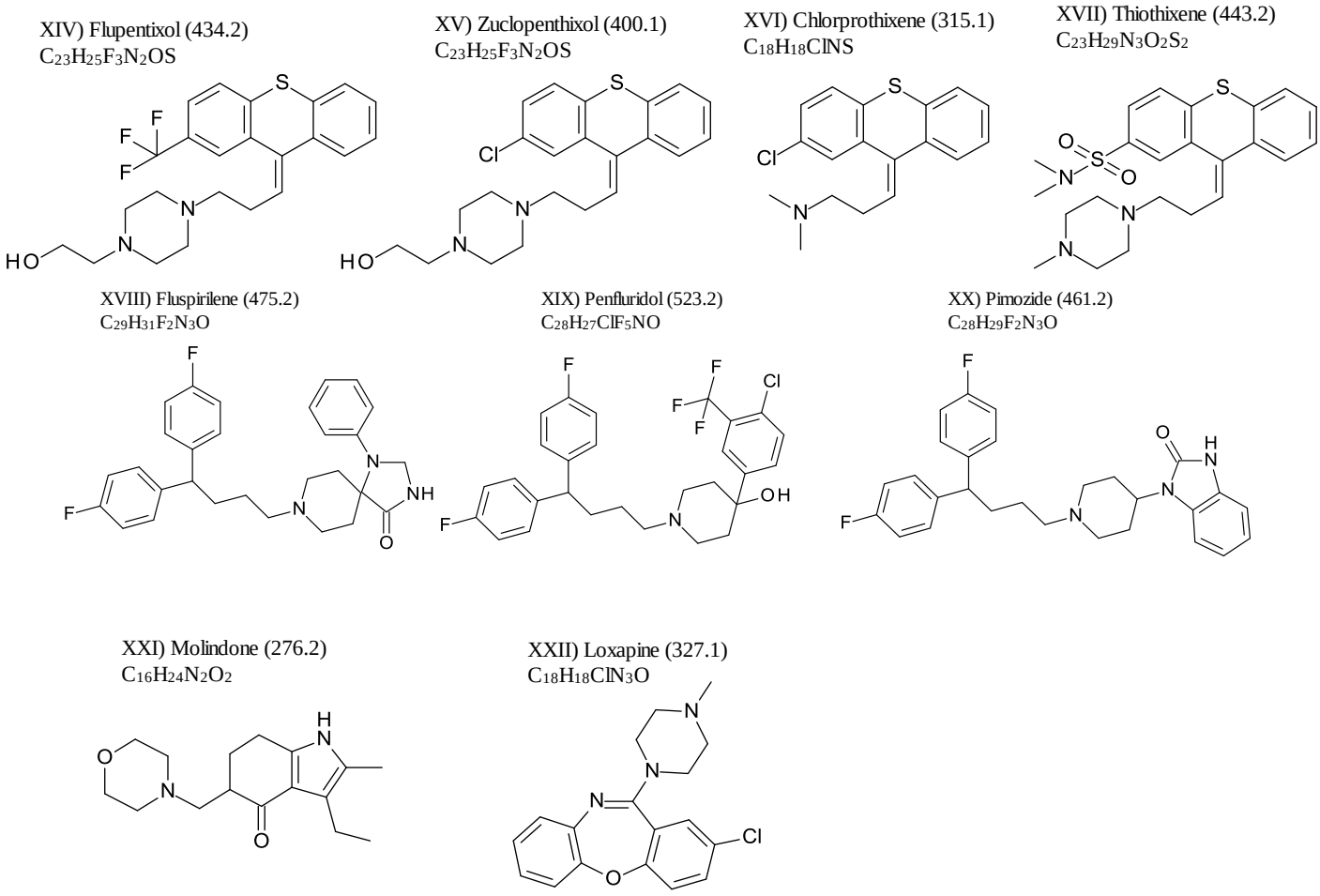


Figure 1: Chemical structures, exact mass in Dalton, and molecular formula of selected typical APs

1.1.2 ATYPICAL ANTIPSYCHOTIC DRUGS

Due to the broad range of side effects associated with typical APs, combined with their inability to improve all symptoms of psychotic disorders, a new generation of APs was introduced in the 1970s. These APs are generally referred to as "second generation" or atypical APs. This group includes indoles (e.g. ziprasidone and sertindole (Figure 2I - 2II)) benzamides (e.g. amisulpride, sulpiride (Figure 2III - 2IV)) diazepines/oxazepines/thiazepines (e.g. clozapine, olanzapine (OLZ), quetiapine (Figure 2V - 2VII)) and others (e.g. aripiprazole, risperidone (RIS), buspirone, paliperidone, zotepine (Figure 2VIII - 2XII)).

Clozapine, a tricyclic dibenzodiazepine derivative, was the first atypical AP to be approved by the FDA in 1989. It was originally thought that increased affinity to the $5HT_{2A}$ receptor, in combination with a lower or no affinity to the D_2 receptors, might define a compound as an "atypical" AP [12, 13]. However, studies have shown that selective 5HT antagonists do not have antipsychotic properties [14]. Some antagonism at the D_2 receptor appears to be mandatory for any antipsychotic effect. More recent studies have presented an alternative to this theory.

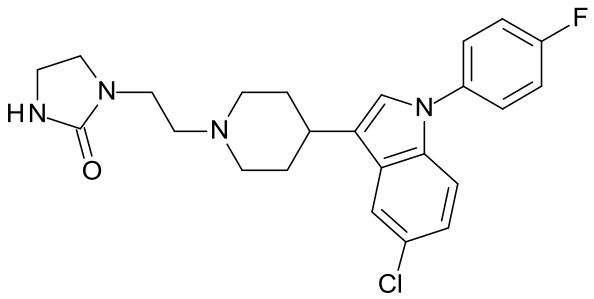
While typical APs bind with high affinity to the D_2 receptors, these newer atypical APs possess only moderate affinity to D_2 receptors and high dissociation constants. They initially occupy D_2 receptors and then rapidly dissociate to allow normal DA neurotransmission, which ultimately decreases extrapyramidal side effects [15, 16].

Although it was initially thought that atypical APs may be linked with fewer side effects than their typical counterparts, this is unlikely to be the case; they have shown to have a similar

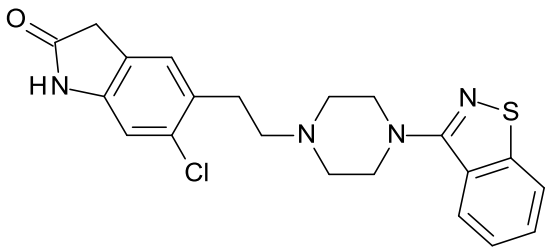
risk of sudden cardiac death [17] as the first generation APs and appear to cause even more problems in regards to metabolic syndrome (e.g. obesity, type 2 diabetes mellitus) [18].

As different APs possess different advantages and disadvantages (not just depending on the group they belong to but on their individual properties) it is usual practice to select antipsychotic medication depending on a patient's individual needs. Therefore, combination therapy is a common occurrence amongst patients treated with APs [19] highlighting the need for detection methods that consist of a large variety of these drugs.

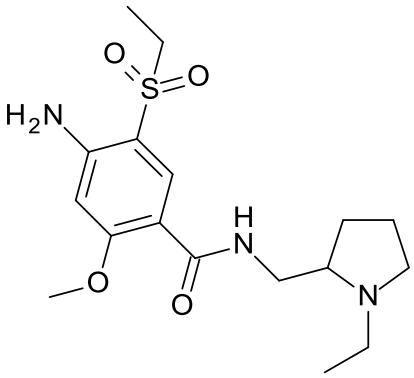
I) Sertindole (440.2)
$C_{24}H_{26}ClFN_4O$



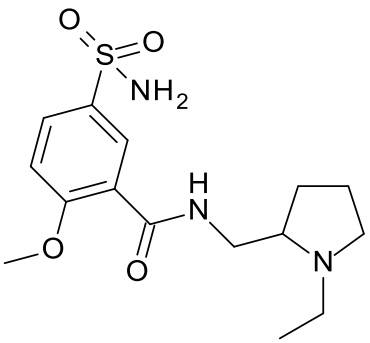
II) Ziprasidone (412.1)
$C_{21}H_{21}ClN_4OS$



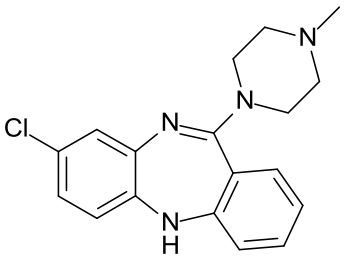
III) Amisulpride (369.2)
$C_{17}H_{27}N_3O_4S$



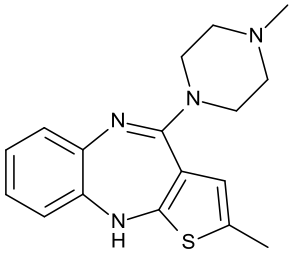
IV) Sulpiride (341.1)
$C_{15}H_{23}N_3O_4S$



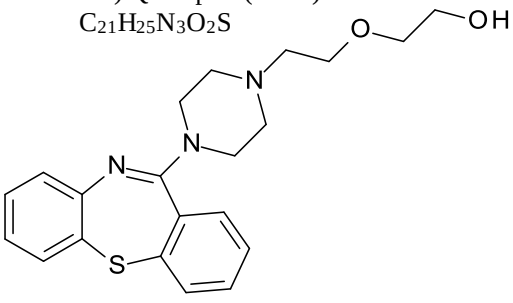
V) Clozapine (326.1)
$C_{18}H_{19}ClN_4$



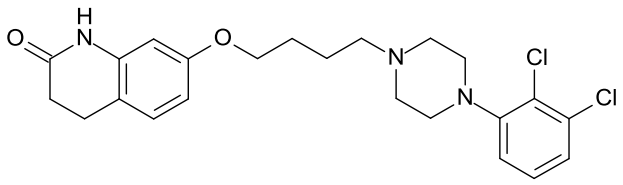
VI) Olanzapine (312.1)
$C_{17}H_{20}N_4S$



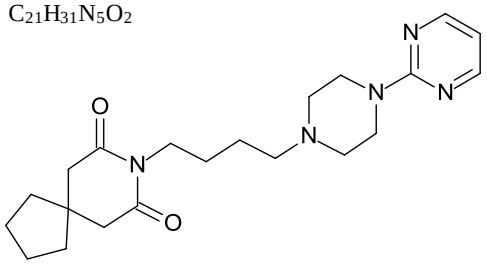
VII) Quetiapine (383.2)
$C_{21}H_{25}N_3O_2S$



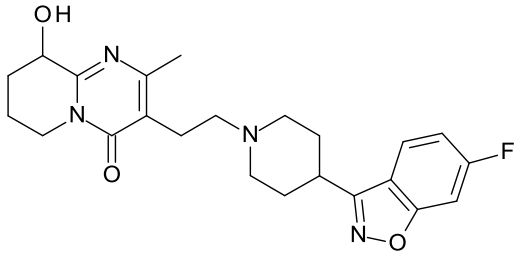
VIII) Aripiprazole (447.1)
$C_{23}H_{27}Cl_2N_3O_2$



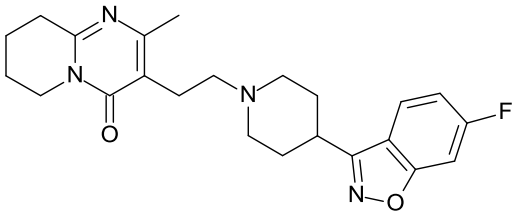
IX) Buspirone (385.2)
$C_{21}H_{31}N_5O_2$



X) Paliperidone (426.2)
$C_{23}H_{27}FN_4O_3$



XI) Risperidone (410.2)
$C_{23}H_{27}FN_4O_2$



XI) Zotepine (331.1)
$C_{18}H_{18}ClNOS$

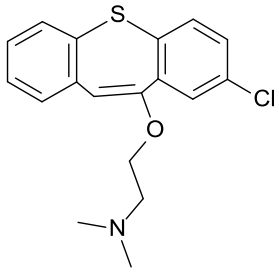


Figure 2: Chemical structures, exact mass in Dalton, and molecular formula of selected atypical APs

1.2 PRESCRIBING TRENDS OF ANTIPSYCHOTIC DRUGS

In order to determine whether AP prescriptions were increasing for youths, Aparasu *et al.* examined prescription data from 1997-2002 [20]. The analysis involved 11 first-generation agents (chlorpromazine, fluphenazine, haloperidol, loxapine, mesoridazine, molindone, perphenazine, promazine, thioridazine, thiothixene, and trifluoperazine) and six second-generation agents (aripiprazole, clozapine, OLZ, quetiapine, RIS, and ziprasidone) prescribed for patients less than 20 years of age. There was a noticeable increase in prescriptions of APs among youths throughout the six-year period, mainly due to the increased use of second-generation APs.

During this period, 83 % of the practitioner visits that involved APs resulted in the prescribing of second-generation agents. RIS and OLZ were the most commonly prescribed APs, accounting for 29 % and 27 % of all prescriptions, respectively [21].

The high prescription rate of second-generation APs may be due to their efficacy in treating non-psychotic disorders [22].

Although research on the paediatric use of second-generation APs is limited, they are frequently prescribed for the treatment of Tourette's Disorder, pervasive developmental disorders (PDD), bipolar disorder and mental retardation. Furthermore, patterns of behaviour such as aggression, self-injury, and impulsivity are commonly treated with second-generation APs [22].

There is no sufficient data on the efficacy and safety of the paediatric use of APs as very few published reviews have targeted the clinical use of atypical APs in children [23-26].

The number of prescriptions of APs in adults has also dramatically increased in recent years, both in Australia as well as the USA [27]. Additionally, atypical APs have largely replaced

typical APs. A study conducted in Australia in 2002 by Mond *et al*. aimed to determine trends in the use of oral typical, depot and atypical antipsychotic medications over a period of approximately six years (July 1995 - December 2001) [27]. Prescription data obtained from the Health Insurance Commission of Australia showed a 12 - fold increase in the use of atypical APs over this timeframe, whereas the use of oral conventional and depot injection medications decreased. At the end of the study, the number of patients receiving one standard daily dose of any type of atypical medication had increased nearly 15 - fold. The total prescriptions for APs –mainly due to the large increase in atypical APs- rose by a factor 8.4 over the period.

A different study conducted in the USA in 2002 using National probability sample survey data from 1998 – 2002 found that the number of antipsychotic-related visits roughly doubled during that period, with the number of visits for second-generation APs increasing almost threefold. The increase in the number of visits involving APs over the six-year period was substantial (120 %) in visits with non-psychiatrist physicians. However this was not the case in visits involving psychiatrists. This development supports the theory already postulated by Cheng-Shannon et al. in 2004 that APs are quite commonly prescribed for non-psychotic disorders.

A recent study by Nishtala *et al.* in 2009 was conducted in order to analyse the determination of antipsychotic medication prescribed in Australia, among older people living in aged care facilities. In Australia, the determination of antipsychotic medication is particularly interesting considering the Therapeutics Goods Administration (TGA) approved the use of RIS for "behavioural disturbances characterised by psychotic symptoms and aggression in patients with dementia where non-pharmacological methods have been unsuccessful" [28].

This development is distinct from the UK and USA, where there are no atypical APs indicated for this condition.

Data collected from 500 residents in 62 aged care homes in Sydney was analysed, demonstrating that over more than 22% of all residents received some antipsychotic medication. Of these, over 80 % received only atypical medications, with RIS the most commonly prescribed AP in more than 58 % of all cases, followed by OLZ in over 11 %. The odds for a patient receiving antipsychotic medication were not only significantly higher for psychotic disorders such as paranoia and psychosis, but also for agitation, challenging behaviour, and dementia [28].

1.3 OFF-LABEL USE

The practice of prescribing pharmaceuticals for an unapproved indication is called "off-label use". While this article mainly highlights the use of APs for psychotic disorders such as schizophrenia, it must be noted that there is a significant off-label use of these drugs worldwide [29]. This is largely caused by heterogeneous indications of different APs in different countries. Fleischhacker *et al.* [30] presented an overview of the indications of relevant atypical APs and haloperidol in ten European countries. Variations in the indication and labelling of APs should be considered in analytical screening procedures applied in clinical and forensic toxicology.

1.4 ADMINISTRATION OF ANTIPSYCHOTIC DRUGS

The two main administration routes for APs are per-oral (p.o.) and intramuscular (i.m.). P.o. administration results in a significant first-pass effect, therefore there is considerable loss of drug after the first liver passage. I.m. preparations are usually synthesised by esterification of the drugs with fatty acids [31] and can be divided into short-acting i.m. antipsychotic medications (SAIM) and long acting i.m. antipsychotic medications (LAIM). SAIM are used for the treatment of agitation and aggressive behaviour of patients experiencing an acute psychotic phase [32, 33]. LAIM are referred to as "depot" injections. I.m. formulations have several advantages such as a higher bioavailability due to the lack of first-pass metabolism and are preferred in the treatment of patients where compliance issues are likely.

1.5 DISPOSITION OF ANTIPSYCHOTIC DRUGS

The volume of distribution (VD) quantifies the distribution of a drug between plasma and the rest of the body after oral or parental application. Drugs which only distribute in blood show a VD of ~ 0.05 L/kg whereas drugs distributed in extracellular water have a VD of ~ 0.2 L/kg; the total body water volume is 0.55 L/Kg. APs are lipid soluble weak bases which are easily taken up into body fat and organs, therefore generally show a large VD. Plasma protein binding, also referred to as the "fraction bound" (Fb), is the percentage of a drug bound to plasma proteins after its admission. Only the unbound fraction of a drug will cause a pharmacological effect and can be detected using analytical methods. Despite their high VD, most common APs show a high Fb; both parameters significantly contribute to low or undetectable blood concentrations.

Therapeutic blood concentrations are usually based on studies that relate to drug concentrations measured at steady state of patients treated with recommended daily doses of a drug. However, the same dose of a drug can result in considerably different plasma concentrations in different individuals, depending on factors such as diet, lifestyle, co-medication and genetic makeup resulting in altered absorption, distribution, and elimination of drugs.

The terminal elimination half-life ($t_{1/2}$) of a drug is not dose-dependent and can range from a few days up to several weeks for APs ($t_{1/2}$ of up to 16 weeks with a very large variation has been described for Flupentixol-decanoate [34]). Importantly, the time required to reach steady state depends only on $t_{1/2}$ of a compound. Steady state is usually reached after five $t_{1/2}$. It is usually not advisable to measure plasma concentrations before this equilibrium is reached except for drugs with very long $t_{1/2}$such as depot-formulations. This is done in order to ensure that patients with impaired metabolism or excretion are not at risk of reaching above-therapeutic drug concentrations at their initial dosage regimen.

1.6 TOXICITY OF ANTIPSYCHOTIC DRUGS

Multiple studies have been conducted in the USA and Europe to determine the cardiotoxic potential of APs and their possible contribution to sudden cardiac death [10, 11, 17, 35-38]. A recent study by Ray *et al.* in 2009 found current users of typical and of atypical APs had significantly higher rates of sudden cardiac death than did nonusers of APs. The incidence-rate ratio for users of atypical APs was even higher than for users of typical APs [17]. This

study demonstrated that the risk for users increased significantly with increasing dose, regardless of the class of AP consumed.

Psychiatric illnesses such as schizophrenia carry a significantly increased risk of self-poisoning and suicide. Low-potency APs such as thioridazine and chlorpromazine have greater cardiotoxicity than more potent APs when taken in overdose [39]. In fact, the main manufacturer of thioridazine (Novartis®) announced a worldwide discontinuation of the drug thioridazine in 2005 due to concerns that it caused increased risk of cardiac arrhythmias and sudden death. In 2007, the main distributor of thioridazine tablets in Australia (Alphapharm®) followed the actions of international manufacturers in announcing the discontinuation of thioridazine, since reliable supply of the active thioridazine could no longer be obtained.

Established risks resulting from treatment with APs include:

1) A risk of CNS depression due to the combination of APs with several central nervous system (CNS)-active substances (for example, antidepressants or benzodiazepines). CNS depression is defined by three major symptoms: decreased respiration, decreased heart rate, and loss of consciousness; all of which can be fatal.

2) The risk of unintentionally increasing the blood concentrations of APs with the administration of other CNS-active drugs, such as antidepressants. This means a drug concentration that is normally considered “therapeutic” can actually result in a “toxic” concentration. This phenomenon occurs because APs and a number of CNS-active drugs are metabolised by the CYP2D6 enzyme [40]. Paroxetine and fluoxetine (both antidepressants of the selective serotonin reuptake inhibitor (SSRI) class) have the potential to increase the plasma concentrations of APs that are CYP2D6 substrates, such as perphenazine,

haloperidol, thioridazine and RIS in patients who are CYP2D6 extensive ("normal") metabolisers [41]. Several studies have shown this for the typical APs perphenazine and haloperidol with paroxetine respectively [42]. Fluvoxamine (another SSRI) is a potent inhibitor of CYP1A2. It is therefore capable of inhibiting the metabolism of clozapine, resulting in higher plasma concentrations [43].

3) An increased risk of neuroleptic malignant syndrome (NMS). NMS is a life-threatening condition, characterised by multiple symptoms including severe rigidity, tremor, fever, altered mental status, autonomic dysfunction, and elevated serum creatinine phosphokinase and white blood cell count [44]. In 1989, the incidence of NMS was estimated to be as high as 3 % in psychiatric patients receiving APs [45]. However, a more recent report indicated that this number dropped dramatically to 0.01 % - 0.02 % by 2004 [7], probably due to the increased awareness of the disorder and therefore more conservative prescription patterns of APs [46].

Blood is generally the specimen of choice in order to determine if a drug concentration is consistent with therapeutic levels [47]. In the field of post-mortem toxicology, however, interpretation of the blood drug concentration obtained in the laboratory is complicated by a range of post-mortem effects, such as instability of a drug and post-mortem redistribution, which can alter drug blood concentrations after death of an individual. The potential impact of these factors on drug concentration is discussed further in **Chapter 1.7**.

1.7 POST-MORTEM TOXICOLOGY

APs are associated with a number of health risks, in addition to a significantly increased risk of self-poisoning and suicide in people suffering from psychiatric illnesses compared with healthy individuals. It is therefore not surprising that APs are commonly present in post-mortem cases.

The analysis of forensic samples involves additional challenges when compared with clinical or ante-mortem specimens. Firstly, separation of erythrocytes from post-mortem blood is generally not possible and depends on the degree of decomposition that may have already taken place prior to collection [48]. Therefore, methods for the analysis of these samples must be validated using whole blood. Since whole blood is a more complex matrix compared with plasma or serum, the sample preparation step is crucial. A thorough clean-up is necessary, prior to injection of a sample into an analytical system. In this study, extraction efficiencies and matrix effects of common liquid-liquid and solid phase extraction procedures were compared in both ante-mortem and post-mortem specimens for the analysis of 19 APs in whole blood, in order to find the most suitable extraction method for APs [49]. This study revealed that solid-phase extraction (SPE) was less suitable than liquid-liquid extraction (LLE) for whole blood samples containing APs. This was mainly due to lower extraction efficiencies when employing SPE compared with LLE, in addition to the higher cost of using SPE cartridges. Furthermore, due to the complexity of the matrix, issues with regards to blockages of the SPE instrument were frequently observed. The outcomes of this study highlighted the variance in extraction efficiencies and matrix effects that can occur when blood of different ‘qualities’ (ante-mortem compared with decomposed blood) is analysed – a complication that is of lesser concern when working with clinical samples.

Another factor that can complicate the analysis of APs in post-mortem specimens is post-mortem redistribution (PMR). In one of the earliest reports describing this phenomenon in 1977, Vorpahl *et al.* hypothesised that digoxin was released from heart tissue post-mortem, subsequently increasing the heart blood concentration [50]. It has since been established that the two main factors influencing the PMR of a drug are sampling site and time of sampling relative to the time of death. Additionally, PMR is mainly associated with drugs which show a large VD (>3 L/kg) and a high degree of lipophilicity [51-55]. Since APs possess these characteristics, they are likely to be susceptible to PMR. However, the extent to which they undergo PMR has not been studied in detail. Currently published data on the PMR of APs has been obtained from animal studies [56-58] or single case studies [59-66], with the main focus being place differences in drug concentrations caused by site differences. Peripheral blood is generally regarded as more suitable for post-mortem drug testing because of its distance from central organs and the gastrointestinal tract [51]. There has been no study published to date investigating the influence of time on the drug blood concentration of APs in peripheral specimens. In post-mortem toxicology, it is common that specimens for toxicological analysis are not taken immediately after admission of a deceased person. This procedure unfortunately enables potential drug blood concentration changes prior to sampling for analysis, however, the extent of this change has not been explored to date. PMR and its influence in interpretation in toxicological analysis is examined further in **Chapter 5.**

The stability of drugs during storage and potential degradation mechanisms play an important role in post-mortem toxicology. Since the analyst has no influence on potential drug concentration changes that may occur between death and sampling, it is important to be aware of preferred storage conditions that minimise the risk of drug concentration changes

between taking of a sample and toxicological analysis. Determining the long-term stability of drugs in stored specimens is considered a crucial part of method validation, however, only storage at -20 ºC is mandatory according to international guidelines [67]. Stability studies published to date have generally targeted only few drugs and are mainly carried out using a plasma matrix with a recommended storage temperature of -20 ºC [68-71]. Since whole blood is more complex than plasma, it is unlikely that the results from studies using plasma are valid for storage of whole blood samples containing APs. Moreover, in toxicology laboratories, storage at different temperatures such as 4 ºC (standard fridge) or -60 ºC is not unusual, depending on the laboratories' routine procedures. Although not recommended, it is also possible that specimens are subjected to higher storage temperatures, such as room temperature (due to technical errors), delays in processing of samples, or faulty equipment. It is important to be aware of potentially compromising blood samples with inappropriate storage conditions. This is further explored in **Chapter 3**.

1.8 AIMS OF THE THESIS

The aims of this thesis were to investigate the involvement of stability and PMR in the forensic toxicological analysis of APs and in addition to develop a validated method to detect and quantify the most commonly prescribed typical and atypical APs in whole blood.

Specifically, the aims were to;

- Develop a LC-MS/MS detection and quantification method for the most commonly prescribed APs in Australia and worldwide using whole blood in addition to

validation of this method in accordance with international guidelines [72, 73] **(Chapter 2.2)**.

- Investigate the stability and the pattern of break-down of commonly prescribed APs in spiked whole blood samples when stored at 20 ºC, 4 ºC, -20 ºC and -60 ºC for 20 weeks (**Chapter 3)**.
- Investigate the influence of ascorbic acid as an antioxidant on the degradation of OLZ in stored blood samples in addition to employment of LC-MS, GC-MS and TOF technology to determine degradation products of OLZ formed in a) degrading whole blood samples and b) post-extraction on the autosampler under the conditions of analysis (**Chapter 4)**.
- Determine the extent of PMR of selected APs in the femoral vein over a time frame of up to nine days post-mortem, by analysing paired blood samples taken at different time points from deceased individuals, in a cohort of 273 cases (**Chapter 5)**.

An in depth review of the literature in **Chapter 2.1** highlights the lack of appropriate detection methods for APs in post-mortem cases and therefore explains the limited data currently published on post-mortem changes that APs are likely to undergo.

Chapter 2

LC-MS/MS method development

A critical literature review in **Chapter 2.1** highlights a gap in the field of the forensic toxicology of antipsychotic drugs, mainly caused by a lack of appropriately validated detection methods using whole blood. Previous research ("Comparison of extraction efficiencies and LC-MS/MS matrix effects using LLE and SPE methods for 19 antipsychotics in human blood" Saar *et al*., 2009, **Appendix 2.1**) has formed the basis for the development of an analytical LC-MS/MS method enabling the detection and quantification of 30 commonly prescribed antipsychotic drugs, presented in **Chapter 2.2**.

Chapter 2.1

Review: The Analysis of Antipsychotic Drugs in Human Matrices using LC-MS(/MS)

Saar, E., Gerostamoulos, D., Beyer, J., Drummer, O. H.
Drug Testing and Analysis **6** (2012) 376-394.

Monash University

Declaration for Thesis Chapter 2.1

Declaration by candidate

In the case of Chapter 2.1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Located references, reviewed articles and wrote the paper	85 %

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D	Advised candidate regarding direction and content, reviewed and edited article	
Beyer, J	Advised candidate regarding direction and content, reviewed and edited article	
Drummer, O. H	Advised candidate regarding direction and content, reviewed and edited article	

Candidate's Signature	[redacted]	**Date** 27/07/2012

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Victorian Institute of Forensic Medicine**

Signature 1	[redacted]	**Date** 27/7/2012
Signature 2		27/8/2012
Signature 3		[illegible]

ABSTRACT

Antipsychotic drugs (APs) are prescribed for a wide range of psychotic illnesses. With more than 35 APs currently available worldwide, this drug class has rapidly gained importance in both a clinical and forensic setting. On account of their chemical properties, many APs are present in human specimens at very low concentrations, which complicate their detection using standard GC-MS procedures that often cannot provide the required sensitivity. Recent advances in LC-MS(/MS) technology have enabled accurate detection and quantification of these compounds in various human specimens, indicated by the increasing number of published methods. Method validation has been a particular focus of analytical chemistry in recent times. Recommendations set by several guidance documents are now widely accepted by the toxicology community, as reflected by the guidelines drafted by leading toxicological societies. This review provides a critical review of single-stage and tandem LC-MS procedures for the detection and quantification of APs, with a particular emphasis on appropriate method validation.

The quality of published methods is inconsistent throughout the literature. While the majority of authors incorporate some validation experiments in their respective method development, a large number of published methods lack essential components of method validation, which are considered mandatory according to the guidelines. If adapting a method for the detection of APs for use in a laboratory, analysts should ensure successful validation experiments for appropriateness and completeness have been conducted, and perform additional experiments when indicated.

Keywords: antipsychotic drugs, LC-MS(/MS), method validation

Introduction

In the 1950s, the phenothiazine derivative chlorpromazine was the first drug introduced for the treatment of psychotic illnesses, largely replacing electroconvulsive therapy and psychosurgery. Subsequent to the success of chlorpromazine, a large number of compounds were introduced for the treatment of patients suffering from mental illnesses. The main category of neuroleptic drugs are the phenothiazine derivatives, butyrophenones and thioxanthenes, known as "typical antipsychotics (APs)". While these drugs show significant improvement in the symptoms of psychotic illness, they are also associated with unwanted extrapyramidal side-effects resulting from their activity at dopamine receptors. A new generation of APs introduced around 1995 largely overcame these side-effects via decreased activity at dopamine receptors compared with their traditional counterparts. These "second generation" or "atypical APs" now account for the vast majority of APs prescriptions. Reports in the USA indicate a steady increase from 1.0 M prescriptions in 1995 to 13.3 M in 2008, while typical agents decreased significantly over the same timeframe [1]. However, studies in recent years have shown that atypical APs are not free from side-effects. An increased risk of mortality in addition to cardiovascular complications have been reported in patients suffering from dementia when treated with atypical APs [2]. Furthermore, second generation APs do not only increase the risk of diabetes [3] compared with typical agents but also show a similar risk of sudden cardiac death to their typical counterparts [4]. With more than 35 APs currently available worldwide, this drug class has rapidly gained importance in both a clinical and forensic setting, which makes the ability to reliably detect APs in human biological specimens a necessity.

In a clinical environment, the analysis of APs in blood is necessary in order to monitor patient compliance and to maintain drug concentrations within the recommended therapeutic range of the respective drug. The absence of prescribed APs in a clinical case may also indicate non-compliance, a common issue among patients suffering from mental illness. In a forensic setting, the detection of APs is crucial in determining whether these drugs played a role in the cause of death. A sub-therapeutic concentration of an AP in forensic cases may be particularly relevant in cases where mental disturbances have contributed to the death of a person by another, e.g. homicides. Analytically, APs have been traditionally measured using gas chromatography (GC) with mass spectrometer (MS).

Zhang *et al.* [5] presented an overview of bio-analytical methods for the determination of APs up until 2007. The authors focused primarily on GC and liquid-chromatography (LC) methods with various detectors such as ultraviolet (UV), nitrogen phosphorus, fluorescence and electrochemical detection (EC), concluding that LC was the most suitable separation technique for these mostly involatile compounds. MS/MS in combination with LC now dominate the analytical field, providing a particularly convenient tool in the analysis of APs. The high sensitivity of LC-MS/MS methods often allows analysis times to be substantially reduced compared with traditional UV and EC methods, which is particularly useful for a large sample throughput or when fast-around times are required.

Method validation has been a particular focus in recent times, in order to ensure true performance of methods and provide an objective tool to establish whether a method works as intended. The reproducibility of an analytical method is mandatory in preventing serious legal consequences that can result from forensic investigations. Specific guidelines for method validation were published two decades ago [6, 7] and have since been revisited by

the authors [8, 9] to produce contemporary guidelines specifying the minimum requirements for method validation. These guidelines are now widely accepted in the toxicology community, reflected in guidelines drafted by leading toxicology societies such as The International Association of Forensic Toxicologists (TIAFT), The Society of Forensic Toxicologists (SOFT) and The Society of Toxicological and Forensic Chemistry (GTFCh). However, a large number of methods still exist that either lack crucial parts of validation, or that have not adequately performed the obligatory validation experiments.

This review provides a critical review of single-stage and tandem LC-MS procedures for the detection and quantification of APs with a particular emphasis on appropriate method validation.

Methods

Papers for this review were selected following a comprehensive PubMed search for English articles using LC-MS or LC-MS/MS methods for the detection of one or more APs in various human specimens (blood, plasma, serum, urine, hair, saliva, and cerebrospinal fluid (CSF)). Selected papers were reviewed for analytical details and assessed with regard to the extent of validation studies against current guidelines [6, 8, 9].

Choice of Biosamples

Blood is the preferred specimen for AP analysis as it provides the most accurate representation of the relevant pharmacological effects. In a clinical setting, plasma and serum are matrices of choice for drug analysis, as they are the most common specimens used in diagnostic medicine. Therapeutic drugs monitoring (TDM) methods are common and are

more likely to focus on one or very few analytes. Whole blood is the most common specimen used in forensic cases since lysis is common in death investigations and centrifugation shortly after collection is not always possible [10].

Urine is a useful specimen for general unknown screening (GUS) procedures, particularly when overdose is suspected and qualitative results are required. APs are included in most published non-targeted screening procedures as part of big libraries. However, since these methods lack the ability to produce quantitative results, they are less relevant for the detection of APs and will not be discussed in this review [11-13]. Targeted published methods for detection of APs in urine using LC-MS(/MS) are rare and usually include an additional matrix [14-17].

Hair has become an increasingly popular alternative specimen to blood, as drugs and their metabolites are likely to remain in hair samples long after the compounds have been eliminated from the body. Segmental hair analysis in particular can provide an indication of the long-term history of drug use in an individual. While hair analysis is frequently used as a tool in the analysis of drugs of abuse, only a limited number of methods targeting APs in hair using LC-MS(/MS) technology have been published to date [16, 18-22].

Oral fluid is used as an alternative to blood, which has increasingly gained importance due to the relatively short drug detection windows in addition to non-invasive collection of specimens. These factors make oral fluid a useful specimen in circumstances where trained medical staff is not available, such as roadside and workplace drug testing. APs are known to reduce salivary flow rate [23] and may therefore not be ideal for detection in oral fluid. This is reflected in the limited number of published methods for APs [24] to date using this specimen.

CSF is commonly analysed in order to help diagnose various diseases and conditions affecting the central nervous system (CNS) such as meningitis and encephalitis. It is also useful in diagnosing bleeding of the brain or tumors within the CNS. CSF is most commonly obtained by lumbar puncture, a complex and invasive procedure that requires specialised medical staff. While it is likely that drug concentrations in CSF are more closely related to pharmacological effects than blood concentrations, the complicated process of sample collection makes it a less favourable specimen in drug analysis, with only one published method for the detection of APs [25].

General considerations

Sample volume and LLOQ

In published analytical methods, sample volumes below 0.1 mL are rare [24, 26-28], whereas volumes closer to 1 mL are frequently used. When selecting a sample volume for an analytical method targeting APs, several factors must be considered. Using a small sample volume in an analytical method provides several advantages, including easier handling during sample extraction and the ability to conduct analysis in cases where only limited specimens are available, e.g. post-mortem cases. However, APs are mostly lipid-soluble weak bases, which are quickly absorbed into body fat and organs following administration, signifying a large volume of distribution (V_D). Despite their high V_D, most common APs also significantly bind to plasma proteins (Fb). Both the large V_D and high Fb significantly reduce the amount of unbound drug available in the blood for detection. Analytical requirements dictate that the lowest therapeutic blood concentration of a drug must be quantified. This equates to determining the lower limit of quantification (LLOQ), usually involving two

different approaches: a signal-to-noise ratio (S/N) of 10 is considered satisfactory [29] and so is a precision and accuracy of < 20 % at the desired LLOQ [6, 8]. Huang *et al.* [30] reported a S/N of 3 at the LLOQ, which is generally acceptable for a limit of detection (LOD), but not for the LLOQ. However, they conducted validation experiments which confirmed the precision and accuracy at the LLOQ to be within 20 %, and therefore meet acceptance criteria. It needs to be guaranteed that a method is sufficiently sensitive to fulfill at least one of these two criteria when selecting the sample volume. Table 1 shows pharmacokinetic parameters of common APs.

Single-analyte methods vs multi-analyte methods

Single-analyte methods are mostly used in a TDM-setting, where only specific compounds are the target of drug monitoring. Methods targeting the atypical AP RIS should always include its major metabolite 9OH-risperidone (9OH RIS), also referred to as paliperidone. 9OH RIS is formed by cytochrome (CYP) P450 enzymes, specifically CYP2D6, and is likely to contribute to the in vivo effects of RIS [31]. Whilst plasma concentrations of RIS and 9OH RIS show a large variation between individuals [32-34], RIS levels are generally lower than 9OH-RIS levels. In fact, a study measuring plasma concentration of RIS and 9OH-RIS after oral administration of RIS in steady-state found RIS was not detectable at a LLOQ of 0.1 ng/mL in ~ 18 % of all tested individuals, whereas 9OH RIS was detected in all cases [32]. Only measuring the parent compound especially in TDM methods can therefore lead to inaccurate conclusions regarding patient compliance.

While the same risk of interferences exists for single-analyte and multi-analyte procedures, chances are higher that they will be identified during method development when a greater number of analytes are included in the method. Generally multi-analyte procedures are preferred over single-analyte approaches, as the inclusion of a number of analytes in one method saves time and resources.

Sample preparation

Extraction of APs from blood, plasma and serum

Table 2 shows an overview of currently published single-analyte LC-MS(/MS) methods using blood, plasma or serum. Table 3 contains all published multi-analyte studies.

Due to the high specificity of LC-MS methods, it was initially thought that the sample preparation step may not be as crucial as with other analytical methods, particularly for MS/MS methods since transitions greatly reduce the risk of interference from other drugs. However, this view was soon revised. While endogenous components might no longer be detected using LC-MS methods, they can still significantly interfere with the quantification of a drug [35, 36].

Therefore, liquid-liquid extraction (LLE) [25, 30, 37-51] and solid-phase extraction (SPE) [15, 16, 52-59] are still most commonly used as a sample treatment prior to injection into the LC-MS system, as they provide the most thorough sample clean-up. Saar *et al.* systematically evaluated nine different combinations of extraction solvents and buffers in order to find the most suitable LLE method for the extraction of 19 APs [60]. The method showing the best results overall for extraction recoveries and matrix effects used trizma buffer and 1-chlorobutane (BuCl) and was subsequently compared with a standard SPE extraction method. While extraction efficiencies were comparable between LLE and SPE method, blockages of SPE cartridges were a common problem, especially when dealing with post-mortem samples. Nirogi *et al.* [46] applied a similar approach when comparing six organic solvents and their combinations in order to optimise extraction recovery for their method targeting olanzapine (OLZ) in plasma. A mixture of diethylether and dichloromethane (7 : 3, v / v) yielded the highest recovery of OLZ and was therefore used in their detection method. Gutteck *et al*. [48] stated that due to the different "extraction coefficients… and different concentration ranges in human serum", four different extraction procedures had to be applied for determination of 13 antidepressants and five APs. Minor variations in organic solvents used for the LLE, differences in the volumes of the mobiles

phases and varying internal standards mark the differences between the four methods. A more practical approach would have been to have one extraction method and chromatographic conditions that allowed the analysis of all drugs in a single cost-effective method, especially since it is not clear which factors resulted in the development of the four different methods.

Simple protein-precipitation (PP) may be used for "cleaner" matrices such as serum or plasma [26, 27, 61, 62]. It needs to be noted, however, that matrix effects must be investigated closely as PP might fail to remove phospholipids from plasma or serum which might cause interferences.[63, 64] Interestingly, Klose Nielsen *et al*. [65] compared LLE methods with different combinations of organic solvents and SPE techniques prior to the development of their method for the determination of OLZ in whole blood, and found none of them to be functional. However, a simple PP appeared to produce sound results. Few methods employed direct-injection [14, 28], while one published method used direct injection in combination with column switching [24] in order to decrease matrix influences. One published approach uses solid-phase micro-extraction (SPME) as a solvent-free and concentrating extraction technique [17]. While traditionally combined with GC, employing heat assisted desorption from the fiber, a simple interface coupling SPME with LC makes it functional for non-volatile substances. Online-SPE has been applied in order to reduce human error and increase time-efficiency [57]. Upscaling of the extraction is achieved by work-up in the 96 well-format [27, 53].

Extraction of APs from hair

Table 4 shows an overview of methods published for the detection of APs in hair, using LC-MS(/MS).

The Society of Hair Testing recommends that hair be washed prior to analysis (e.g. in MeOH) and subsequently analyse the wash solution for drug content [66]. A high concentration of the drug of interest in the wash solution may indicate external contamination of the hair sample. However to date, a conclusion has not been reached concerning the best decontamination strategy [67-73].

Among the most commonly used extraction procedures for hair analysis are alkaline hydrolysis using NaOH followed by SPE, or extraction with MeOH and aqueous buffer using an ultrasonicator [74]. Whilst both techniques are used for analysis of APs in hair, methods using NaOH appear to be preferable for alkaline-stable drugs such as APs. Josefsson *et al*. [16] did not attempt a full validation of their LC-MS/MS method for the identification of 19 APs and their major metabolites in hair. Incubation with NaOH was performed prior to extraction with BuCl and back extraction into formic acid. Two SRM transitions were chosen per AP (and where possible per metabolite) for identification of the drugs of interest. The authors highlighted the importance of including metabolites of drugs of interest in hair methods. In hair analysis, the issue of incorporation of a drug into the hair from external sources rather than ingestion is a frequent point of discussion, especially in court cases where an accused person denies the use of a drug. For some drugs, the presence of metabolites in a certain ratio to the parent drug can be an additional indication that ingestion of the drug has occurred and facilitate interpretation of results of hair analyses [75].

Nielsen *et al*. [20] tested different combinations and ratios of organic and aqueous solvents prior to the development of their detection and quantification method. This involved 52 common pharmaceuticals and drugs of abuse in hair, including five APs. This "mixed" approach was fully validated in accordance with international guidelines [9]. When extracting basic compounds such as APs from hair, the use of a neutral or slightly acidic aqueous buffer is recommended in order to facilitate ionisation of the compounds prior to transition into the aqueous phase [74]. Mueller *et al*. [19] and Weinmann *et al*. [22] performed ultrasonication with MeOH prior to mixed-mode SPE. Thieme *et al*. [21] divided the initial 50mg segment of hair into individual hairs prior to analysis. 30 fg on column was sufficient to detect clozapine in single hairs. The authors, however, acknowledge the uncertainty associated with hair analysis, mainly resulting from the unknown recovery of drug from hair combined with the uncertainty of the exact length of single hair segments.

Extraction of APs from cerebrospinal fluid, oral fluid and urine

Table 5 shows an overview of published methods for the detection of APs in CSF, oral fluid and urine using LC-MS(/MS).

Several authors have attempted to validate previously developed methods for the detection of APs in plasma or blood for urine [14, 15, 17]. Bogusz *et al*. [76] applied full-scan mode to urine samples of patients treated with OLZ in order to find proposed metabolites. A large number of OLZ metabolites in urine have been confirmed by Kassahun *et al*. in their comprehensive study of the metabolism of OLZ in humans [77]. It was hypothesised that OLZ-10-N-glucuronide and N-desmethyl-OLZ would be present in urine samples following

OLZ ingestion. However, the compounds were not unequivocally identified as a valid reference standard was not available.

To the authors' knowledge, the only method for the detection of APs in oral fluid was published by Flarakos *et al*. in 2004 [24]. Their fully validated method applied online clean-up with column switching for the detection of RIS and 9OH RIS in 25 µL saliva and plasma, aiming to establish a salivary/plasma (S/P) ratio. A wide range of S/P ratios obtained from 13 plasma and saliva samples (seven adults and six children) confirmed that saliva analysis only provided a qualitative tool for the presence of RIS and 9OH RIS but did not allow a conclusion regarding plasma concentrations at the time of sampling.

Josefsson *et al*. applied their detection method for OLZ and N-desmethyl OLZ not only to serum but also to CSF [25]. The authors postulated that the pharmacological effects of OLZ are likely to be more closely related to its concentration in the CNS (i.e., CSF) than in serum. With a LLOQ of 0.2 ng / mL in plasma, the method showed sufficient sensitivity for the expected low concentrations in CSF. The authors postulated a linear correlation between serum and CSF OLZ concentrations ($r^2 = 0.77$). While there were only six individuals included in this study, the developed method was successfully applied to a cohort of 37 individuals. The authors also considered the influence of gender, age, smoking and pharmacogenetics, when investigating the ratio between OLZ and metabolite concentrations in serum and CSF [78].

LC separation

All APs possess hydrophobic properties and as such, all currently published methods for the detection and quantification of APs in biological matrices have employed reversed phase

(RP) stationary phases, with mostly silica-based packings containing C_8 and C_{18} chains. Cabovska *et al*. [40] and de Meulder *et al*. [15] used chiral columns in order to separate the (+) and (-) enantiomers of 9OH-RIS. 9OH-RIS is the main metabolite of the atypical AP RIS and has shown to be almost equipotent to RIS in animal studies [79]. Due to its efficacy, racemic 9OH-RIS (paliperidone) is also marketed as a drug in its own right [80]. The separation of the two enantiomers is useful for kinetic studies, as the formation of the (+)-form appears to be catalysed by CYP2D6, whereas CYP3A4 and CYP3A5 are essential for the formation of the (-)-form [81]. The separation of these enantiomers is usually not essential in routine drug analysis.

Columns packed with < 2 µm particles are referred to as ultra-high pressure LC (UHPLC) columns and are said to reduce analytical run times due to improved compound separation. This is desirable in a TDM environment where a large number of samples are tested for very few compounds. To the authors' knowledge, there are only two methods using UHPLC published to date. Hasselstrom *et al*. [27] used a Zorbax SB-C_8 column with a particle size of 1.8 µm, resulting in the detection and quantification of 13 antidepressants and APs, including 13 deuterated IS over a total analytical run time of 4 min. Remane and collegues [82] covered a total of 62 compounds including 31 APs over a total run time of 26 min, employing a TF Hypersil GOLD Phenyl column with a particle size of 1.9 µm. A recent review however compared the separation power of columns with particle sizes of 1.8 µm and 5 µm at a "fast" (1 mL / L) and a "slow" (0.3 mL / L) flow rate, and concluded that the particle size was less significant than initially proposed. The column particle size appeared to make only a modest difference in the peak height, peak width or resolution, with the difference for each parameter being less than a factor of 2. Higher flow rates distinctively

increased peak height by 6–7-fold and the peak width decreased by about 3-fold when using the faster flow rate [64]. In a post-mortem environment, larger particle sizes (3 – 5 µm) have proven to be favourable due to the higher robustness which is required for more complex matrices such as whole blood [50]. The presented methods show a wide range of isocratic and gradient elutions, including various aqueous and organic elution solvents. Details are shown in the column "Mobile Phase" in Tables 3 and 4.

MS detection

Ionisation of compounds in LC-MS technology is usually achieved with either Electrospray Ionisation (ESI) or Atmospheric-Pressure Chemical Ionisation (APCI). The reason ESI is used in the majority of presented methods for the detection of APs is likely to be associated with the higher sensitivity achieved by ESI. Bhatt and colleagues compared ESI with APCI ionisation, prior to development of their method for the detection of RIS and 9OH RIS in plasma. They found APCI to be less favourable when compared with ESI [62]. In a comprehensive study investigating the influence of anticoagulant and lipemia on matrix effects when analysing OLZ, Chin *et al*. reported that the analyte response with APCI was five times less than with ESI [83]. Therefore, the required LLOQ of 0.05 ng / mL for OLZ was not achieved in APCI mode. The higher sensitivity achieved by ESI, however, was the expense of lower selectivity. Many authors have found matrix effects to be more prominent when applying ESI [84, 85]. Ionisation efficient neutral compounds including matrix particles, co-eluting compounds, or additives such as salts in biological samples, can compete with analytes during the evaporation process. This is likely to lower the ionisation rate of the compounds of interest. It is further suggested that during the evaporation process,

the analyte of interest may precipitate from solution by itself or as a co-precipitate with non-volatile sample components [84]. This highlights the need for thorough sample clean-up prior to MS analysis and the assessment of matrix-effects as a crucial part of method validation. This is discussed later in this article.

Due to the predominantly basic properties of APs, ionisation takes place in the positive mode. The vast majority of published methods apply selected reaction monitoring (SRM) as an easy way for the detection and quantification of APs. International guidelines [86-88] require a minimum of two SRM transitions for reliable identification of an analyte – unfortunately a large component of SRM methods do not comply with this rule. The best example of possible misidentification of a compound due to monitoring a single SRM transition, is the structurally similar O-desmethyl metabolite of the antidepressant venlafaxine and the synthetic opioid tramadol. Due to their almost identical chemical structure, they do not only elute at the same time but also share the most abundant transition (m/z 264.2 → 58.2) [89]. Less common examples in the field of APs include the structural isomers promazine and promethazine (Figures 1a and 1b). These drugs share the most abundant transition (m/z 285 → 86), representing the cleavage of the side chain [50] and also elute at the same time.

The isobaric compounds pipamperone and haloperidol (Figures 2a and 2b) share the two most abundant transitions (m/z 376 → 123 and m/z 376.0 → 165) [50]. If sensitivity can still be maintained, it is recommended to pick a transition with a smaller abundance for one of the two analytes or, alternatively, add a third transition in order to guarantee reliable differentiation.

While MS in the SRM mode certainly provides an efficient tool for compound identification, these examples highlight the need to critically evaluate parameters (such as most abundant transitions) provided by the instrument during compound optimisation. Few authors use screening procedures that allow subsequent quantification of APs of interest [17, 47, 58].

Validation issues

Table 2 and Table 3 present an overview of single-analyte and multi-analyte published methods, respectively, for the detection of APs in blood, plasma and serum using LC-MS(/MS). It is generally accepted that all methods must be validated using internationally accepted guidelines. Specific validation criteria must be met to satisfy the following minimum requirements [7-9]: selectivity, matrix effects, extraction efficiency, process efficiency, processed sample stability, linearity, accuracy, precision and freeze-thaw stability. Although some authors claim to have conducted all/specific components of the method validation experiments, the quality and reputability of these experiments is not consistent across all papers. Parameters which are frequently associated with inconsistencies will be discussed below.

Internal Standard

A variety of internal standards (IS) have been used in the reviewed methods. Preferred internal standards are deuterated compounds of the drug class of interest, such as clozapine-d_3 [27], haloperidol-d_4 [49, 50], olanzapine-d_3 [25], quetiapine-d_8 [27], and ziprasidone-d_8 [27]. If these IS are unavailable to a laboratory, it is recommended to use a deuterated IS from a different drug-class rather than an AP that is in therapeutic use [90]. To the contrary,

it has been suggested that high concentrations of a drug can influence the peak areas of their co-injected deuterated analogues when using APCI mode with isotope peaks (M+1 to M+3) of analytes contributing to the peak area of the IS. This can lead to miscalculation of the IS concentration and subsequently underestimation of the drugs of interest. However, for masses (M+5) and higher, no isotopic contribution was observed [91].

As co-medication and therapeutic use of a compound can never be fully excluded, overestimation of an IS is likely to result in underestimation of a drug concentration. Swart *et al*. [47] did not achieve good results in their detection method for fluspirilene in human plasma when using dimethothiazine as an IS. Their decision not to use an IS at all defies the guidelines of acceptable analytical practice. Particularly in cases where only few analytes are included in a method, a suitable deuterated IS is preferred in all instances. Unfortunately, this is not an isolated event. A large number of analytical methods still use therapeutic drugs as IS [17, 26, 28, 30, 41, 42, 46, 51, 52, 55, 57, 61, 65, 92].

Selectivity

In order to guarantee selectivity of an analytical method, it would be ideal that all possible interferences arising from matrix compounds, other drugs and IS, are excluded. As this is impractical, the analysis of six blank specimens from different sources is widely considered acceptable [6] and is applied by most authors. The testing of ten blank specimens, however, has been employed by some authors [50, 56] and is encouraged for improved selectivity [93]. Josefsson *et al*. [25] performed method validation in accordance with international guidelines in their method for the detection of OLZ and N-desmethyl OLZ in CSF, however selectivity of the method was not investigated. This is surprising, as despite the more invasive nature of

sample collection compared with taking blood, the authors obtained drug-free CSF samples from six different patients. Several authors do not state clearly how many different sources of blank specimens were tested for interferences [30, 48]. Klose Nielsen *et al*. [65] examined the interferences from other possible drugs in forensic samples by spiking blank blood samples with 66 common drugs such as benzodiazepines, analgesics, antidepressants, APs, β-blockers, narcotics and stimulants. Two "zero" samples (blank sample containing IS) should be included in validation-experiments in order to exclude possible interferences of the IS on the selectivity of the method.

Calibration

Linearity is an important part of method validation whenever quantification of analytes via a standard curve is carried out, which is the case in the vast majority of all published methods. An alternative is presented by Rittner *et al*. in their method for the detection of 70 psychoactive drugs, where they semi-quantify several analytes using the method of standard addition [58].

Peters *et al*. [7] comprehensively summarised the requirements for an adequate calibration model in their review paper (which is beyond the scope of this article). The calibration range should cover at least the therapeutic range of the drug of interest, however as long as linearity can be assured, a greater range can be included.

Arinobu *et al*. [14] include 14 calibrators in order to cover the wide calibration range of 1 ng / mL – 800 ng/mL for the detection of haloperidol and its metabolites in plasma and urine, measuring 10 replicates per calibrator. Moody *et al*. [45] could not guarantee linearity of calibration curves in their method targeting risperidone and 9OH RIS when using ESI. As

the calibration curves started to plateau above 10 ng / mL when using ESI, APCI was used to continue the method validation. The plateau could be caused by saturation of the detector. This is, however, unlikely as the concentrations injected are not very high with the highest calibrator at 25 ng / mL. Furthermore, the problem of the plateau does not exist in APCI mode, confirming that detector saturation is not the reason. A more likely cause is a saturation of the droplets during the ionisation process; a problem not occurring in APCI mode as the ionisation of compounds takes place in the gas-phase.

Matrix effects

The investigation of matrix effects is considered to be an essential part of method development. As discussed earlier, ESI appears to cause greater matrix effects than APCI, however no new method should be accepted without appropriate investigation of matrix effects. Two approaches for the evaluation of matrix effects have been accepted by the analytical community: the post-column infusion approach presented by Bonfiglio *et al*. [94] and the post-extraction spike method by Matuszewski *et al*. [85].

While the evaluation process of matrix effects using these methods is considered to be common knowledge, there is some inconsistency throughout the literature when it comes to interpreting the details. When Matuszewski *et al*. [85] stated that an appropriate IS can compensate for matrix effects "assuming the relative matrix effect exhibits the same pattern for the drug and the internal standard in all lots studied", some authors [40] unfortunately misinterpret this observation by stating that a deuterated IS can compensate for matrix effects. Firstly, it must be confirmed that the matrix effects are equivalent for a drug and the respective IS, which is more likely if they show a similar chromatography and elute close to

each other. Secondly, when it comes to low drug concentrations, ion suppression may lower the concentration of a drug below the LOD, in which case a positive case may be missed despite the concentration of the IS being lowered by the same percentage.

Berna *et al*. report to have investigated matrix effects in both their methods for the detection of OLZ in plasma and serum [53] and whole blood [39], however, do not report any outcomes. Swart *et al*. [47] conclude it is "doubtful" that matrix effects are present in their method for the detection of fluspirilene in plasma as their calibration curves appear to be "fairly linear". There is no evidence to suggest that linear calibration curves give an indication of possible matrix effects, this assumption is therefore unjustified.

Stability

Processed sample stability

Prior to progressing to further validation experiments, the stability of the drugs of interest in processed samples must be verified. Extracted samples should not be stored longer than the stability in processed samples has been tested and assured. 24 h [39, 40, 52, 53, 62] is the most commonly investigated timeframe as runtimes are unlikely to exceed one day. Nevertheless, it can be useful to obtain stability information for a longer period of time in cases where instrument issues may cause samples to be re-run on the next day [7, 8].

There are three ways the result can be reported. Either as a percentage loss over a defined timeframe (given as the mean with S.D.) [42]; a comparison between the initial drug concentration and the concentration after storage using a paired *t*-test [40]; or as more frequent injections over the investigated timeframe, a curve is generated and (after regression analysis) a negative slope significantly different from zero ($p < 0.05$) indicates instability

[56]. Kratzsch *et al*. accurately plotted absolute peak areas as opposed to relative peak areas against the time of injection, in order to prevent the IS from correcting for eventual losses [56]. Some authors followed the recommendations of testing two concentrations (one low and one high of the calibration range) [42, 44, 47, 50, 55, 56], whereas others improved on this by including an additional concentration [15, 30, 54]. Josefsson *et al*. investigated processed sample stability and found sample extracts to be unstable over 24 h, with significant losses for both OLZ and N-desmethyl OLZ [25]. This outcome is not surprising as significant stability issues in processed samples containing OLZ have been reported in other matrices such as whole blood [50]. If processed sample stability is not guaranteed over 24 h, it is recommended that analysis is completed prior to degradation of OLZ taking place.

Freeze-thaw stability

Assuring that multiple cycles of freezing and thawing do not compromise the integrity of tested samples is crucial in routine toxicological analysis. A blood sample is likely to be tested for different groups of analytes and therefore be thawed and frozen again several times. Experimental factors should be selected based on the conditions that are intended to be used on real cases, i.e., the temperature at which routine samples are being stored should be the temperature applied in the freeze-thaw (F/T) experiments. Shah *et al*. recommended the testing of at least three F/T cycles and two concentration levels in triplicate [6, 8]. While there are variations in the number of concentration levels and F/T cycles tested by some authors, it is most concerning that there is still a large number of methods where no F/T stability experiments were conducted at all [14, 16, 17, 26-28, 37, 38, 40, 43, 46-49, 57-59, 61, 65, 92, 95].

Conclusions

Currently, there are more than 35 different APs available worldwide for the treatment of a range of psychotic illnesses. Over the past 15 years, recent advances in LC-MS(/MS) technology has enabled the detection and quantification of these drugs in exceptionally low concentrations; the newer generation APs in particular. This has led to the development of numerous LC-MS(/MS) methods for the analysis of APs in human biological specimens. A requirement for the success of such detection methods is that they are suitably sensitive to cover the low therapeutic range in which APs are usually present. Proficiency with LC-MS(/MS) technology has increase dramatically over the past decade. Aspects of method development that require particular attention in order to guarantee reproducible results are identified and summarized in various method validation guidelines [7-9]. However, the quality of published methods with regard to validation criteria is not always consistent. The most significant issues relate to the evaluation of selectivity, linearity, matrix effects and stability. Addressing these issues in future analytical studies is mandatory to accurately detect APs in biological specimens and, consequently, to better understand this increasingly prevalent class of drugs.

Table 1: Pharmacokinetic parameters of common APs

Drug	Common daily oral dose range in adults (mg)[1]	Blood concentrations expected following therapeutic use (ng/mL)[2]	$t_{1/2}$ (h)[3]	V_D (L/Kg)[4]
9OH-Risperidone*	3 - 12	10 - 100	23	U/K
Amisulpride	400 - 1200	50 - 400	11 - 27	13 - 16
Aripiprazole	10 - 30	50 - 350	60 - 90	4.9
Bromperidol	1 - 15	1 - 20	15 - 35	U/K
Buspirone	20 - 30	1 - 10	3 - 12	5 - 6
Chlorpromazine	200 - 600	30 - 300	7 - 119	10 - 35
Chlorprothixene	40 - 80	20 - 200	8 - 12	11 - 23
Clozapine	300 - 450	200 - 800	6 - 17	2 - 7
Flupentixol	3 - 6	1 - 15	19 - 39	14.1
Fluphenazine	1 - 5	2 - 20	13 - 58	220
Fluspirilene¥	2 - 5 (i.m)	U/K	21 days (decanoate)	U/K
Haloperidol	1 - 15	5 - 50	18	18 - 30
Levomepromazine	25 - 50	15 - 60	15 - 30	30
Loxapine	20 - 100	10 - 100	3 - 4	U/K
Melperone	100 - 400	5 - 40	2 - 4	7 - 10
Mesoridazine	100 - 400	15 - 100	2 - 9	3 - 6
Molindone	50 - 100	~ 500	1.2 - 2.8	3 - 6
Olanzapine	5 - 20	10 - 100	21 - 54	10 - 20
Penfluridol	20 - 60 (once per week)	4 - 25	70	U/K
Perazine	50 - 600	100 - 230	8 - 15	U/K
Pericyazine	15 - 60	5 - 60	U/K	U/K
Perphenazine	12 - 24	0.6 - 2.4	8 - 12	10 - 35
Pimozide	7 - 10	15 - 20	28 - 214	11 - 62

Drug	Common daily oral dose range in adults (mg)[1]	Blood concentrations expected following therapeutic use (ng/mL)[2]	$t_{1/2}$ (h)[3]	V_D (L/Kg)[4]
Pipamperone	80 - 120	100 - 400	12 - 30	U/K
Prochlorperazine	15 - 40	10 - 500	14 - 27	13 - 32
Promazine	200 - 800	10 - 400	7 - 17	27 - 42
Quetiapine	300 - 450	70 - 170	6 - 7	8 - 12
Risperidone	2 - 6	10 - 100	3 - 20	0.7 - 2.1
Sertindole	12 - 20	50 - 500	U/K	20 - 40
Sulpiride	400 - 600	50 - 400	4 - 11	2.7
Thioridazine	150 - 300	200 - 2000	26 - 36	18
Thiothixene	6 - 30	U/K	12 - 36	U/K
Trifluoperazine	15 - 20	1 - 50	7 - 18	U/K
Triflupromazine	165 - 375	30 - 100	U/K	U/K
Ziprasidone	40 - 160	50 - 120	2 - 8	1.5 - 2.3
Zotepine	75 - 300	5 - 300	12 - 30	50 - 168
Zuclopenthixol	20 - 50	5 - 100	12 - 28	15 - 20

[1]: Common daily oral dose data for the treatment of schizophrenia, psychoses or bipolar disorder from Drugdex® Evaluations in the Micromedex® Internet database [96]. Where the drug is indicated for other disorders (e.g. depressive disorders), dosages may vary.
[2]: Blood concentrations expected following therapeutic use obtained from TIAFT guidelines [97]
[3]: terminal elimination half-life and; [4]: Volume of distribution from Baselt [98]
*: also referred to as "Paliperidone"
¥: only available as i.m injection
U/K: unknown

Table 2: Summary of single-analyte methods for the detection of APs in blood (a), plasma (b) and serum (c) using LC-MS/MS

a)

Author (Year)	Volume [mL]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Klose Nielsen et al.[65] (2009)	0.19	olanzapine	dibenzepine	acidic MeOH-induced PP	Zorbax Extend C_{18} (50 x 2.1 mm, 5µm)	gradient with 5mM ammonium hydroxide in ACN and ACN	ESI, positive mode, SRM, MS/MS	linearity, selecticity, matrix effects, recovery, LLOQ, precision, accuracy, PS stability, LT stability
Kollroser et al.[43]◊ (2001)	1	zuclopenthixol	flupentixol	LLE (ammonia solution and ethylacetate)	Symmetry C_{18} Waters (3.0 x 150 mm, 5µm)	gradient with ACN and 0.1% formic acid	ESI, positive mode, SRM, MS/MS	linearity, accuracy, precision, LOD

b)

Author (Year)	Volume [mL]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Aravagiri et al.[37] (2001)	0.5	clozapine, norclozapine, clozapine-N-oxide	"a derivative of risperidone"	LLE (ethyl acetate, methylene chloride, pentane)	Phenomenex C_{18} (50 x 4.6mm, 5µm)	isocratic with 60mM ammonium acetate MeOH and ACN	ESI, positive mode, SRM	precision, accuracy
Aravagiri et al.[38] (2000)	0.5	risperidone, 9OH-risperidone	R68808	LLE (0.5 mL sat solution of sodium carbonate (pH = 10.5) 15 % methylene chloride in pentane	Phenomenex phenyl hexyl column (5µm, 50 x 4.6mm)	isocratic with 0.15mM ammonium acetate, MeOH, and ACN	ESI, positive mode, SRM	precision, accuracy,
Arinobu et al.[14] ## (2002)	1	haloperidol, reduced haloperidol, 4-(4-chlorophenyl)-4-hydroxypiperidine	4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-(4-chlorophenyl-1-butanone	addition of 3 mL of dH_2O with 0.09% formic acid and 20 mM ammonium acetate, freezing, thawing, centrifugation, injection of 20 µL of supernatant	Mspak GF-310 4B (50 x 4.6mm)	gradient with formic acid and 20mM ammonium acetate in dH_2O and ACN	SSI, positive mode, MS	linearity, LOD, precision, accuracy

Author (Year)	Volume [mL]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Barret et al.[52] (2007)	0.5	quetiapine	clozapine	SPE	Atlantis dC_{18} (100mm x 3mm, 3µm)	isocratic with ACN-MeOH-0.01M ammonium acetate	ESI, positive mode, SRM, MS/MS	selectivity, LOD, LLOQ, recovery, matrix effects, linearity, precision, F/T and LT stability, PS stability
Bhatt et al.[62] (2006)	0.1	risperidone, 9OH-risperidone	methyl risperidone	PP (ACN)	Betasil C_{18} column (3 µm, 100 x 3 mm)	isocratic with ammonium acetate and ACN	ESI, positive mode, SRM MS/MS	selectivity, linearity, LLOQ, precision, accuracy, recovery, F/T and LT stability, PS stability
De Meulder et al.[15] ## (2006)	0.2	risperidone, 9OH-risperidone	$^{2}H_{2}$-$^{13}C_{2}$-risperidone and $^{2}H_{2}$-$^{13}C_{2}$-9OH-risperidone	SPE (mixed mode)	Chiralcel OJ column (50 mm x 4.6, 10µm)	gradient with hexane, 0.01mM ammonium acetate in isopropanol, 0.01mM ammonium acetate in ethanol	ESI, positive mode, SRM, MS/MS	selectivity, precision, accuracy, recovery, F/T and LT stability, PS stability
Flarakos et al.[24] ## (2004)	0.025	risperidone, 9OH-risperidone	R068808	online cleanup, column switching	Zorbax SB_{18} (30 x 2.1mm, 3.5µm)	isocratic with 10mM ammonium acetate/ACN	SRM, MS/MS	linearity, selectivity, precision, accuracy, recovery, matrix effects, F/T and LT stability
Gschwend et al.[42] (2006)	0.25	amisulpride	sulpiride	LLE (diisopropylether:dichloromethane, 1:1)	Phenomenex Synergi Polar-RP analytical column (75 mm x 4.6mm, 4µm)	isocratic with 5mM ammonium formate/ACN	ESI, positive mode, SRM, MS/MS	linearity, selectivity, recovery, precision, accuracy, F/T and LT stability, PS stability
Kubo et al.[44] (2005)	0.4	aripiprazole, OPC-14857	OPC-14714	LLE (diethylether)	RP Chemcobond ODS-W (150 x 2.1 mm, 5µm)	isocratic with dH_2O /ACN	ESI, positive mode, SRM, MS/MS	selectivity, linearity, accuracy, precision, recovery, F/T and LT stability, PS stability

Author (Year)	Volume [mL]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Moody et al.[45] (2004)	1	risperidone, 9OH-risperidone	RO68808	LLE (pentane/methylene chloride)	Intersil 5 ODS3 (150 x 2.1 mm)	gradient with dH_2O and ACN	APCI, positive mode, SRM, MS/MS	selectivity, extraction efficiency, accuracy, precision, F/T stability, LT stability
Nirogi et al.[46] (2006)	0.5	olanzapine	Loratadine	LLE (diethylether:dichloromethane)	Inertsil ODS column (3µm, 100 x 3mm)	isocratic with 10mM ammonium acetate:ACN	ESI, positive mode, SRM, MS/MS	linearity, precision, accuracy, LLOQ, recovery, F/T and LT stability, PS stability
Remmerie et al.[54] (2003)	0.5	risperidone, 9OH-risperidone	Method A: R068809 Method B: $^{2}H_2$-$^{13}C_2$-risperidone and $^{2}H_2$-$^{13}C_2$-9OH-risperidone	SPE (10cc/130mg Bond Elut Certify)	3-µm C_{18} BDS-Hypersil column (100 x 4.6mm)	gradient with 0.01 M ammonium formate and ACN	ESI, positive mode, SRM, MS/MS,	linearity, selectivity, accuracy, precision, recovery, PS stability, LT stability, F/T stability, matrix effects
Swart et al.[47] (1998)	1	fluspirilene	?	LLE (4 % isoamyl alcohol in hexane)	Phenomenex Luna C_{18} 5µm, 150 x 2.1mm)	isocratic with MeOH and dH_2O	ESI, positive mode, scanning product ion spectrum from m/z 130 - 500	selectivity, recovery, LLOQ, accuracy, precision, PS stability, LT stability

c)

Author (Year)	Volume [mL]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Huang et al.[30] (2008)	0.3	risperidone	paroxetine	LLE (ACN)	Alltima-C_{18} (2.1mm x 100 mm, 3µm)	isocratic with formic acid/ACN	ESI, positive mode, SRM, MS/MS	selectivity, linearity, precision, accuracy, recovery, PS stability, F/T stability
Josefsson et al. [25]## (2010)	0.2	olanzapine, N-desmethylolanzapine	olanzapine-d_3	LLE (tert-butyl-methyl-ether)	Synergi Hydro-RP (50mm x 2mm, 2.5µm)	gradient with 10mM ammonium formate with formic acid and MeOH with formic acid	ESI, positive mode, SRM, MS/MS	linearity, LLOQ, precision, accuracy, recovery, matrix effects, F/T stability, LT stability
Nozaki et al.[26] (2009)	0.03	zotepine	imipramine	PP (ACN)	Tosoh ODS-100V (50 mm x 2mm, 5µm)	gradient with 10 mM ammonium formate containing ACN and 10 mM ammonium formate containing 90 % ACN	ESI, positive mode, EC-MS/MS	linearity, LLOQ, accuracy, precision, recovery, matrix effects

Abbreviations: ACN: acetonitrile, APCI: atmospheric pressure chemical ionization, dH_2O: deionized water, EC: electrochemistry, ESI: electrospray ionization, F/T: freeze/thaw, LLOQ: lower limit of quantification, LOD: limit of detection, LT: long term, m/z: mass over charge ratio, MeOH: methanol, SRM: selected reaction monitoring, MS/MS: tandem mass spectrometry, PP: protein precipitation, PS: processed sample, SPE: solid phase extraction, SSI: sonic spray ionization

#: more drugs are included in this method but do not belong to the group of APs
##: this method was applied to more than one matrix
◊: post-mortem specimens were analysed in this method
?: an IS appears to have been used but is not specified

Table 3: Summary of multi-analyte methods for the detection of APs in blood (a), plasma (b) and serum (c) using LC-MS/MS

a)

Author (Year)	Sample [mL]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Josefsson et al.[16] ◊ ## (2003)	1	buspirone, chlorpromazine, chlorprothixene, clozapine, dixyrazine, flupentixol, fluphenazine, haloperidol, hydroxyzine, levomepromazine, melperone, olanzapine, perphenazine, pimozide, prochlorperazine, risperidone, thioridazine, ziprasidone, zuclopenthixol#	N/A	SPE	Zorbax Stable Bond Cyano column (50 x 2.1mm, 3.5µm)	gradient with different ratios of MeOH:ACN:20mM ammonium formiate	ESI, positive mode, SRM, MS/MS	N/A
Kumazawa et al.[17] ## (2000)	1	perazine, thioridazine, prochlorperazine, perphenazine, trifluoperazine, flupentixol, fluphenazine, thioproperazine#	propericiazine	SPME (polyacrylate-coated fiber)	Capcell Pak C_{18} UG120, S-5µm, 2.0 x 150 (Shiseido)	gradient with 10 mM ammonium acetate and ACN	ESI, full scan m/z 50-500, SRM, MS/MS	linearity, precision, accuracy,
Roman et al.[49] ◊ (2008)	1	buspirone, fluphenazine, flupentixol, perphenazine, risperidone, 9OH-risperidone, ziprasidone, zuclopenthixol	haloperidol-d_4	LLE (trizma buffer, methyl t-butyl ether)	Zorbax Stable Bond Cyano column (50 x 2.1mm, 3.5µm)	gradient with different ratios MeOH, ACN, 20 mM ammonium formate	ESI, positive mode, SRM, MS/MS	selectivity, linearity, LLOQ, precision, recovery, matrix effects
Saar et al.[50] (2010)	0.1	9OH risperidone, amisulpride, aripiprazole, bromperidol, buspirone, chlorpromazine, chlorprothixene, clozapine, droperidol, fluphenazine, fluspirilene, haloperidol, levomepromazine, loxapine, melperone, mesoridazine, olanzapine, perazine, pericyazine, perphenazine, pimozide, pipamperone, prochlorperazine, promazine, promethazine, quetiapine, risperidone, sulpiride, thioridazine, trifluoperazine, triflupromazine, ziprasidone, zotepine, zuclopenthixol	haloperidol-d_4	LLE (trizma buffer, 1-chlororbutane)	Zorbax Eclipse XCB-C_{18} (4.6 x 150, 5µm)	gradient with ammonium formate and ACN	ESI, positive mode, SRM, MS/MS	selectivity, linearity, accuracy, precision, PS stability, LT stability, LLOQ, extraction efficiencies, matrix effects, process efficiencies, F/T stability

Author (Year)	Sample [mL]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Seno et al.[95] (1999)	1	flupentixol, perazine, prochlorperazine, trifluoperazine, thioproperazine, perphenazine, fluphenazine, propericiazine, thioridazine#	?	SPE	Capcell Pak C_{18} UG80, S-5µm, 1.0 x 250 mm (Shiseido)	gradient with 10mM ammonium acetate and ACN	ESI, positive mode, SRM, MS/MS (for Flupentixol)	linearity, recovery,
Verweij et al.[59] (1994)	1	chlorprothixene, flupentixol, thiothixene, zuclopenthixol	N/A	SPE (Bond Certify 3cc column (Varian)	HP 5µm Asahipak ODP-50, 4.0 x 125 mm	isocratic with ACN and 50 mM ammonium acetate in dH_2O (85:15)	ESI, comparison of fullscan and SRM	selectivity, linearity

b)

Author (Year)	Sample [mL]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Choong et al.[55] (2009)	0.5	aripiprazole, clozapine, olanzapine, sertindole, dehydroaripiprazole, norclozapine, dehydrosertindole#	remoxipride	SPE (mixed mode support)	Xbridge C_{18} column (2.1 mm x 100, 3.5µm)	gradient with ammonium acetate 20mM and ACN	ESI, positive mode, MS, SIM	selectivity, repeatability, precision, trueness, accuracy, matrix effects, F/T and LT stability, PS stability
Kollroser et al.[28] (2002)	0.05	clozapine, desmethylclozapine, olanzapine	dibenzepine	direct injection procedure, HPLC-integrated sample clean-up with Oasis® HLB extraction column (50 mm x 1.3, 5µm)	Symmetry C_{18} Waters (3.0 x 150 mm, 5µm)	isocratic with ACN/formic acid	ESI, positive mode, SRM, MS/MS	selectivity, linearity, recovery, LLOQ, accuracy, precision,
Kratzsch et al.[56] (2003)	0.5	amisulpride, bromperidol, clozapine, droperidol, flupentixol, fluphenazine, haloperidol, melperone, olanzapine, perazine, pimozide, risperidone, sulpiride, zotepine, zuclopenthixol, norclozapine, clozapine-N-oxide, 9OH risperidone	trimipramine-d3	SPE	Merck LiChroCART column (125 x 2mm)	gradient with 5 mM aqueous ammonium formate and ACN	APCI, positive mode, MS/MS, SRM	selectivity, linearity, accuracy, precision, F/T stability, LT stability, PS stability,

Author (Year)	Sample [mL]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Remane et al. [82] (2011)	0.5	9OH risperidone, amisulpride, aripiprazole, benperidol, bromperidol, chlorpromazine, clozapine, clozapine-N-oxide, droperidol, flupentixol, fluphenazine, fluspirilene, haloperidol, levomepromazine, melperone, norclozapine, perazine, perphenazine, pimozide, pipamperone, promazine, prothipendyl, quetiapine, risperidone, sulpiride, thioridazine, ziprasidone, zotepine#	citalopram-d_6, norclozapine-d_8, nordazepam-d_5, trimipramine-d3, zolpidem-d_6	LLE (butyl acetate/ ethyl acetate)	TF Hypersil GOLD Phenyl column (100 x 2.1 mm, 1.9µm)	Gradient with 10 mM aqueous ammonium formate plus 0.1% formic acid (pH=3.4) and ACN plus 0.1% formic acid	APCI, positive mode, MS/MS, SRM	selectivity, linearity, accuracy, precision, ion suppression/enhancement of co-eluting analytes, PS stability, LT stability, LLOQ, extraction efficiencies, matrix effects, process efficiencies, "crosstalk", F/T stability
Zhou et al.[51] (2004)	0.5	clozapine, olanzapine, risperidone, quetiapine	diazepam	LLE (ether)	Macherey-Nagel C18 (2 mm x 125 mm, 3µm)	isocratic with dH_20 (formic acid: 2.7mmol/l, ammonium acetate: 10mmol	ESI, SRM,	accuracy, precision, LT stability, F/T stability

c)

Author (Year)	Sample [mL]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Gutteck et al.[48]	1	flupentixol, fluphenazine, pipamperone, thioridazine, zuclopenthixol	imipramine-d3, doxepine-d3, chlorohaloperidol	LLE (n-hexane/dichloromethane 4:1) or dichloromethane	Silice Uptisphere column RP C_{18} (12.5cm x 2mm, 5µm)	isocratic with four different combinations of 50mM acetate buffer and ACN	ESI, positive mode, MS, SIM	linearity, selectivity, precision, accuracy, recovery, LLOQ

Author (Year)	Sample [mL]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Hasselstrom et al.[27]	0.06	clozapine, quetiapine, ziprasidone	clozapine-d3, quetiapine-d8, ziprasidone-d8	Zinc sulphate, MeOH, 96-well plate	Zorbax SB-C_8 (2.0 x 50mm, 1.8µm)	gradient with formic acid in dH_2O and formic acid in MeOH	ESI, positive mode, MRM MS/MS,	selectivity, recovery, matrix effects, LLOQ, precision, trueness, LT stability
Kirchherr et al.[61]	0.1	amisulpride, aripiprazole, benperidol, chlorpromazine, chlorprothixene, olanzapine, flupentixol, fluphenazine, haloperidol, 9OH risperidone, levomepromazine, olanzapine, perazine, perphenazine, pimozide, pipamperone, quetiapine, risperidone, sulpiride, thioridazine, ziprasidone, zotepine, zuclopenthixol	clonidine, methylrisperidone, MBHZ	PP (ACN:MeOH)	Chromolith Speed ROD C_{18} (50 mm x 4.6 mm, 5µm)	gradient with MeOH and acetic acid	ESI, positive mode, MRM, MS/MS	linearity, accuracy, precision, LLOQ, recovery, matrix effects
Niederlaender et al.[57]	N/A	clozapine, desmethylclozapine, clozapine-N-oxide	Mirtazapine	SPE (online)	Zorbax Eclipse XDB-C_{18} (4.6 x 150 mm, 5µm)	isocratic with MeOH-aqueous ammonium acetate buffer (25mM)	ESI, positive mode, MS, SIM	linearity, recovery, accuracy, precision. LLOQ
Rittner et al.[58]	1	clozapine, haloperidol, levomepromazine, perazine, pimozide, sulpiride#	flunitrazepam-d3	SPE	Symmetry WAT C_{18} (1.0 x 150 mm, 3.5µm)	gradient with ACN, dH_2O , MeOH	ESI, positive mode, MS fullscan mode (m/z = 100-650)	N/A

Abbreviations: ACN: acetonitrile, APCI: atmospheric pressure chemical ionization, dH_2O: deionized water, ESI: electrospray ionization, F/T: freeze/thaw, LLOQ: lower limit of quantification, LOD: limit of detection, LT: long term, m/z: mass over charge ratio, MeOH: methanol, MRM: multiple reaction monitoring, MS: single stage mass spectrometry, MS/MS: tandem mass spectrometry, PP: protein precipitation, PS: processed sample, SIM: single ion monitoring, SPE: solid phase ectraction, SPME: solid-phase micro-extraction

#: more drugs are included in this method but do not belong to the group of APs
##: this method was applied to more than one matrix
◊: post-mortem specimens were analysed in this method
?: an IS appears to have been used but is not specified

Table 4: Summary of methods for the detection of APs in hair

Author (Year)	Sample [g]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Josefsson et al.[16] ## (2003)	0.01-0.02	buspirone, chlorpromazine, chlorprothixene, clozapine, dixyrazine, flupentixol, fluphenazine, haloperidol, hydroxyzine, levomepromazine, melperone, olanzapine, perphenazine, pimozide, prochlorperazine, risperidone, thioridazine, ziprasidone, zuclopenthixol#	N/A	Incubation for 15min in 1 M NaOH, 25mM trizma buffer, extraction with BuCl, back extraction into formic acid	Zorbax Stable Bond Cyano column (50 x 2.1 mm, 3.5µm)	Gradient with MeOH-ACN-20mM ammonium formate and MeOH-ACN-20mM ammonium formate	ESI, positive mode, MS/MS, SRM	N/A
McClean et al.[18] (2000)	0.5	chlorpromazine, flupentixol, trifluoperazine, risperidone	trimipramine	MeOH, NaOH, 4M hydrochloric acid, final extraction with hexane	Phenomenex Luna C_{18} (150 x 4.6 mm)	Isocratic with 0.02 mol/L ammonium acetate/0.1 % acetic acid in dH_2O and ACN	ESI, positive mode, MS/MS, SRM	linearity, LOD, recovery
Mueller et al.[19] (2000)	0.05	pipamperone#	doxepine-d_3	MeOH, SPE (mixed mode)	RP-C_8-select B (2 mm x 125 mm, 5µm)	Gradient with ACN 25% aqueous ammonia and formic acid	ESI/CID-MS, ProdI scan, positive mode, MS/MS, SRM	N/A
Nielsen et al.[20] (2010)	0.01	chlorprothixene, clozapine, levomepromazine, promethazine, quetiapine#	mianserin- d_3	Incubation with MeOH:ACN:ammonium formate (2 mM, 8 % ACN, pH = 5.3) at 37 ºC for 18 h, Mini-Uniprep vials (PTFE filter)	Waters 100 mm x 2.1 mm ACQUITY HSS T3 1.8µm C_{18}	Gradient with 0.05 % formic acid and MeOH	ESI, positive mode, TOF-MS	LOD, LLOQ, matrix effects, selectivity, carry-over, linearity, trueness, precision
Thieme et al.[21] (2007)	0.05 (divided into single hairs for segmentation)	clozapine, norclozapine	5-(4-methylphenyl)-5-phenyl hydantoine	Decontamination with 5 mL petroleum benzene, Ultrasonication with 3 mL MeOH for 3 h, reduce to single hairs, segmentation, 3 h ultrasonication in 30 uL dH_2O /MeOH (50/50)	Synergy Polar-RP (Phenomenex, 75mm x 2.0mm, 4µm)	Isocratic with ammonium acetate buffer in (50:50) water and ACN	ESI, ProdI, MS/MS, SRM	N/A
Weinmann et al.[22] (2002)	0.02-0.05	clozapine, norclozapine, haloperidol, penfluridol, thioridazine, northioridazine, flupentixol, zuclopenthixol, de-(hydroxyethyl)-zuclopenthixol	doxepine-d_3	Ultrasonication with 4 mL MeOH for 2 h, SPE (mixed mode)	RP-C_8-select B (2 mm x 125 mm, 5µm)	Gradient with 1 mM ammonium formate/0.1 % formic acid, and ACN/0.1 % formic acid	ESI, ProdI, MS/MS, SRM	linearity, LOD, LLOQ, recovery, precision

Abbreviations: ACN: acetonitrile, BuCL: 1-chlorobutane, dH_2O: deionized water, CID: collision induced dissociation ESI: electrospray ionization, LLOQ: lower limit of quantification, LOD: limit of detection, MeOH: methanol, SRM: selected reaction monitoring, MS: single stage mass spectrometry, MS/MS: tandem mass spectrometry, NaOH: sodium hydroxide, ProdI: Product Ion Scan, SPE: solid phase extraction, TOF: time of flight

#: more drugs are included in this method but do not belong to the group of APs
##: this method was applied to more than one matrix

Table 5: Summary of methods for the detection of APs in CSF, saliva, and urine using LC-MS/MS

Author (Year)	Matrix	Sample [g]	Drugs	IS	Extraction	Stationary Phase	Mobile Phase	Detection mode	Validation data
Arinobu et al.[14] ## (2002)	urine	1	haloperidol, reduced haloperidol, 4-(4-chlorophenyl)-4-hydroxypiperidine	4-[4-(4-chlorophenyl) -4-hydroxy-1-piperidinyl]-(4-chlorophenyl-1-butanone	addition of 3mL of dH_2O with 0.09 % formic acid and 20mM ammonium acetate, freezing, thawing, centrifugation, injection of 20 µL of supernatant	Mspak GF-310 4B (50 x 4.6mm)	gradient with formic acid and 20mM ammonium acetate in dH_2O (A) and ACN (B)	SSI, positive mode, MS	LOD, precision, accuracy
De Meulder et al.[15] ## (2008)	urine	0.2	risperidone, 9OH risperidone	$^{2}H_2$-$^{13}C_2$-risperidone and $^{2}H_2$-$^{13}C_2$-9OH-risperidone	SPE (mixed-mode)	Chiralcel OJ column (50 mm x 4.6, 10µm)	gradient with hexane, 0.01mM ammonium acetate in isopropanol, 0.01mM ammonium acetate in ethanol	ESI, positive mode, SRM, MS/MS	selectivity, precision, accuracy, recovery, F/T and LT stability, PS stability
Flarakos et al.[24] ## (2004)	saliva	0.025	risperidone, 9OH risperidone	R068808	online cleanup, column switching	Zorbax SB_{18} (30 x 2.1mm, 3.5µm)	isocratic with 10mM ammonium acetate/ACN	N/A	linearity, selectivity, precision, accuracy, recovery, matrix effects, F/T and LT stability
Kumazawa et al.[17] ## (2000)	urine	1	perazine, thioridazine, prochlorperazine, perphenazine, trifluoperazine, flupentixol, fluphenazine, thioproperazine#	propericiazine	SPME (polyacrylate-coated fiber)	Capcell Pak C_{18} UG120, S-5µm, 2.0 x 150 (Shiseido)	gradient with 10 mM ammonium acetate and ACN	ESI, full scan m/z 50-500, SRM, MS/MS	linearity, precision, accuracy,

Author (Year)	Matrix	Sample [g]	Drugs	IS	Extraction	Stationary Phase	Mobile Phase	Detection mode	Validation data
Josefsson et al.[25] ## (2010)	CSF	0.2	olanzapine, N-desmethylolanzapine	olanzapine-d_3	LLE (tert-butyl-methyl-ether)	Synergi Hydro-RP (50mm x 2mm, 2.5µm)	gradient with 10mM ammonium formate with formic acid and MeOH with formic acid	ESI, positive mode, SRM, MS/MS	linearity, LLOQ, precision, accuracy, recovery, matrix effects, F/T stability, LT stability
Bogusz et al.[76] ## (1999)	urine	1	olanzapine	LY170222	SPE	Super Spher RP $_{18}$ (125 x 3 mm; 4µm) (Merck)	isocratic with ACN/ammonium formate, OLZ metabolites with Gradient	APCI, positive mode, MS	recovery, LLOQ, precision, linearity, selectivity, F/T and LT stability
Josefsson et al. [16]_ENREF_17 ## (2003)	urine	0.5	buspirone, chlorpromazine, chlorprothixene, clozapine, dixyrazine, flupentixol, fluphenazine, haloperidol, hydroxyzine, levomepromazine, melperone, olanzapine, perphenazine, pimozide, prochlorperazine, risperidone, thioridazine, ziprasidone, zuclopenthixol	N/A	SPE	Zorbax Stable Bond Cyano column (50 x 2.1mm, 3.5µm)	Gradient with different ratios of MeOH:ACN:20mM ammonium formiate	ESI, positive mode, SRM, MS/MS	N/A

Legend: ACN: acetonitrile, APCI: atmospheric pressure chemical ionization, dH_2O: deionized water, ESI: electrospray ionization, F/T: freeze/thaw, LLOQ: lower limit of quantification, LOD: limit of detection, LT: long term, m/z: mass over charge ratio, MeOH: methanol, SRM: selected reaction monitoring, MS: single stage mass spectrometry, MS/MS: tandem mass spectrometry, PS: processed sample, SIM: single ion monitoring, SPE: solid phase extraction, SPME: solid-phase micro-extraction, SSI: sonic spray ionization

#: more drugs are included in this method but do not belong to the group of APs
##: this method was applied to more than one matrix

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Chapter 2.2

Identification and Quantification of 30 Antipsychotics in Blood using LC-MS/MS

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J Mass Spectrom, 2010. **45**(8): p. 915-25

Monash University

Declaration for Thesis Chapter 2.2

Declaration by candidate

In the case of Chapter 2.2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conducted literature review, performed method development and validation studies. Wrote article	85 %

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D	Advised candidate regarding direction and content, reviewed and edited article	
Drummer, O. H	Advised candidate regarding direction and content, reviewed and edited article	
Beyer, J.	Advised candidate regarding direction and content, reviewed and edited article	

Candidate's Signature [redacted] **Date** 27/07/2012

Declaration by co-authors

The undersigned hereby certify that:

(7) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(8) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(9) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(10) there are no other authors of the publication according to these criteria;
(11) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(12) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) **Victorian Institute of Forensic Medicine**

Signature 1 [redacted] **Date** 27/7/2012
Signature 2 27/7/2012
Signature 3 27/7/2012

ABSTRACT

Over the last decade the prescription rates of antipsychotic drugs (APs) have increased worldwide. Studies have shown that the risk of sudden cardiac death is 3 fold higher among patients treated with APs.

In order to investigate the presence of APs in post mortem cases, a LC-MS/MS method was developed using only 100 µL of blood with 10 µL of the internal standard (haloperidol-d_4, 1 µg/mL). After the addition of 200 µL of Trizma buffer, the blood was extracted using LLE with 1 mL of 1-chlorbutane for 5 min on a shaker at 1500 rpm. After centrifugation at 12000 rpm for 1 min, the separated solvent layer was transferred to an autosampler vial and evaporated to dryness under N_2. The residue was reconstituted in 50 µL acetonitrile containing 0.1 % formic acid, vortexed for 30 sec and an additional 450 µL 50 mmol/L ammonium formate pH = 3.5 was added and the sample vortexed. 100 µL of the final extract was injected into a Shimadzu Prominence HPLC system, with detection of drugs achieved using an Applied Biosystems 3200 Q-TRAP® LC-MS/MS system equipped with a Turbo V ion source (ESI, MRM mode).

The method has been fully validated according to international guidelines and was found to be selective for all tested compounds. Calibration was satisfactory for all drugs except olanzapine from sub-therapeutic to toxic concentrations The LLOQs corresponded to the lowest concentrations used for the calibration curves. With the exception of olanzapine, accuracy data were within the acceptance interval of ±15 % (±20 % at the LLOQ) of the nominal values for all drugs.

The method has been proven to be useful for the routine analysis of antipsychotics in post-mortem blood samples.

Keywords: antipsychotics, quantification, detection, LC-MS/MS, blood

Introduction

Antipsychotic drugs (APs) are widely prescribed for the treatment of schizophrenia and psychosis. The so called "first generation" or "typical" APs were developed in the 1950s and show severe side-effects such as extrapyramidal symptoms due to their pharmacological action on d_2 and d_4 receptors. As a measure of reducing these severe side effects, a range of "second generation" APs were developed. These newer generation drugs act considerably less on d_2 and d_4 receptors and therefore exhibit less extrapyramidal side-effects.

Over the last decade the prescription rate of APs (more notably second generation) has increased worldwide [1-3], especially among young adults and children [1-4]. However, second generation APs are not entirely free of side-effects. Irrespective of their generation, it has been shown that these drugs can increase the risk of sudden cardiac death with studies showing that the risk of sudden cardiac death is increased 3-fold among patients treated with APs [5-7]. There are several published multi-analyte procedures for the detection of APs in human blood [8-15]. Methods using high performance liquid chromatography coupled with UV detection (HPLC-UV) [8-10] and gas chromatography coupled with nitrogen phosphorous detection (GC-NPD) [11] show that these techniques do not provide the required sensitivity or selectivity for the detection of low dose APs in post-mortem blood. Currently, the use of liquid chromatography coupled with mass spectrometry (LC-MS) has replaced some of the more traditional GC-MS assays showing superior selectivity and sensitivity [12-15]. Validated methods have been published by Kirchherr *et al.* [12] and Kratzsch *et al.* [13] covering a wide range of APs, however neither method is suitable for post-mortem blood which is subject to decomposition and change in matrix effects. In addition, the method published by Kirchherr *et al.* [12] used only one transition for

compound identification despite contrary international recommendations. Roman *et al.* [15] described the detection of seven low dose APs in post-mortem blood, however, this study did not allow the simultaneous detection of other common APs. Although a study published by Josefsson *et al.* [14] covers the 19 most common APs in post-mortem samples, this method was not validated.

Our unit is monitoring the presence of APs in forensic cases and we are not only interested in the detection but also the relative safety of these drugs either alone and/or in combination with other therapeutic agents. Therefore, a reliable, sensitive and validated quantitation method for a range of APs in blood samples has been developed. This paper describes the detection and validated quantification of 30 APs in post-mortem blood samples. The method has been validated according to internationally accepted criteria and guidelines [16, 17].

Materials and methods

Chemicals and reagents

Bromperidol, chlorpromazine, fluspirilene, haloperidol, pipamperone, prochlorperazine, thioridazine, trifluoperazine, triflupromazine and Trizma base were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Buspirone, chlorprothixene, mesoridazine, olanzapine, promazine, promethazine, risperidone, and zuclopenthixol were obtained from the Division of Analytical Laboratories, (Lidcombe, NSW, Australia). Droperidol, fluphenazine, loxapine, pericyazine, perphenazine, pimozide, and sulpiride were provided by Australian Government Analytical Laboratories (Pymble, NSW, Australia). Levomepromazine, melperone, perazine and zotepine was obtained from Phast GmbH (Homburg/Saar, Germany). Amisulpride, aripiprazole, quetiapine, and ziprasidone were

purchased from National Institute of Forensic Science (Melbourne, VIC, Australia). Clozapine was provided by Sandoz (Pyrmont, NSW, Australia); 9-OH-Risperidone was obtained from Janssen–Cilag (North Ryde, NSW, Australia). The isotope-labeled internal standard haloperidol-d_4 was purchased from Cerilliant (Round Rock, TX, USA). Acetonitrile, ammonium formate, 1-chlorobutane, methanol, and formic acid were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from BDH Chemicals (Kilsyth, VIC, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia).

Trizma buffer (pH 9.2) was prepared by dissolving 242 g Trizma base in 1 l water.

Specimens

Blood for calibration purposes was obtained from drug-free volunteers. Samples were collected into spray-coated K_2EDTA preserved plastic tubes (BD Australia, North Ryde, NSW). Post-mortem blood samples were submitted to the authors' laboratory for routine toxicological analysis. The post-mortem blood samples were regarded as drug-free if none of the existing tests showed the presence of the studied drugs in any specimen (including blood, liver, and urine). All post-mortem blood samples were collected into plastic tubes containing 1 % fluoride–oxalate. This is the standard collection tube in the laboratory. All blood samples were stored at −20 °C prior to analysis.

Apparatus

The LC-MS/MS system consisted of an Applied Biosystems 3200 Q-TRAP® linear ion-trap quadrupole mass spectrometer (Applied Biosystems, Melbourne, VIC, Australia) equipped

with a Turbo V ion source, operated in the electron spray ionization (ESI) mode, and an Agilent Technologies (AT) 1200 Series HPLC system (Agilent, Melbourne, VIC, Australia) which consisted of a degasser, a binary pump, and an autosampler.

HPLC conditions

Gradient elution was performed on an Agilent Zorbax Eclipse XDB-C_{18} (4.6 mm × 150 mm, 5 μm particle size; Biolab, Scoresby, VIC, Australia). The mobile phase consisted of 50 mmoL/L aqueous ammonium formate adjusted to pH = 3.5 with formic acid (eluent A) and acetonitrile containing 0.1 % formic acid (eluent B). During use, the mobile phase was degassed by the integrated Agilent 1200 degasser. Before starting the analysis, the HPLC system was equilibrated for 10 min with a mixture of 90 % eluent A and 10 % eluent B. The HPLC system was additionally equilibrated for 4 min prior to each run. The flow rate and gradient were programmed as follows: equilibration time (−4.00 min – 0.00 min) 10 % eluent B, flow rate 1.4 mL/min; 0.00 – 1.00 min: 10 % eluent B, flow rate 1.4 mL/min; 1.01–18.00 min: gradient increase to 100 % eluent B, flow rate increase to 2.2 mL/min; 18.01 – 20.00 min: 100 % eluent B, flow rate 2.2 mL/min.

The column oven was set at 60 °C. The autosampler was operated at room temperature; the autosampler needle was rinsed using a wash vial filled with a mixture of eluent A and eluent B (90 : 10).

MS/MS conditions

For detection and quantification, the following ESI inlet conditions were applied: gas 1, nitrogen (90 psi; 620.5 kPa); gas 2, nitrogen (90 psi; 620.5 kPa); ion-spray voltage, 5500 V;

ion-source temperature, 750 °C; curtain gas, nitrogen (10 psi; 68.9 kPa). The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with the collision gas was set at medium. The dwell times were optimized using scheduled MRM algorithm incorporated in Analyst® software 1.5. The MRM detection window was set at 60 sec, the target scan time was 1.5 sec. All other settings were analyte-specific and were determined using Analyst® software in the quantitative optimization mode (Table 1). The most abundant MRM transition for each analyte was considered as quantifier ion.

Preparation of stock solutions, calibration standards, and control samples

Stock solutions of amisulpride, bromperidol, buspirone, chlorpromazine, chlorprothixene, fluphenazine, fluspirilene, haloperidol, levomepromazine, loxapine, melperone, mesoridazine, perazine, pericyazine, perphenazine, pipamperone, promethazine, quetiapine, sulpiride, thioridazine, trifluperazine, ziprasidone, zotepine, and zuclopenthixol were prepared at a concentration of 1 mg/mL by separate weighings using methanol. Stock solutions of 1 mg/mL of 9-OH-risperidone, aripiprazole, clozapine, droperidol, olanzapine, pimozide, prochlorperazine, promazine, risperidone, and triflupromazine were prepared using eluent B. The preparation of stock solutions in acetonitrile with formic acid was necessary due to the lack of solubility of these drugs in methanol.

Table 1: Analytes, multiple reaction monitoring (MRM) transitions and parameter settings including declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell energy (CE) and collision cell exit potential (CXP) used in LC-ESI-MS/MS.

ID	Q1 Mass [Da]	Q3 Mass [Da]	Retention Time [min]	DP [V]	EP [V]	CEP [V]	CE [V]	CXP [V]
9-OH Risperidone (Quant)	427.0	207.2	6.6	61	4.5	18	39	4
9-OH Risperidone (Qual)		110.2		61	4.5	18	39	4
9-OH Risperidone (Qual)		69.1		61	4.5	18	39	4
Amisulpride (Quant)	370.1	242.2	5.0	61	8	32	41	4
Amisulpride (Qual)		195.9		61	8	32	55	4
Amisulpride (Qual)		112.1		61	8	32	39	4
Aripiprazole (Quant)	448.0	285.2	8.9	71	9.5	20	33	4
Aripiprazole (Qual)		176.1		71	9.5	20	43	4
Aripiprazole (Qual)		98.2		71	9.5	20	51	4
Bromperidol (Quant)	422.0	123.1	8.5	1	12	50	59	4
Bromperidol (Qual)		165.1		1	12	50	37	4
Bromperidol (Qual)		95		1	12	50	103	4
Buspirone (Quant)	386.1	122.2	7.2	71	10	32	43	4
Buspirone (Qual)		79		71	10	32	105	4
Buspirone (Qual)		95.2		71	10	32	75	4
Chlorpromazine (Quant)	319.1	86.1	9.5	46	5	14	31	4
Chlorpromazine (Qual)		58.2		46	5	14	55	4
Chlorpromazine (Qual)		246.1		46	5	14	33	4
Chlorprothixene (Quant)	316.0	271.1	9.6	51	3.5	18	23	4
Chlorprothixene (Qual)		231		51	3.5	18	39	4
Chlorprothixene (Qual)		221.2		51	3.5	18	49	4
Clozapine (Quant)	327.1	270.2	7.8	51	4.5	30	29	4
Clozapine (Qual)		192.2		51	4.5	30	59	4
Clozapine (Qual)		164.1		51	4.5	30	95	4
Droperidol (Quant)	380.1	123.1	7.3	41	5.5	16	63	4
Droperidol (Qual)		194.2		41	5.5	16	21	4
Droperidol (Qual)		165.1		41	5.5	16	39	4
Fluphenazine (Quant)	438.1	171	10.1	61	6.5	60	25	4
Fluphenazine (Qual)		100		61	6.5	60	63	4
Fluphenazine (Qual)		143.1		61	6.5	60	57	4
Fluspirilene (Quant)	476.1	98.2	10.4	61	7.5	24	47	4
Fluspirilene (Qual)		371.3		61	7.5	24	25	6
Fluspirilene		55.1		61	7.5	24	85	4
Haloperidol (Quant)	376.0	123.1	8.3	56	4.5	26	57	4
Haloperidol (Qual)		165.2		56	4.5	26	35	4
Haloperidol (Qual)		95		56	4.5	26	93	4
Levomepromazine (Quant)	329.1	58.1	9.1	41	6	34	59	4
Levomepromazine (Qual)		100.2		41	6	34	25	4
Levomepromazine (Qual)		242.1		41	6	34	29	4
Loxapine (Quant)	328.1	271.1	8.5	41	3.5	30	33	50
Loxapine (Qual)		84.2		41	3.5	30	33	4

ID	Q1 Mass [Da]	Q3 Mass [Da]	Retention Time [min]	DP [V]	EP [V]	CEP [V]	CE [V]	CXP [V]
Loxapine (Qual)		164		41	3.5	30	85	4
Melperone (Quant)	264.0	123.1	6.9	116	4	42	43	4
Melperone (Qual)		165.2		116	4	42	19	4
Melperone (Qual)		95.3		116	4	42	63	4
Mesoridazine (Quant)	387.7	98.2	7.6	51	8	56	51	4
Mesoridazine (Qual)		126.1		51	8	56	35	4
Mesoridazine (Qual)		70		51	8	56	87	4
Olanzapine (Quant)	313.1	256.1	4.9	56	4.5	14	31	4
Olanzapine (Qual)		198.1		56	4.5	14	53	4
Olanzapine (Qual)		84.2		56	4.5	14	33	4
Perazine (Quant)	340.0	141.2	9.0	56	9	32	27	4
Perazine (Qual)		113.1		56	9	32	39	4
Perazine (Qual)		70		56	9	32	57	4
Pericyazine (Quant)	365.8	114.2	8.2	56	5.5	34	43	4
Pericyazine (Qual)		142.1		56	5.5	34	33	8
Pericyazine (Qual)		44.1		56	5.5	34	77	4
Perphenazine (Quant)	404.0	171.1	9.6	56	10.5	18	31	4
Perphenazine (Qual)		143.2		56	10.5	18	39	4
Perphenazine (Qual)		100.2		56	10.5	18	57	4
Pimozide (Quant)	462.1	109.1		396	10.5	56	71	4
Pimozide (Qual)		328.3		396	10.5	56	33	4
Pimozide (Qual)		147.1		396	10.5	56	55	4
Pipamperone (Quant)	376.2	123.2	6.2	51	12	16	65	4
Pipamperone (Qual)		165.2		51	12	16	37	4
Pipamperone (Qual)		98.2		51	12	16	39	4
Prochlorperazine (Quant)	374.1	141.4	10.1	46	7.5	40	27	4
Prochlorperazine (Qual)		113.1		46	7.5	40	35	4
Prochlorperazine (Qual)		70.2		46	7.5	40	63	4
Promazine (Quant)	285.1	86.2	8.4	46	4.5	34	27	4
Promazine (Qual)		58.1		46	4.5	34	53	4
Promazine (Qual)		180.1		46	4.5	34	51	4
Promethazine (Quant)	285.1	86.1	8.5	36	4.5	32	27	4
Promethazine (Qual)		198.1		36	4.5	32	35	4
Promethazine (Qual)		71.2		36	4.5	32	57	4
Quetiapine (Quant)	384.1	253.2	7.9	61	5	18	29	4
Quetiapine (Qual)		221.3		61	5	18	53	4
Quetiapine (Qual)		279.2		61	5	18	53	4
Risperidone (Quant)	411.1	191.2	7.1	56	9	18	41	4
Risperidone (Qual)		110.2		56	9	18	69	4
Risperidone (Qual)		82.2		56	9	18	81	4
Sulpiride (Quant)	342.0	112.2	3.0	66	4.5	40	37	4
Sulpiride (Qual)		214.1		66	4.5	40	45	4
Sulpiride (Qual)		84.1		66	4.5	40	57	4
Thioridazine (Quant)	371.1	126.2	10.2	51	8.5	16	33	4

ID	Q1 Mass [Da]	Q3 Mass [Da]	Retention Time [min]	DP [V]	EP [V]	CEP [V]	CE [V]	CXP [V]
Thioridazine (Qual)		98.3		51	8.5	16	47	4
Thioridazine (Qual)		70		51	8.5	16	87	4
Trifluoperazine (Quant)		70		61	5	34	67	4
Trifluoperazine (Qual)	408.0	113.2	10.6	61	5	34	39	4
Trifluoperazine (Qual)		141.3		61	5	34	31	4
Triflupromazine (Quant)		58.1		56	1	12	55	4
Triflupromazine (Qual)	353.0	86.3	10.0	56	1	12	33	4
Triflupromazine (Qual)		280.2		56	1	12	31	4
Ziprasidone (Quant)		194		66	8.5	22	41	4
Ziprasidone (Qual)	413.0	130	7.7	66	8.5	22	91	4
Ziprasidone (Qual)		159.2		66	8.5	22	55	4
Zotepine (Quant)		72.1		16	5.5	14	39	4
Zotepine (Qual)	332.1	42.2	9.7	16	5.5	14	109	6
Zotepine (Qual)		72.6		16	5.5	14	31	58
Zuclopenthixol (Quant)		231.2		66	4.5	38	55	4
Zuclopenthixol (Qual)	401.0	221.1	9.7	66	4.5	38	69	4
Zuclopenthixol (Qual)		271		66	4.5	38	37	4
Haloperidol-d_4 (Quant)	380.1	169.2	8.3	41	5	18	33	4
Haloperidol-d_4 (Qual)		127.1		41	5	18	57	4

Working solutions of each analyte were prepared using methanol by independent dilution from each stock solution at the following concentrations: 0.1 mg/mL, 0.01 mg/mL, and 0.001 mg/mL. All solutions were stored at -60 °C.

The calibration standards were prepared using pooled blank blood and spiking solutions prepared from the working solutions as mixtures of the 34 APs in at concentrations 10 times higher than the corresponding calibration standards. The quality control samples were prepared using pooled blank blood and independently prepared mixtures of the 34 APs at concentrations 100 times higher than the concentrations of the corresponding quality control samples and stored at -60 °C.

The final blood concentrations of the calibration standards and quality control sample are given in Table 2.

Extraction procedure

In a 2 mL Eppendorf tube (Eppendorf Australia, North Ryde, NSW), 0.1 mL blood was mixed with 10 µL of the internal standard (IS) haloperidol-d_4 at a concentration of 1 µg/mL. To the blood, 0.2 mL of Trizma buffer and 1 mL of 1-chlorobutane were added and mixed thoroughly. The sample was extracted for 5 min on a VXR basic IKA® Vibrax shaker at 1500 rpm. After a brief centrifugation to separate layers, the solvent layer was transferred to an autosampler vial and evaporated to dryness using a Ratek dry block heater DBH10 operated at room temperature.

The residue was reconstituted in 50 µL of eluent B, and diluted with 450 µL of eluent A. 0.1 mL of the final extract were injected into the LC-MS/MS system.

Table 2: Concentrations of calibration standards and quality control samples of all studied analytes as well as respective described therapeutic blood concentrations. All concentrations are given in µg/L.

Drug	S*1	S2	S3	S4	S5	S6	S7	QC LOW	QC MED	QC HIGH	THERAPEUTIC LEVEL [µg/L]
Bromperidol	0.1	0.5	7.5	15	22.5	30	40	0.3	20	35	1 – 20 [13]
Buspirone	0.1	0.5	7.5	15	22.5	30	40	0.3	20	35	1 – 10 [18]
Perphenazine	0.1	0.5	7.5	15	22.5	30	40	0.3	20	35	0.6 – 2.4 [12]
Droperidol	1	5	20	40	60	80	100	3	50	90	5 – 50 [13]
Fluphenazine	1	5	20	40	60	80	100	3	50	90	2 – 20 [13]
Fluspirilene	1	5	20	40	60	80	100	3	50	90	N/A[#]
Haloperidol	1	5	20	40	60	80	100	3	50	90	5 – 50 [13]
Levomepromazine	1	5	20	40	60	80	100	3	50	90	15 – 60 [12]
Pericyazine	1	5	20	40	60	80	100	3	50	90	5 – 60 [18]
Pimozide	1	5	20	40	60	80	100	3	50	90	15 – 20 [12]
Trifluoperazine	1	5	20	40	60	80	100	3	50	90	1 – 50 [18]
9-OH-Risperidone	1	5	40	80	120	150	200	3	100	175	10 – 100 [13]
Loxapine	1	5	40	80	120	150	200	3	100	175	10 – 100 [12]
Olanzapine	1	5	40	80	120	150	200	3	100	175	10 – 100 [13]
Risperidone	1	5	40	80	120	150	200	3	100	175	10 – 100 [13]
Zuclopenthixol	1	5	40	80	120	150	200	3	100	175	5 – 100 [13]
Aripiprazole	10	50	150	250	350	450	600	30	300	500	50 – 350 [12]
Chlorpromazine	10	50	150	250	350	450	600	30	300	500	30 – 300 [12]
Chlorprothixene	10	50	150	250	350	450	600	30	300	500	20 – 200 [12]
Perazine	10	50	150	250	350	450	600	30	300	500	100 – 230 [12]
Quetiapine	10	50	150	250	350	450	600	30	300	500	70 – 170 [12]
Triflupromazine	10	50	150	250	350	450	600	30	300	500	30 – 100 [18]
Ziprasidone	10	50	150	250	350	450	600	30	300	500	50 – 120 [12]
Amisulpride	10	50	200	350	500	650	800	30	400	700	50 – 400 [13]
Melperone	10	50	200	350	500	650	800	30	400	700	50 – 400 [13]
Pipamperone	10	50	200	350	500	650	800	30	400	700	100 – 400 [18]
Promethazine	10	50	200	350	500	650	800	30	400	700	50 – 400 [18]
Sulpiride	10	50	200	350	500	650	800	30	400	700	50 – 400 [13]

Drug	S*1	S2	S3	S4	S5	S6	S7	QC LOW	QC MED	QC HIGH	THERAPEUTIC LEVEL [µg/L]
Clozapine	10	50	250	500	800	1200	1600	30	650	1400	200 - 800 [13]
Mesoridazine	10	50	300	700	1100	1600	2000	30	900	1800	150 – 1000 [18]
Prochlorperazine	1	50	200	350	500	750	1000	3	450	850	10 – 500 [18]
Promazine	1	5	150	300	450	650	800	3	400	700	10 – 400 [18]
Thioridazine	10	600	1100	1800	2500	3200	4000	30	2150	3600	200 - 2000 [12]
Zotepine	1	5	150	300	400	500	600	3	350	550	5 – 300 [13]

*S – Standard, all concentrations in µg/l.

No therapeutic concentration available

Validation experiments

Selectivity

Selectivity experiments were carried using post-mortem and ante-mortem blood samples sent to the authors' laboratory for toxicological analysis. 10 post-mortem and 5 ante-mortem samples were extracted as described previously without the addition of IS. The samples were analysed to exclude any interference with endogenous peaks. Additionally, 2 zero samples (blank sample + IS) were analysed to check for absence of analyte ions in the respective peaks of the IS.

Linearity

Aliquots of blank blood samples were spiked at concentrations given in Table 2 and extracted as described previously to obtain calibration standards. The chosen concentrations ranged from half the lowest described therapeutic concentration of each AP to double the highest described therapeutic concentration.

Replicates ($n = 6$) at each of the 7 concentration levels were analysed. Daily calibration curves using the same concentrations (single measurements per level) were prepared with each batch of validation and authentic samples.

Accuracy and precision

Quality control (QC) samples "QC LOW", "QC MEDIUM', and "QC HIGH" were prepared at concentration described in Table 2. 2 samples of each QC concentration were measured over a period of eight consecutive days. Daily calibration curves were used to calculate the concentration of the QCs. Accuracy was calculated for each analyte as bias determined by

calculating the percent deviation of the mean of all calculated concentration values at a specific level from the respective nominal concentration. Precision data (given as relative standard deviations, RSD) for within-day (repeatability), and time-different intermediate precision (combination of within and between day effects) of the method were calculated according to Beyer *et al.*[19, 20] using one-way ANOVA with the grouping-variable 'day'. The acceptance intervals of within-day (repeatability) and intermediate precision were ≤ 15 % RSD (≤ 20 % RSD at "QC LOW") and ± 15 % for bias (± 20 % at "QC LOW") of the nominal values [21].

Table 3: Mean values and ranges of recoveries and matrix effects using different sources of blank blood (*n=5*) spiked at "QC LOW" and "QC HIGH" concentrations. Data sets where the range is more than ± 20 % difference of the mean value (not acceptable) are marked in bold and italics.

Drug	**RECOVERY**		**MATRIX EFFECTS**	
	LOW Mean [range]	**HIGH** Mean [range]	**LOW** Mean [range]	**HIGH** Mean [range]
9-OH-Risperidone	105 [102 - 108]	95 [82 - 103]	53 [46 - 62]	76 [69- 88]
Amisulpride	68 [57 - 82]	72 [66 - 77]	91 [77 - 97]	104 [93 - 117]
Aripiprazole	105 [101 - 112]	97 [87- 108]	90 [75 – 102]	107 [93 - 115]
Bromperidol	***96 [61 - 116]***	110 [100 – 124]	***245 [166 - 337]***	104 [92 - 113]
Buspirone	***120 [98 - 171]***	100 [89 - 110]	91 [65 - 115]	105 [89 - 124]
Chlorpromazine	91 [84- 97]	86 [80 – 97]	96 [81 - 104]	106 [89 - 118]
Chlorprothixene	100 [91 - 108]	93 [84 - 105]	100 [86 - 112]	108 [96- 112]
Clozapine	108 [99 - 115]	98 [91 – 109]	104 [84 - 115]	105 [93- 117]
Droperidol	101 [85.6 - 112]	94 [79 - 105]	93 [83 - 104]	108 [92 - 122]
Fluphenazine	100 [92 - 107]	94 [83 – 110]	98 [83 – 110]	98 [83 - 114]
Fluspirilene	98 [87 - 110]	85 [73 - 93]	92 [85 - 100]	102 [94 - 109]
Haloperidol	110 [90 - 127]	99 [91 – 110]	112 [105 - 119]	108 [90 - 116]
Levomepromazine	91 [76 - 99]	85 [78 - 97]	99 [82 - 111]	107 [94 - 120]
Loxapine	107 [95 - 123]	89 [87 – 92]	88 [81 - 97]	109 [95 - 116]
Melperone	104 [88 - 116]	90 [85 - 100]	108 [95 - 121]	***97 [66 - 117]***
Mesoridazine	101 [93 - 112]	98 [95 – 101]	93 [83 - 105]	102 [93 - 110]
Olanzapine	79 [64 - 91]	83 [70 - 92]	***237 [172 - 292]***	140 [122 - 157]
Perazine	104 [100 - 110]	92 [90 – 94]	101 [91 - 109]	106 [91 - 121]
Pericyazine	84 [81 - 86]	88 [81 - 93]	116 [100 - 135]	102 [92 - 115]
Perphenazine	***132 [91 - 199]***	89 [80 – 96]	79 [56 - 95]	102 [83 - 118]
Pimozide	93 [92- 93]	89 [83 - 96]	87 [72- 117]	105 [95 - 122]
Pipamperone	104 [95- 118]	94 [89 - 99]	85 [76 - 97]	100 [90 - 107]
Prochlorperazine	97 [90 - 103]	95 [89 - 101]	97 [86 - 106]	97 [87 - 108]
Promazine	93 [78 - 103]	93 [91 - 94]	101 [92 - 116]	106 [92 - 119]
Promethazine	98 [91 - 103]	93 [86 - 100]	100 [84 - 112]	105 [96 - 114]
Quetiapine	107 [102 - 114]	102 [88 - 111]	95 [82 - 105]	105 [88 - 122]

Drug	RECOVERY		MATRIX EFFECTS	
	LOW Mean [range]	**HIGH** Mean [range]	**LOW** Mean [range]	**HIGH** Mean [range]
Sulpiride	6 [5 - 7]	8 [7 - 8]	103 [91 - 119]	103 [90 - 118]
Thioridazine	96 [89 - 102]	93 [88 - 102]	96 [85 - 104]	100 [87 - 110]
Trifluoperazine	96 [89 - 102]	84 [82 - 86]	93 [92 - 96]	102 [88 - 120]
Triflupromazine	88 [85 - 93]	81 [78 - 86]	96. [82 - 110]	105 [94 - 120]
Ziprasidone	101 [95 - 106]	88 [81 - 97]	93 [87 - 98]	105 [97 - 115]
Zotepine	94 [90 - 99]	89 [80 - 98]	109 [107 - 111]	102 [88 - 111]
Zuclopenthixol	111 [93 - 126]	91 [90 - 92]	104 [101 - 108]	***95 [77 - 115]***

Processed sample stability

For estimation of stability of the processed samples under the conditions of LC-MS/MS analysis, "QC LOW" and "QC HIGH" samples (*n=8* each) were extracted as described previously. The resulting extracts at each concentration level were pooled. Aliquots of these pooled extracts at each concentration level were transferred to autosampler vials and injected into the LC-MS/MS system and analysed under conditions given previously. The time intervals between the analyses of the QC samples were extended to 2 hours by the injection of 5 blank samples. Stability of the extracted analytes was tested by regression analysis plotting absolute peak areas of each analyte at each concentration versus injection time. The instability of the processed samples was indicated by a negative slope, significantly different from zero ($p \leq 0.05$) [16].

Freeze/thaw and bench top stability

Combined freeze/thaw and bench top stability were evaluated by analysis of QC samples (6 replicates at each concentration) prior to (control samples) and after 4 freeze/thaw cycles

(stability samples). For each cycle, the samples were kept at -60 °C for 22.5 hours. The thawed samples were kept at room temperature for 1 hour prior to the next freeze cycle to incorporate bench top stability. The experiments were carried out together with the accuracy and precision experiments and the concentrations of the control and stability samples were calculated via daily calibration curves. Stability was tested against an acceptance interval of 90 – 110 % for the ratio of the means (stability samples vs control samples) and an acceptance interval of 80 – 120% from the control samples' mean for the 90 % confidence interval (CI) of stability samples [16].

Long-term stability

Experimental design for the study of long-term stability was similar to the freeze/thaw stability. Analyte stability for long-term storage was evaluated by analysis of QC samples (*n=6* at each concentration*)* before (control samples) and after storage for 6 weeks at -20 °C (stability samples). Stability was measured against an acceptance interval of 90 – 110 % for the ratio of the means (stability samples vs control samples) and an acceptance interval of 80 – 120 % from the control samples' mean for the 90 % CI of stability samples [16].

Lower limits of quantification

The LLOQ in the MRM mode was defined as the lowest point of the calibration curve (see Table 2 for concentrations) and fulfilled the requirement of LLOQ signal-to-noise ratio of 10 : 1 [16, 17]. The limit of detection (LOD) was not systematically evaluated.

Extraction efficiencies, matrix effects, and process efficiencies

Extraction efficiencies, matrix effects, and process efficiencies were estimated in a previously published study using 500 µL of blank blood for extraction [22]. The experiments were repeated under the conditions described previously using 0.1 mL of blank blood and compared with the previously published results.

Application to authentic samples

Applicability experiments were carried using post-mortem blood samples sent to the authors' laboratory for toxicological analysis. A total of 183 samples have been analysed using the described method.

Results and discussion

Extraction procedure

In a previous study [22] different extraction procedures for APs were compared in terms of the extraction efficiencies and matrix effects. Based on the results of this study, a liquid-liquid extraction procedure (LLE) using Trizma buffer and 1-chlorobutane was chosen. Although this extraction procedure showed considerably lower extraction efficiencies for sulpiride, the method gave overall the best results in terms of extraction efficiencies and matrix effects.

Table 3 shows mean values of recoveries and matrix effects including ranges for this method. Data sets where the range is greater than ±20 % difference of the mean value (not acceptable) are marked bold. The extraction recoveries of most analytes (other than sulpiride and to a lesser extent amisulpride) exceed 80 % which is acceptable for this method. Other than

melperone and zuclopenthixol at high concentrations, as well as bromperidol and olanzapine at low concentration, the variations of matrix effects over 5 different blood samples were acceptable. Overall, this method has shown to have less matrix effects than the previously published study [22] most likely due to lower blood sample volume utilised for analysis.

In toxicological analysis, a lack of sample volume provided may reduce the possible number of tests able to be conducted. In this study, a small sample volume of 0.1 mL provided the required sensitivity. As lowest calibrator, at least half the lowest described therapeutic concentration was able to be detected.

Preliminary experiments showed that the reconstitution of samples was critical for the performance of the assay. The composition of the reconstitution solvent usually matches the start conditions of the mobile phase in order to avoid chromatographic changes for early eluting drugs. A pre-mixed solution of eluent A and eluent B (90 : 10) did not offer the lipophilic properties to re-dissolve some compounds. Therefore, 50 µL of eluent B were used to dissolve the APs. This solution was diluted using 450 µL of eluent A to provide the constitution required for the chromatographic conditions.

Ante-mortem blood was chosen as the matrix for the calibration standards rather than post-mortem blood due to a number of reasons. Firstly, excess “blank” post-mortem blood from deceased persons is difficult to obtain ethically for assay calibration purposes, whereas ante-mortem blood is readily available through blood banks. Secondly, post-mortem blood is often of variable quality and depending upon post-mortem change, can lead to unknown matrix effects and variable recovery which could affect assay results if used as a calibration matrix. The validation data has clearly shown that post-mortem blood does not seriously

affect background signals and matrix effects until it is quite decomposed. Unfortunately the degree of putrefaction cannot be quantified in individual cases.

Detection

After extraction from blood, the drugs were separated using gradient elution on a XBD C_{18} column. Preliminary experiments showed increased chromatographic robustness using a large size column and considerably high flow rates (data not shown). The increase of flow rate over the run improved the separation and peak shape of lipophilic compounds. For the detection of the APs, 3 MRM transitions were used for each analyte; their use and their respective peak area ratios enabled unambiguous identification of all APs included in the assay and showed no inference in a number of drug-free samples.

The potential described in Table 1 were chosen using Analyst software and additionally critically reviewed. Extreme values such as the declustering potential (DP) of bromperidol (DP = 1) and pimozide (DP = 396) and the entrance potential (EP) of triflupromazine (EP = 1) were identified as the best given option.

The structural isomers promazine and promethazine (formula and product ion spectra shown in Supplement 1) show both the presence of a most abundant transition 285 → 86, which represents the cleavage of the side chain. This cleavage can occur despite the different side chain structures. However, the cleavage of the side chain in alpha position of the side chain nitrogen results in a fragment *m/z* = 58 in case of promazine, and a fragment *m/z* = 71 in case of promethazine. The resulting transition using this fragmentation allows the differentiation of the 2 structural isomers. A sample chromatogram showing the different MRM transitions for promazine and promethazine is given in Figure 1.

The isobaric compounds pipamperone and haloperidol (structures and product ion spectra shown in Supplement 1) also show a similar fragmentation pattern. Due to the significantly different retention times and the use of scheduled MRM, transitions of the structurally-related compound are not monitored in the respective expected retention time range. An example chromatogram of a sample including both compounds can be seen in Figure 2.

The fragmentation pattern of zotepine did not provide 3 fragments with sufficient sensitivity. The software assisted optimisation process selected two transitions (332.1 → 72.1 and 332.1 → 72.6) which are likely to reflect the same fragment. This needs to be considered when applying the method to routine casework. It is however still possible to identify the presence of zotepine according to internationally accepted guidelines as the requirement of two transitions and their ratio is fulfilled.

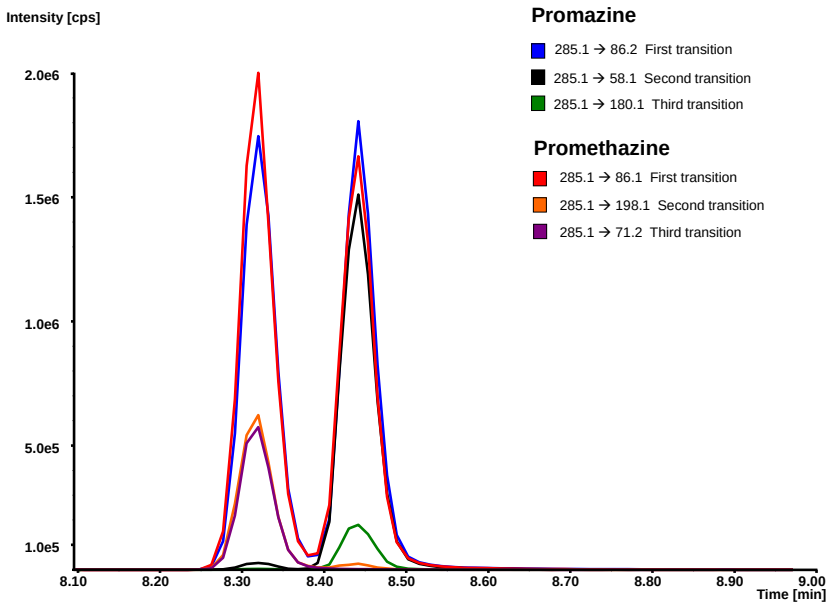


Figure 1: Chromatogram of promazine and promethazine recorded in the scheduled MRM mode showing three MRM transitions of each analyte.

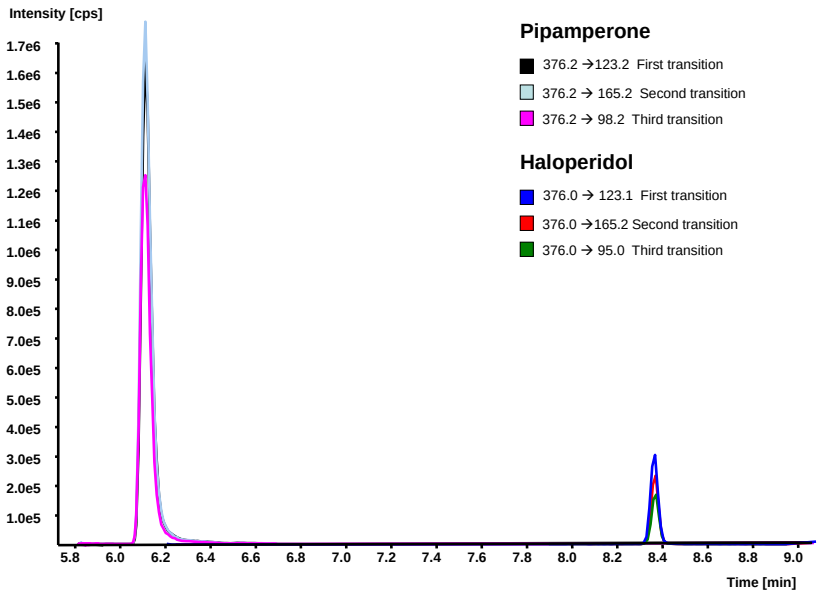


Figure 2: Chromatogram of haloperidol and pipamperone recorded in the scheduled MRM mode showing three MRM transitions of each analyte.

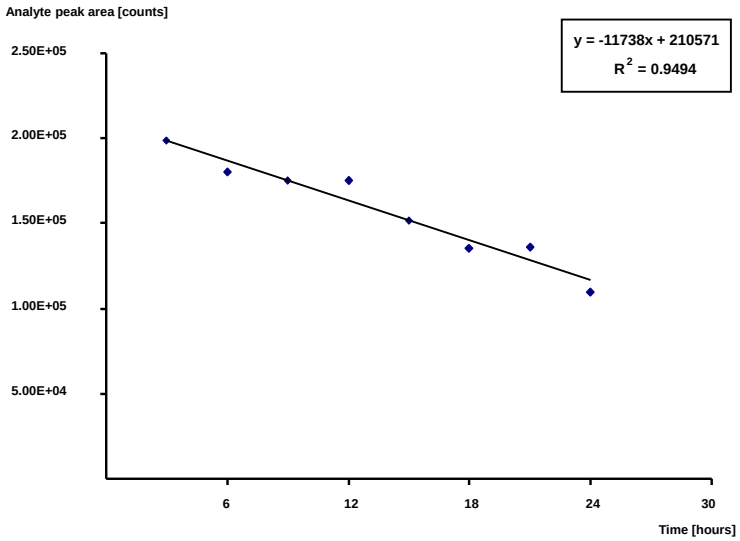


Figure 3. Peak area plot over time in autosampler stability of olanzapine.

Validation experiments

The described procedure was validated according to internationally accepted recommendations [2, 16, 17]. The assay was found to be selective for all tested compounds, no interfering peaks were observed in the extracts of the different post-mortem and ante-mortem blank blood samples. A comparison of sample chromatograms of a blank blood sample, a zero sample and a lowest calibrator are shown in Supplement 2a, 2b, and 2c. The MRM transition signals observed in the blank blood are non-significant as their intensities are considerably lower than the intensities in the lowest calibrator. The MRM transitions of the internal standard are of similar intensity, therefore only one peak is visible in the zero sample (Supplement 2b).

Calibration curves were linear in the range given in Table 2. All analytes were visually checked for a linear fit, a weighted second order model fit and a quadratic fit. A linear fit was used for 9OH-risperidone, haloperidol, levomepromazine, loxapine, olanzapine, perphenazine, and zuclopenthixol. Linear regression ($1/x^2$ weighting) was used for

aripiprazole, bromperidol, buspirone, chlorpromazine, chlorprothixene, droperidol, fluphenazine, fluspirilene, melperone, perazine, pericyazine, pimozide, pipamperone, prochlorperazine, sulpiride, trifluprom azine, and ziprasidone. A quadratic fit was used for amisulpride, clozapine, mesoridazine, promazine, promethazine, quetiapine, risperidone, thioridazine, trifluoperazine, and zotepine. The calibration fit showed a coefficient of determination of $r^2 > 0.99$ for all drugs.

With the exception of olanzapine, all drugs appeared to be stable for up to 24 hours when stored in the autosampler. Figure 3 shows the autosampler degradation rate of olanzapine with time. As previously described, olanzapine is unstable in blood samples [23]. This instability was also confirmed in our processed sample stability experiments. In freeze/thaw stability experiments, all drugs appeared to be stable however low concentrations of buspirone, bromperidol and perphenazine (Table 4) showed variability in detection at low concentrations. As already mentioned previously, due to the lack of processed sample stability, olanzapine could not be quantified reliably, hence the inability to determine freeze/thaw stability.

All drugs appeared to be stable over a period of 6 weeks when stored at -20 °C, with the exception of olanzapine which showed losses of approximately 80 % compared with control samples at all 3 concentrations. The LLOQs corresponded to the lowest concentrations used for the calibration curves with a signal-to-noise ratio of at least 10. With the exception of olanzapine, accuracy data were within the acceptance interval of ±15% (±20% at the LLOQ) of the nominal values for all drugs. Within day (repeatability) and intermediate precision data were within the required limits of 15 % RSD (20 % RSD at LLOQ) with the exception of low concentrations of buspirone, bromperidol and perphenazine (Table 4).

As olanzapine showed instability in several validation experiments and its detection could therefore not be reliably performed, it was excluded from this method.

Applicability of the previously described method was demonstrated by the analysis of post-mortem blood samples. The results obtained from the analysis of 183 post-mortem blood samples including the mean concentrations are given in Table 5. Cases that were above the standard curve were diluted appropriately to provide accurate results. These concentrations vary depending on the type of death which range from therapeutic use to suicidal ingestion (high concentration). The use of this method has importantly enabled the detection of a large range of APs simultaneously and accurately. The minimal use of blood (0.1 mL) is also advantageous and combined with the use of LC-MS/MS has led to significant progress towards a single assay for detection of numerous typical and atypical APs. In summary, this method is robust, reliable, sensitive and validated for the measurement of APs in blood samples.

Table 4: Accuracy, intermediate precision, and repeatability data of the LC-MS/MS assay for the studied analytes. Values greater than international acceptance criteria are highlighted in bold. Quality control samples were prepared at concentrations given in Table 2.

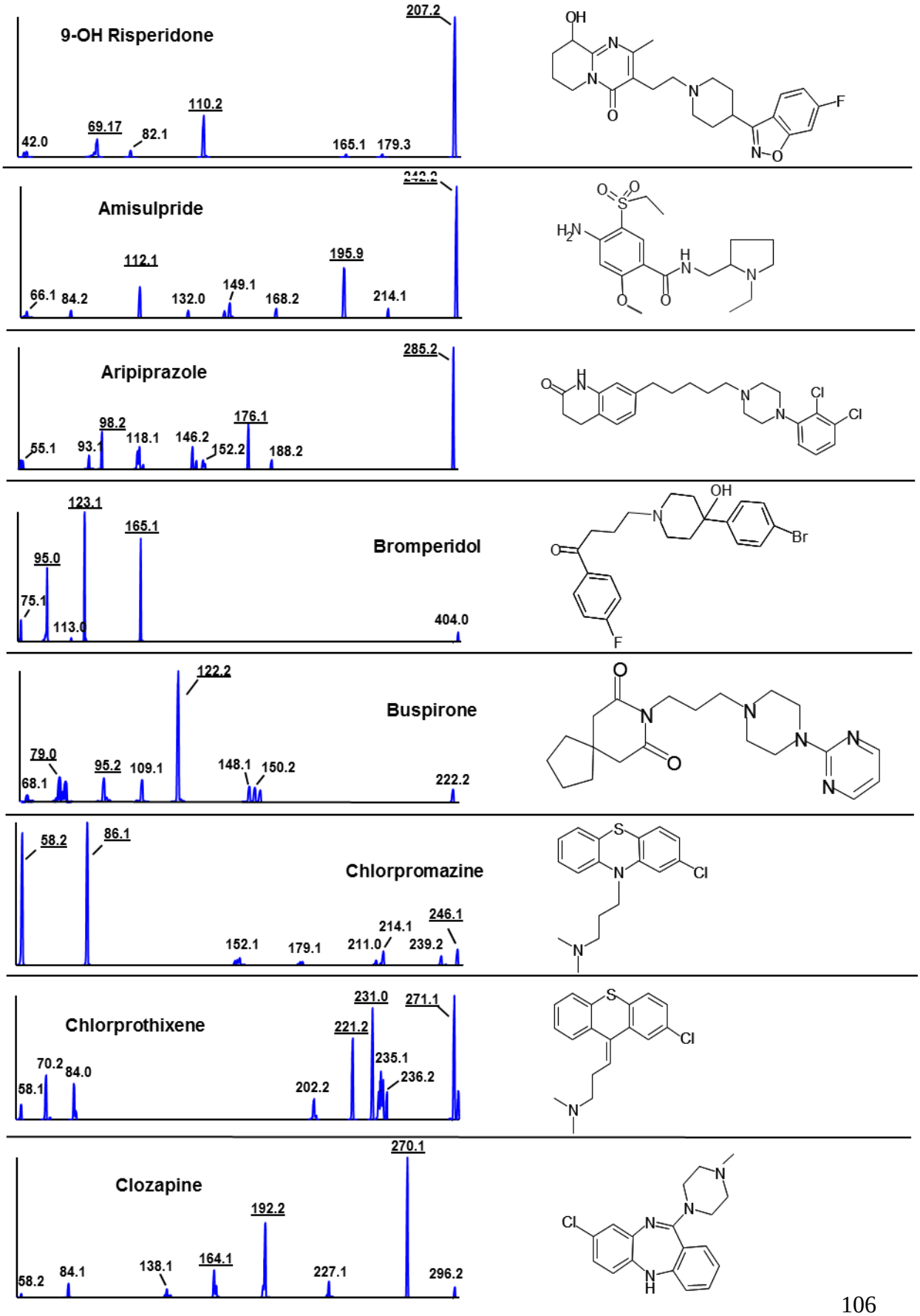
Drug	Validation Experiment	QC LOW[#]	QC MED[#]	QC HIGH[#]
9-OH-Risperidone	Repeatability	12.8	4.4	6.4
	Precision	13.7	12.2	10.5
	Accuracy	8.7	-0.5	-4.2
Amisulpride	Repeatability	8.7	4.6	10.6
	Precision	18.1	14.6	11.9
	Accuracy	7.9	12.8	-4.1
Aripiprazole	Repeatability	13.7	5.6	7.2
	Precision	13.7	10.8	11.3
	Accuracy	-2.5	0.3	-5.0
Bromperidol	Repeatability	**57.1**	8.5	8.8
	Precision	**57.1**	10.9	11.4
	Accuracy	6.5	12.0	0.0
Buspirone	Repeatability	**29.0**	5.3	5.3
	Precision	**29.0**	12.4	12.1
	Accuracy	10.6	-7.7	-5.4
Chlorpromazine	Repeatability	8.4	4.6	7.2
	Precision	10.2	12.0	9.9
	Accuracy	7.3	7.0	5.5
Chlorprothixene	Repeatability	9.1	3.1	4.1
	Precision	10.0	10.1	10.8
	Accuracy	19.1	13.9	14.0
Clozapine	Repeatability	13.9	4.3	6.0
	Precision	13.9	12.5	11.0
	Accuracy	13.9	0.7	-2.3
Droperidol	Repeatability	14.5	11.2	11.7
	Precision	16.3	11.2	13.3
	Accuracy	-6.6	-6.6	-7.0
Fluphenazine	Repeatability	15.6	3.7	8.2
	Precision	16.6	8.5	9.7
	Accuracy	6.6	11.1	9.8
Fluspirilene	Repeatability	17.2	6.6	8.9
	Precision	18.5	14.1	13.3
	Accuracy	-7.7	-2.9	3.0
Haloperidol	Repeatability	14.6	4.1	7.6
	Precision	14.6	10.3	11.1
	Accuracy	2.9	0.6	-1.1
Levomepromazine	Repeatability	13.4	6.6	6.9
	Precision	14.8	14.1	11.4
	Accuracy	-9.8	-3.9	-0.7
Loxapine	Repeatability	10.6	6.2	6.7

Drug	Validation Experiment	QC LOW[#]	QC MED[#]	QC HIGH[#]
	Precision	13.8	12.0	11.1
	Accuracy	18.5	3.4	2.7
Melperone	Repeatability	10.0	10.3	12.6
	Precision	11.6	14.7	14.5
	Accuracy	8.3	-4.6	4.0
Mesoridazine	Repeatability	8.4	3.1	7.8
	Precision	12.9	12.5	14.6
	Accuracy	-7.1	-5.0	-11.0
Olanzapine	Repeatability	17.8	7.8	7.5
	Precision	18.2	11.8	11.3
	Accuracy	-16.7	-0.9	-2.2
Perazine	Repeatability	8.0	4.5	5.2
	Precision	10.0	10.0	7.5
	Accuracy	1.1	8.5	6.4
Pericyazine	Repeatability	12.6	6.7	7.9
	Precision	15.4	12.1	9.9
	Accuracy	-7.0	-7.5	-4.1
Perphenazine	Repeatability	**54.0**	5.2	7.0
	Precision	**54.0**	13.0	9.5
	Accuracy	3.8	9.6	3.3
Pimozide	Repeatability	11.2	11.5	11.4
	Precision	18.5	14.7	14.2
	Accuracy	13.3	-5.5	-0.2
Pipamperone	Repeatability	5.0	4.4	7.1
	Precision	12.1	9.6	12.3
	Accuracy	5.0	-0.7	-1.4
Prochlorperazine	Repeatability	9.5	3.6	7.6
	Precision	14.6	11.5	9.8
	Accuracy	6.6	1.2	1.0
Promazine	Repeatability	6.0	4.6	8.0
	Precision	14.0	12.4	10.7
	Accuracy	5.9	-0.9	-1.3
Promethazine	Repeatability	9.9	4.9	6.1
	Precision	12.5	12.2	11.4
	Accuracy	-0.9	2.2	0.9
Quetiapine	Repeatability	7.3	4.9	6.3
	Precision	14.9	9.7	9.1
	Accuracy	-0.4	2.2	-5.3
Risperidone	Repeatability	6.6	4.4	7.0
	Precision	10.3	9.9	9.9
	Accuracy	-3.9	-4.6	-5.2
Sulpiride	Repeatability	8.9	6.4	11.6
	Precision	14.5	12.7	12.0
	Accuracy	-10.1	-6.1	-13.9

Drug	Validation Experiment	QC LOW[#]	QC MED[#]	QC HIGH[#]
Thioridazine	Repeatability	6.7	6.5	8.2
	Precision	7.8	14.2	13.4
	Accuracy	-7.2	11.4	6.0
Trifluoperazine	Repeatability	14.0	4.5	5.5
	Precision	14.6	8.8	8.3
	Accuracy	-18.4	-7.9	-8.0
Triflupromazine	Repeatability	9.4	6.5	5.6
	Precision	16.0	12.6	12.8
	Accuracy	8.9	3.9	5.0
Ziprasidone	Repeatability	9.8	4.6	7.7
	Precision	11.4	10.2	11.1
	Accuracy	13.7	2.4	1.5
Zotepine	Repeatability	7.0	5.3	8.8
	Precision	8.4	13.0	9.6
	Accuracy	-2.7	-1.1	-13.6
Zuclopenthixol	Repeatability	11.7	3.2	6.3
	Precision	15.8	13.0	12.0
	Accuracy	-6.1	-3.8	-3.7

Conclusions

The LC-MS/MS assay presented described is a suitable procedure for separation, detection, and quantification of 30 APs in blood samples. It has proven to be selective, linear, accurate, and precise for all studied drugs. However olanzapine must be analysed promptly as it can degrade quite rapidly after extraction. The presented LC-MS/MS assay has been found to be applicable for clinical and forensic toxicological casework.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42.0
165.1
179.3
Amisulpride
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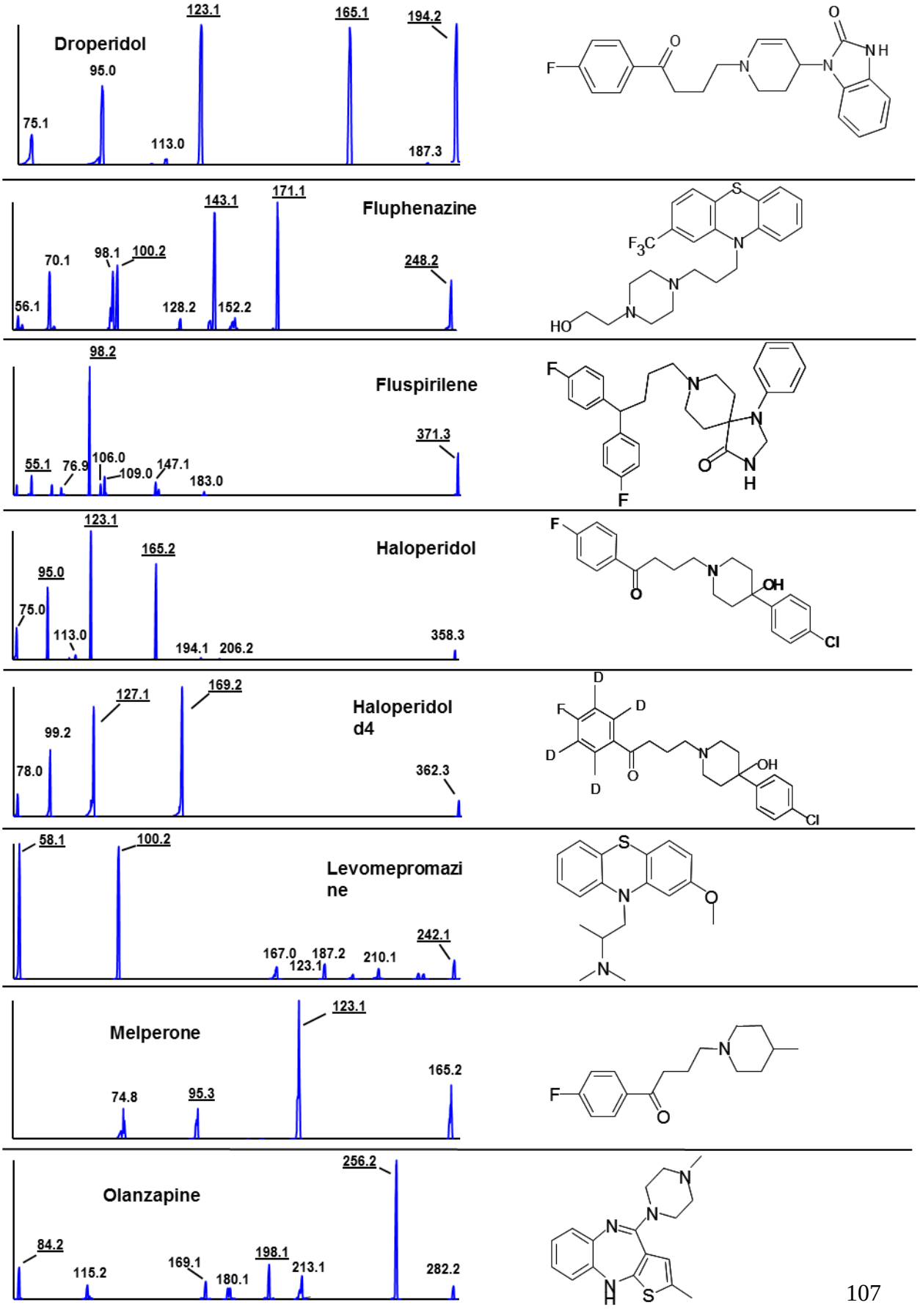
195.9
66.1
84.2
132.0
149.1
168.2
214.1
Aripiprazole
285.2
98.2
176.1
55.1
93.1
118.1
146.2
152.2
188.2
Bromperidol
123.1
165.1
95.0
75.1
113.0
404.0
Buspirone
122.2
79.0
95.2
109.1
148.1
150.2
68.1
222.2
Chlorpromazine
58.2
86.1
152.1
179.1
214.1
211.0
239.2
246.1
Chlorprothixene
231.0
271.1
221.2
235.1
70.2
84.0
58.1
202.2
236.2
Clozapine
270.1
192.2
58.2
84.1
138.1
164.1
227.1
296.2


Droperidol
123.1
165.1
194.2
95.0
75.1
113.0
187.3
Fluphenazine
143.1
171.1
70.1
98.1
100.2
248.2
56.1
128.2
152.2
Fluspirilene
98.2
371.3
55.1
76.9
106.0
109.0
147.1
183.0
Haloperidol
123.1
165.2
95.0
75.0
113.0
194.1
206.2
358.3
Haloperidol d4
127.1
169.2
99.2
78.0
362.3
Levomepromazine
58.1
100.2
242.1
167.0
187.2
123.1
210.1
Melperone
123.1
165.2
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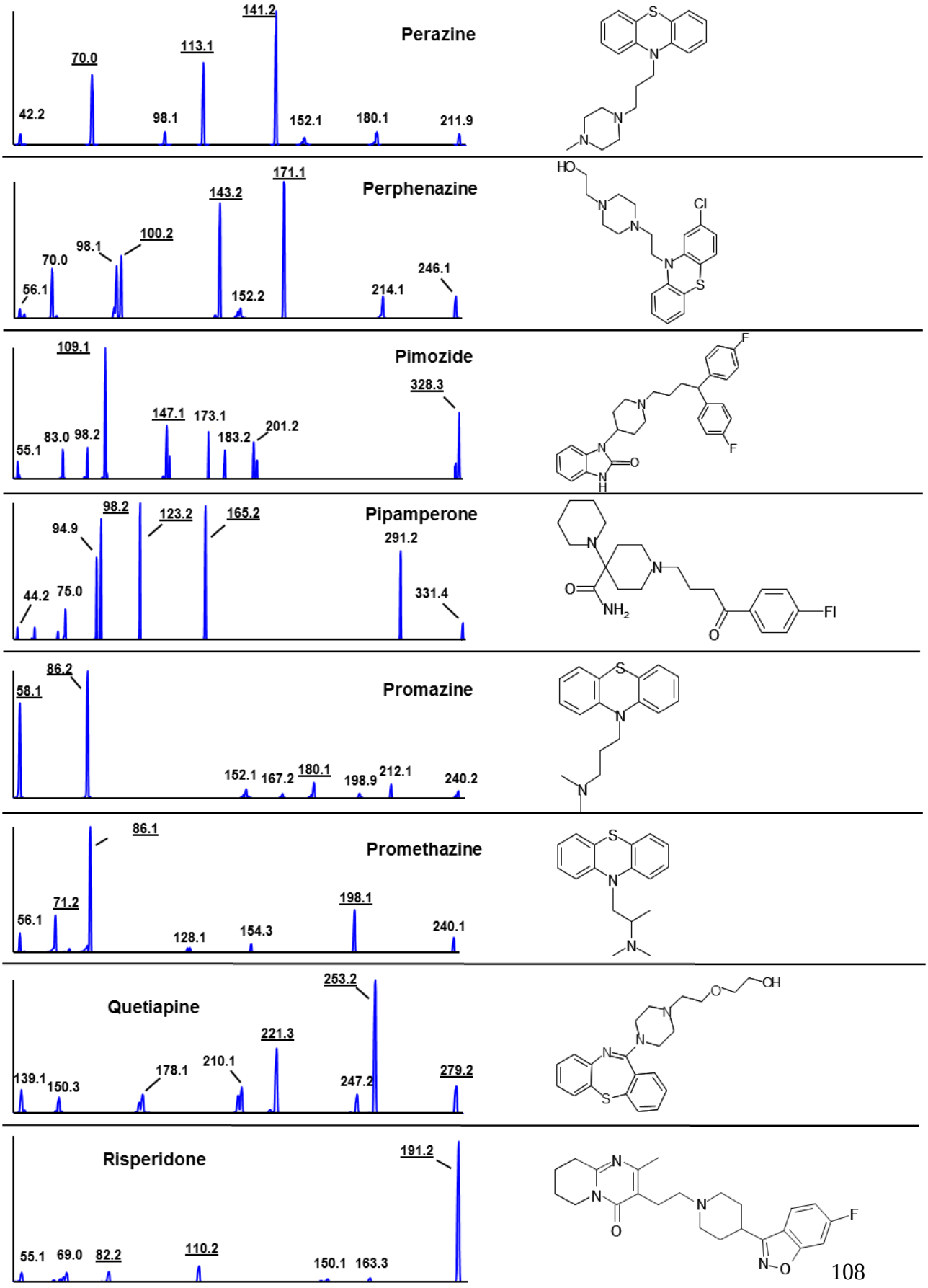
169.1
198.1
180.1
213.1
282.2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98.1
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Perphenazine
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100.2
98.1
70.0
56.1
152.2
214.1
246.1
Pimozide
109.1
328.3
147.1
173.1
183.2
201.2
55.1
83.0
98.2
Pipamperone
98.2
123.2
165.2
94.9
291.2
44.2
75.0
331.4
Promazine
86.2
58.1
152.1
167.2
180.1
198.9
212.1
240.2
Promethazine
86.1
71.2
198.1
56.1
128.1
154.3
240.1
Quetiapine
253.2
221.3
178.1
210.1
139.1
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279.2
Risperidone
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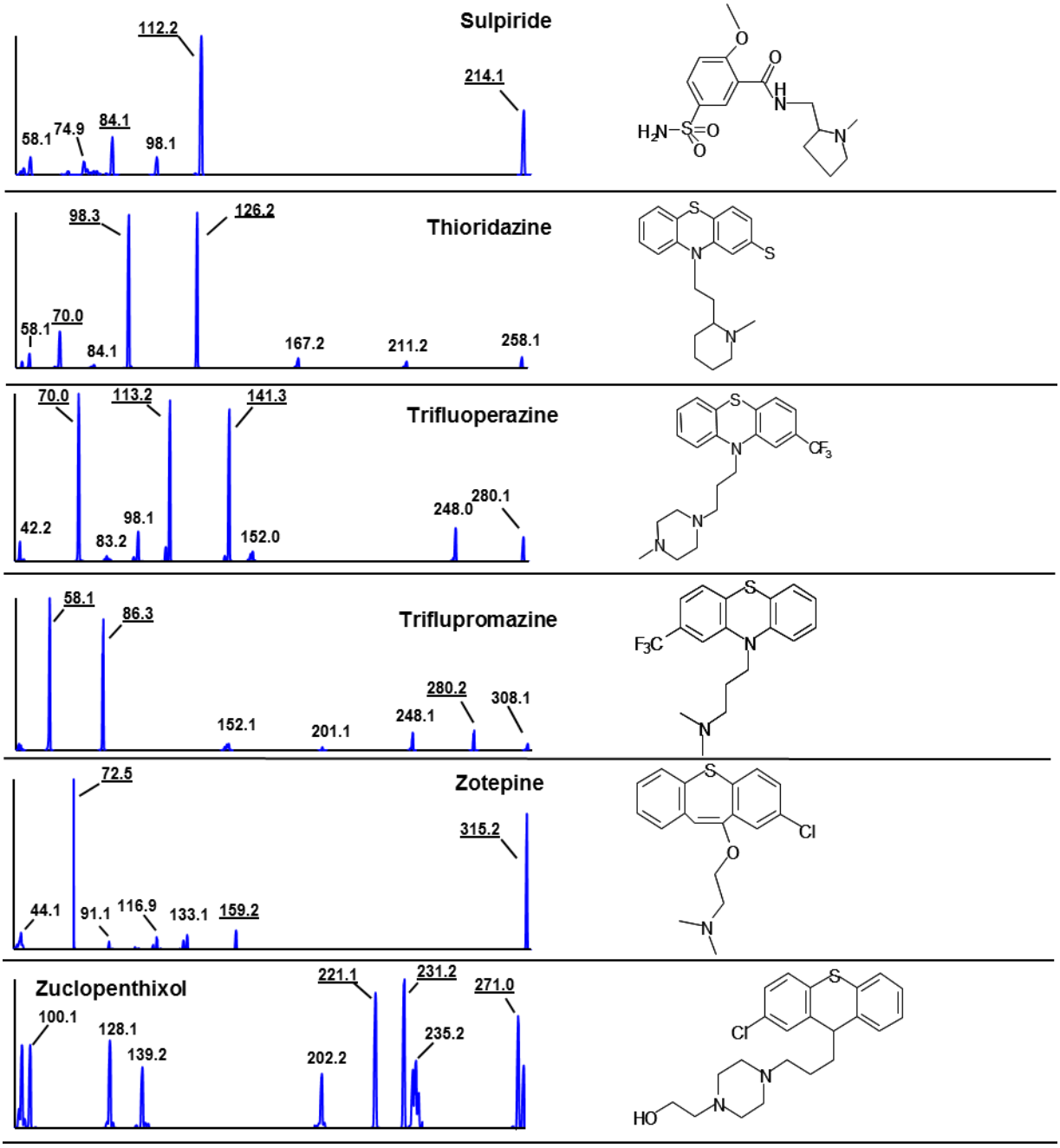
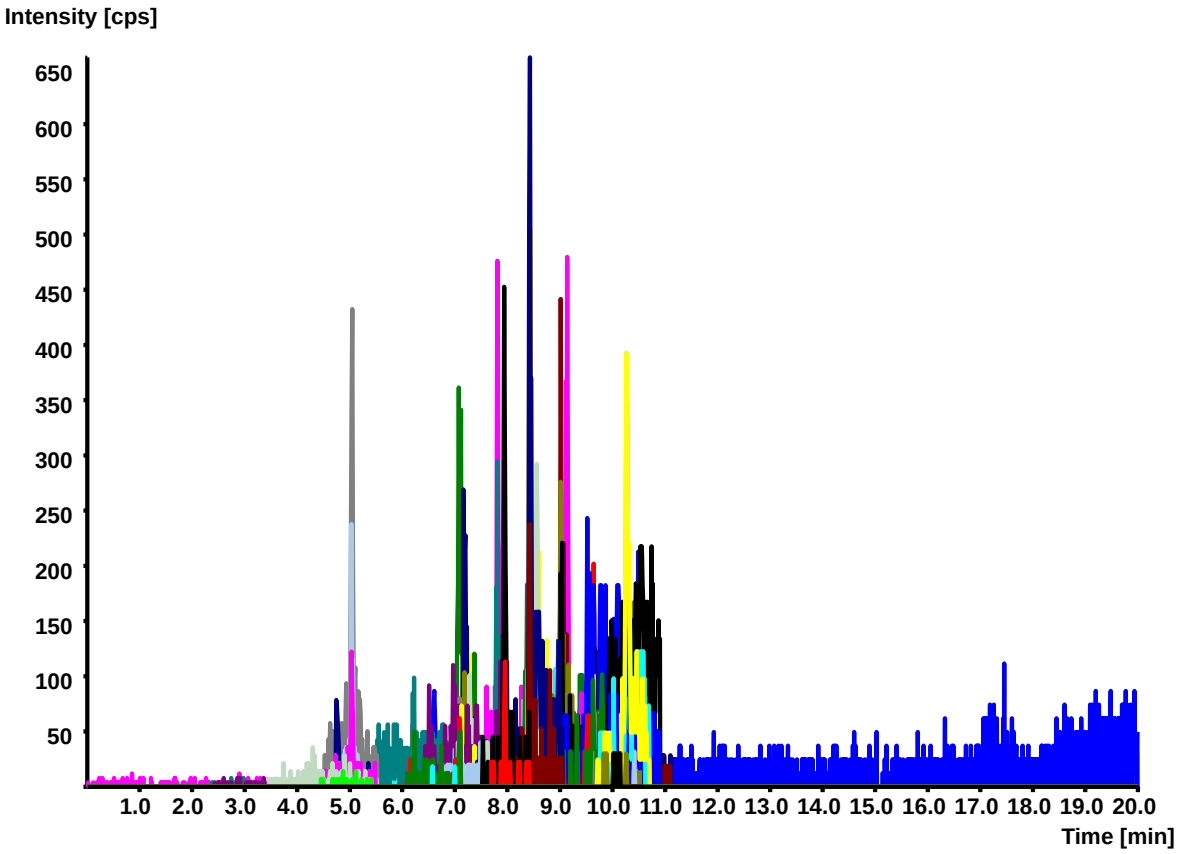
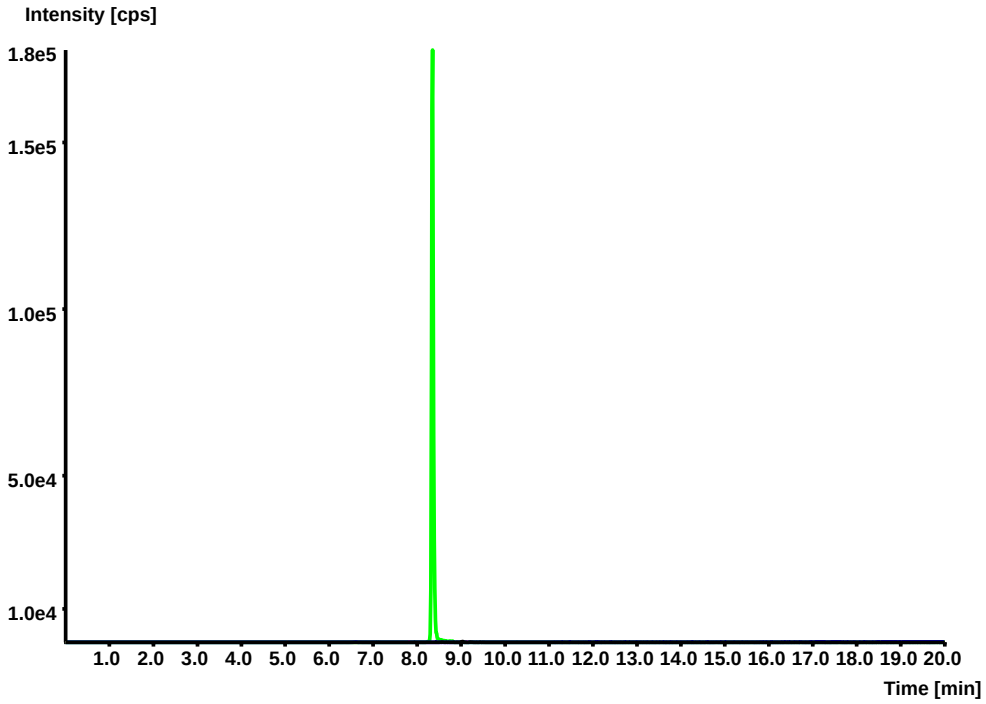
55.1
69.0
82.2
110.2
150.1
163.3



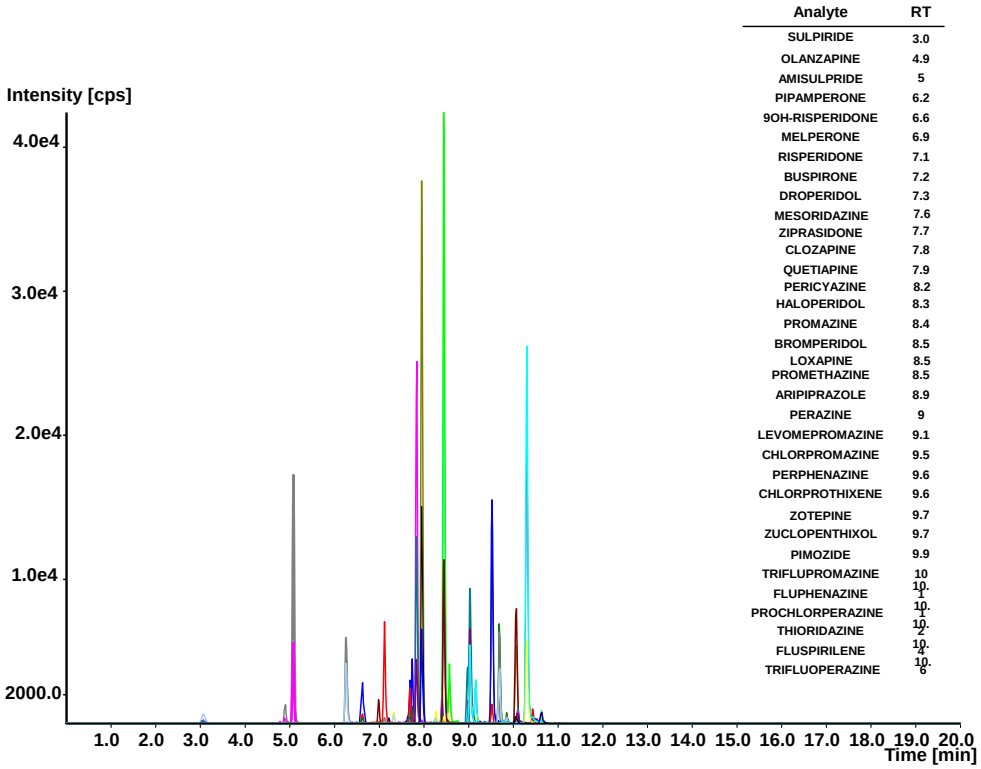
Supplement 1: Chemical structures and product-ion spectra of all studied analytes and the internal standard recorded in the product ion scan mode.



Supplement 2a: Chromatogram of a blank blood sample recorded in the scheduled MRM mode showing all recorded transitions



Supplement 2b: Chromatogram of a zero sample recorded in the scheduled MRM mode showing all recorded transitions



Supplement 2c: Chromatogram of a lowest calibrator (concentrations given in Table 2) recorded in the scheduled MRM mode showing all recorded transitions

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Chapter 3

Stability of Antipsychotic drugs

The critical literature review in **Chapter 2.1** highlighted the stability of antipsychotic drugs as one of the underexplored aspects of the forensic toxicology of these drugs. Additionally, the outcomes of the stability studies as part of the method validation in **Chapter 2.2** identified the atypical antipsychotic drug olanzapine as particularly unstable, subsequently leading to the research in **Chapter 3.1**. A comprehensive stability study was conducted, investigating the influence of time and storage temperature on the stability of 30 commonly prescribed antipsychotic drugs.

Chapter 3.1

Assessment of the Stability of 30 Antipsychotic Drugs in Stored Blood Specimens

Saar, E., Gerostamoulos, D., Drummer, O.H., Beyer, J.
Forensic Sci Int, 2012. **215** (1-3): p. 152-8.

Monash University

Declaration for Thesis Chapter 3.1

Declaration by candidate

In the case of Chapter 3.1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conducted literature review, performed stability experiments and interpreted data. Wrote article.	85 %

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D	Advised candidate regarding direction and content, reviewed and edited article	
Drummer, O. H	Advised candidate regarding direction and content, reviewed and edited article	
Beyer, J.	Advised candidate regarding direction and content, reviewed and edited article	

Candidate's Signature	[redacted]	**Date** 27/07/2012

Declaration by co-authors

The undersigned hereby certify that:

(13) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(14) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(15) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(16) there are no other authors of the publication according to these criteria;
(17) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(18) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Victorian Institute of Forensic Medicine**

Signature 1	[redacted]	**Date** 27/7/12
Signature 2		27/7/12
Signature 3		27/7/2012

ABSTRACT

The stability of 30 common antipsychotics (APs) in spiked whole blood was investigated over ten weeks in a preliminary experiment (designated "P experiment"). Pools of blank blood spiked with drugs at two different therapeutic levels were stored at four different temperatures: 20 ºC, 4 ºC, -20 ºC, and -60 ºC and extracted once weekly in duplicate, using a previously published method. A loss of > 15 % of the initial drug concentration was considered to indicate possible instability and the respective drugs were selected for further investigation in a final experiment (designated "F experiment"). Eight APs (chlorpromazine, chlorprothixene, fluspirilene, droperidol, olanzapine, thioridazine, triflupromazine, and ziprasidone) were incorporated into F experiment. The same conditions were used in both experiments, however only a high therapeutic drug concentration was chosen for the F experiment and the storage time was extended to 20 weeks. All drugs of interest in the F experiment showed significant losses after 20 weeks of storage under at least one storage condition. The most notable results involved olanzapine, where losses of almost 100 % in all storage temperatures were observed. Drug degradation in fluspirilene samples was significant after 20 weeks under all storage conditions. Overall, extensive degradation was seen with approximately 80 % drug loss when stored at 20 ºC and 4 ºC with samples also seriously affected by degradation of up to 50 % when stored at -20 ºC and -60 ºC, respectively. Ziprasidone remained stable when stored at 4 ºC, -20 ºC, and -60 ºC over nine weeks, however significant degradation was observed when stored at 20 ºC, with a loss of almost 100 % after 20 weeks of storage.

The time period and temperature of storage of biological samples can have a significant influence on the stability of several APs. It is therefore important to be aware of potential changes in drug concentrations during storage when interpreting analytical results.

Keywords: Antipsychotic drugs, LC-MS/MS, stability, whole blood, toxicology

1. Introduction:

An increase in the number of prescriptions of antipsychotics drugs (AP) in recent years [1, 2] in addition to mounting evidence suggesting that these drugs can increase the risk of sudden cardiac death [3-5] may present an explanation for the high prevalence of AP in forensic cases. The advent of liquid chromatography with tandem mass spectrometry (LC-MS/MS) has greatly facilitated the identification of these drugs, however the interpretation of their concentrations in biological specimens still requires further research.

In forensic toxicology, potential post-mortem changes such as post-mortem redistribution (PMR) or instability of the target drugs provide an additional challenge. The atypical AP olanzapine has been shown previously to be unstable in blood [6-9].Various conclusions have been drawn from these studies but the extent to which this drug degrades remains uncertain. There is little information published on any other AP.

Evaluation of stability of drugs in bio-analytical methods is often performed using four different experiments: long-term stability in the sample matrix, freeze/thaw stability, bench-top stability, and stability in the prepared samples under conditions of analysis [10]. Stability studies have targeted either single or a few drugs and are mainly carried out using a plasma matrix [11-14]. In contrast, there is little information available on the stability of AP in whole blood [15]. Furthermore, stability data is often only collected as part of method validation and thus information concerning different storage conditions and data over longer periods of time is often inadequate or completely absent. Unfortunately, the definition and evaluation of ‘stability’ is not very consistent throughout the literature. In 1998 Hartmann *et al*. defined ‘stability’ as the “absence of an influence of time on the concentration of the analyte in a sample” [16]. Despite the various definitions and wide-ranging requirements to

determine the long term stability of an analyte in a sample matrix [16-19], most of the approaches show a similar experimental setup. In order to determine long term stability of drugs in the sample matrix it is frequently suggested to analyze a set of samples ("control samples") at the beginning of the stability study and an additional set of samples ("stability samples") after a certain time of storage. Various statistical tests have been used to determine if the drug concentration differs significantly between control samples and stability samples, consequently providing an indication of stability problems. Unfortunately these approaches do not provide any information about the pattern of break down. A similar experiment design has been used in two very recent publications by Nilson *et al.* [20, 21] looking at the hypnotic drug zopiclone and providing useful information regarding storage requirements of this particular drug.

The aim of this study is to determine the stability of 30 APs in stored blood samples at different temperatures at a number of time intervals over a 10 - 20 week period.

2. Material and Methods

2.1 Chemicals and reagents

Bromperidol, chlorpromazine, fluspirilene, haloperidol, pipamperone, trifluoperazine, triflupromazine and Trizma base were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Buspirone, chlorprothixene, olanzapine, promazine, promethazine, risperidone and zuclopenthixol were obtained from the Division of Analytical Laboratories (Lidcombe, NSW, Australia). Droperidol, fluphenazine, perphenazine, pimozide and sulpiride were provided by Australian Government Analytical Laboratories (Pymble, NSW, Australia).

Levomepromazine, melperone, perazine and zotepine were obtained from Phast GmbH (Homburg/Saar, Germany). Amisulpride, aripiprazole, quetiapine, and ziprasidone were purchased from the National Institute of Forensic Science (Melbourne, VIC, Australia). Clozapine was provided by Sandoz (Pyrmont, NSW, Australia) and 9-OH Risperidone (paliperidone) was obtained from Janssen–Cilag (North Ryde, NSW, Australia). The isotope-labeled internal standard haloperidol-d_4 was purchased from Cerilliant (Round Rock, TX, USA). Acetonitrile, ammonium formate, 1-chlorobutane, methanol and formic acid were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from BDH Chemicals (Kilsyth, VIC, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia). Eluent A consisted of 50 mmol/L aqueous ammonium formate adjusted to pH 3.5 with formic acid. 2 M Trizma buffer (pH = 9.2) was prepared by dissolving 242 g Trizma base in 1 L water.

2.2 Specimens

Whole blood for stability experiments was obtained from the local blood bank in lithium heparin-coated plastic bags (500 mL). The blood was aliquoted into 10 mL polypropylene tubes containing 200 mg sodium fluoride and 30 mg potassium oxalate. Blood was analysed using a previously published method [22] and was found to be “drug-free”. All blood samples were stored at −20 °C prior to analysis.

3. Methods of Detection

3.1 Apparatus

The LC-MS/MS system consisted of an Applied Biosystems 3200 Q-TRAP® linear ion-trap quadrupole mass spectrometer (Applied Biosystems, Melbourne, VIC, Australia) equipped with a Turbo V ion source, operated in the electron spray ionization (ESI) mode, and an Agilent Technologies (AT) 1200 Series HPLC system (Agilent, Melbourne, VIC, Australia) which consisted of a degasser, a binary pump and an autosampler.

3.2 Preparation of stock solutions and extraction

Preparation of stock solutions, calibration standards and stability samples and extraction procedures were performed as published previously [22]. The extraction consisted of a liquid-liquid extraction of the AP from whole blood using butylchloride.

4. Stability experiments

4.1 Preliminary experiment

Preliminary samples (designated "P") were prepared at two concentration levels: "LOW" and "HIGH" (at a low therapeutic concentration and a supratherapeutic concentration of the respective drug [22]. The P LOW samples were prepared using 20 mL of blank blood pooled in a volumetric flask and an independently prepared mixture of the 30 AP in methanol at a concentration 100 times higher than the concentrations of the corresponding P LOW samples. After inversion for 30 min on a rotary wheel, 100 µL aliquots were transferred to 2 mL Sarstedt tubes (Sarstedt, Mawson Lakes, SA,). P LOW samples (*n = 160*) were divided into four groups (n=40 samples) of different storage temperatures (20 °C, 4 °C, -20 °C, and -60 °C). P HIGH samples were prepared according to the same protocol, but using a "high"

therapeutic concentration of the respective drugs instead. Sample extraction was performed twice weekly in duplicate over a period of 10 weeks and the samples were analysed according to the procedure described previously [22]. The concentrations of the analytes in the P samples were calculated using the daily calibration curves included in each assay.

4.2 Final experiment

From the P experiment, haloperidol and risperidone were shown to be stable under all conditions and were used as control references, as they are also commonly detected in cases sent to the authors' laboratory for toxicological testing. Eight drugs (chlorpromazine, chlorprothixene, droperidol, fluspirilene, olanzapine, thioridazine, triflupromazine and ziprasidone) which did not meet the acceptance criteria for stability under at least one storage condition, were selected for further investigation and designated as "F" samples. F samples were prepared at the respective HIGH concentration of each drug using 100 mL of blank pooled blood aliquoted in 10 x 10 mL volumetric flasks. Each blood aliquot was spiked with a methanolic solution of one of the drugs of interest at a concentration 100 times higher than the concentrations of the corresponding F sample. After inversion for 30 min on a rotary wheel 100 μL aliquots of each flask were transferred to 2 mL Sarstedt tubes (*n = 800*) and labelled accordingly. F samples of each drug (*n = 80*) were divided into four groups (*n = 20* samples) at different storage temperatures (20 °C, 4 °C, -20 °C, and -60 °C).

Sample extraction was performed once weekly (n=1 per drug and storage temperature) over a period of 20 weeks and the samples were analysed according to the procedure described above. The concentrations of the analytes in the F samples were calculated via the daily calibration curves included in each assay.

4.3 Stability evaluation / Acceptance criteria

As the degradation of a drug over time was of interest, fewer replicates were analysed with more frequency in contrast with papers that seek to establish a statistically significant difference between "control" samples at the start of the experiment and "stability" samples at the end of the experiment. In order to determine all possible instabilities of the target drugs, the approach introduced by Wieling *et al.* in 1996 for the determination of autosampler stability was used in the experiments [23]. The calculated drug concentration was plotted versus time and a curve of best fit was visually evaluated. Outliers have been determined via a residual plot using a 95 % CI and removed from the data prior to interpretation of the results. As all drugs included in this study (with the exception of bromperidol, buspirone and perphenazine) had proven to fulfill the acceptance criteria for accuracy of being ± 15 % of the target value in the previously published method validation [22] a loss of > 15 % of the initial drug concentration in the P experiment was considered a possible instability and the drug was incorporated in the F experiment for further investigation.

Olanzapine was of great interest in this study as it is known to be an exceptionally unstable drug. Since olanzapine was excluded from a previously published method validation [22] due to difficulties mainly regarding processed sample stability, the authors attempted to overcome this problem by reducing the time of olanzapine samples on the autosampler to a minimum (< 1 h). However, the reader must be aware that the measured concentrations can only be seen as approximate values with a higher variation than the other drugs included in this study.

5. Results

Table 1 shows the standardised losses of the P experiment: A loss < 15% is referred to as "1" (acceptable), a loss ≤ 30 % - ≥ 15 % is referred to as "2" (acceptable if present only at 20 ºC), a loss ≥ 30 % is referred to as "3" (unacceptable). Therefore, for drugs that only showed losses at 20 °C, a loss of > 30 % ("3") (twice the minimum accuracy requirement) was considered unacceptable.

In the P experiment the majority of the 30 drugs (*n = 24*) appeared to be stable at 4 ºC and lower temperatures. An additional six drugs showed some losses (< 30 %) when stored at 20 ºC. Table 1 shows the standardised results of the P experiment. Haloperidol and risperidone were chosen as stability controls as they did not show significant losses under any storage condition after 10 weeks. Since instability was either found to be independent of concentration or else only the "HIGH" concentration was affected by degradation in the P experiment, the F experiment was performed using only a "HIGH" concentration of the respective drug. Towards the end of the P experiment, an error in storage conditions of the sample set P LOW -60 ºC was discovered. Therefore, these samples were excluded from this study as their integrity was compromised.

Table 2 lists all drugs that were included in the F experiment and their respective concentrations over 20 weeks of storage while Table 3 shows their respective best fits. An instrument failure during the F experiment rendered the data collected in week 15 and 17 unusable; therefore these weeks were excluded prior to data analysis.

Haloperidol showed losses of ~ 25 % after 20 weeks of storage at 20 ºC and > 15 % degradation after 20 weeks of storage at 4 ºC. None of the other storage temperatures seemed to have an effect on the haloperidol concentration, even after 20 weeks of storage.

Risperidone revealed losses of 15 – 20 % under all storage conditions after 10 weeks of storage, with losses increasing up to ~ 35 % after 20 weeks of storage under all storage conditions.

Similar to the P experiment, chlorpromazine showed losses of ~ 40 % after 10 weeks of storage at 20 ºC, with the concentration decreasing by another 10 % over the following 10 weeks.

Table 1: Results of the P experiment, showing standardised* losses after 10 weeks of storage at 20 ºC, 4 ºC, -20 ºC and -60 ºC

	20 ºC		4 ºC		-20 ºC		-60 ºC
ID	LOW	HIGH	LOW	HIGH	LOW	HIGH	HIGH
9-OH Risperidone	**2**	**2**	1	1	1	1	1
Amisulpride	1	1	1	1	1	1	1
Aripiprazole	1	1	1	1	1	1	1
Bromperidol	1	1	1	1	1	1	1
Buspirone	1	1	1	1	1	1	1
Chlorpromazine	**2**	**3**	**2**	**2**	1	**2**	1
Chlorprothixene	**2**	**3**	**3**	**2**	1	1	1
Clozapine	1	1	1	1	1	1	1
Droperidol	**2**	**3**	1	1	1	1	1
Fluphenazine	1	1	1	1	1	1	1
Fluspirilene	**3**	**3**	**3**	**3**	1	1	1
Haloperidol	1	1	1	1	1	1	1
Levomepromazine	**2**	**2**	1	1	1	1	1
Melperone	1	1	1	1	1	1	1
Olanzapine	**3**	**3**	**3**	**3**	1	1	**3**
Perazine	**2**	**2**	1	1	1	1	1
Perphenazine	**2**	**2**	1	1	1	1	1
Pimozide	1	1	1	1	1	1	1
Pipamperone	1	1	1	1	1	1	1
Promazine	**2**	**2**	1	1	1	1	1
Promethazine	**2**	**2**	1	1	1	1	1
Quetiapine	1	1	1	1	1	1	1
Risperidone	1	1	1	1	1	1	1
Sulpiride	1	1	1	1	1	1	1
Thioridazine	1	**2**	1	**2**	1	1	1
Trifluoperazine	1	1	1	1	1	1	1
Triflupromazine	**2**	**3**	1	**2**	1	1	1
Ziprasidone	**3**	**3**	1	1	1	1	1
Zotepine	1	1	1	1	1	1	1
Zuclopenthixol	1	1	1	1	1	1	1

Drugs in bold indicate that at least one acceptance criteria** for stability was not met and drug was therefore included in the F experiment.

* Losses: 1 = stable (< 15 %)
2 = some losses (≥ 15 % - ≤ 30 %)
3 = unstable (≥ 30 %)

** 1 = acceptable
2 = unacceptable, unless only 20 ºC is affected
3 = unacceptable

Storage at 4 ºC confirmed the observation from the P experiment with losses of ~ 35 – 40 % after 20 weeks of storage. Interestingly, the most extensive losses of chlorpromazine (~ 65 – 70 %) could be seen after 20 weeks of storage at -20 ºC with the main degradation occurring after 17 weeks. Chlorprothixene and droperidol showed comparable losses to chlorpromazine when stored at 20 ºC and 4 ºC. Interestingly, droperidol appeared to be stable when stored at -20 ºC but showed losses similar to storage at 4 ºC and 20 ºC (~ 35 %) when stored at -60 ºC. Degradation of fluspirilene in samples was significant after 20 weeks under all storage conditions. Extensive degradation was seen with ~ 80 % drug loss when stored at 20 ºC and 4 ºC, but samples were also significantly affected by degradation of up to 50 % when stored at -20 ºC and -60 ºC, respectively (Figure 1).

Olanzapine showed more extensive losses in the F experiment compared with the P experiment. All storage temperatures were affected by severe degradation of up to almost 100 % after 20 weeks. The observation for storage of ziprasidone samples at 20 ºC for 10 weeks was consistent with the P experiment and ~ 85 % of degradation and a clear pattern of break-down. The concentration decreased further to almost 100 % loss of the drug after 20 weeks of storage. Storage at 4 ºC, -20 ºC and -60 ºC seemed favourable with ~ 30 -40 % losses at the end of the experiment (Figure 2).

Table 2: Relative concentrations of drugs in F experiment stored at 20 °C, 4 °C, -20 °C and -60 °C over 20 weeks.

		Storage Temperature			
		20°C	4°C	- 20°C	-60°C
Drug	Time (Weeks)				
Risperidone	0	100 (95)	100 (104)	100 (100)	100 (90)
	1	99	114	109	102
	2	86	89	84	77
	3	83	79	-	82
	4	82	-	91	77
	5	90	107	102	75
	6	-	103	92	89
	7	81	94	89	84
	8	101	107	96	91
	9	87	91	77	67
	10	**77 (82)**	**77** (88)	**75 (84)**	**68 (79)**
	11	89	90	93	93
	12	85	94	-	79
	13	66	77	86	68
	15	68	67	74	59
	17	78	72	61	64
	18	75	90	74	86
	19	65	64	62	69
	20	**69 (69)**	**76 (72)**	**63 (68)**	**72 (68)**
Haloperidol	0	100 (90)	100 (100)	100 (99)	100
	1	101	107	93	100
	2	85	93	90	87
	3	85	88	92	96
	4	83	-	90	100
	5	87	105	113	102
	6	95	111	110	102
	7	87	95	95	80
	8	78	86	89	99
	9	81	99	107	106
	10	**77 (83)**	86 (92)	95 (95)	99 (92)
	11	72	83	94	84
	12	73	100	104	99
	13	74	76	87	83
	15	80	88	97	80
	17	74	86	84	82
	18	83	92	80	78
	19	82	86	96	89
	20	87 **(75)**	89 **(84)**	93 (91)	99 (86)

		Storage Temperature			
		20°C	4°C	- 20°C	-60°C
Drug	Time (Weeks)				
Chlorpromazine	0	100 (98)	100 (99)	100 (93)	100 (91)
	1	95	100	100	102
	2	-	-	67	75
	3	-	68	72	95
	4	71	77	74	88
	5	69	86	88	102
	6	72	80	94	93
	7	57	63	68	-
	8	-	62	67	78
	9	56	50	59	-
	10	**50 (60)**	**61 (60)**	**71 (65)**	**81** (87)
	11	50	50	50	70
	12	50	69	82	85
	13	50	54	58	67
	15	64	72	-	98
	17	50	55	71	80
	18	51	68	30	78
	19	48	57	26	93
	20	**47 (52)**	**60 (65)**	**30 (36)**	97 **(83)**
Chlorprothixene	0	100 (91)	100 (84)	100 (108)	100 (110)
	1	90	90	115	-
	2	93	70	80	110
	3	90	77	99	119
	4	68	87	104	98
	5	81	75	118	99
	6	81	93	105	-
	7	67	61	94	101
	8	71	60	96	119
	9	59	45	74	89
	10	**60 (68)**	**53 (67)**	**85 (83)**	90 (101)
	11	61	59	63	99
	12	62	70	74	111
	13	54	57	49	95
	15	66	61	81	103
	17	58	60	-	90
	18	49	69	102	99
	19	50	53	72	80
	20	**46 (45)**	**52 (50)**	**62 (57)**	101 (93)

		Storage Temperature			
		20°C	4°C	- 20°C	-60°C
Drug	Time (Weeks)				
Droperidol	0	100 (86)	100 (92)	100 (110)	100 (92)
	1	85	107	122	85
	2	72	91	100	93
	3	72	69	98	84
	4	67	72	89	86
	5	81	76	100	87
	6	67	77	106	80
	7	75	95	126	72
	8	78	83	120	85
	9	70	99	121	99
	10	**62 (66)**	**68 (79)**	94 (100)	**78 (80)**
	11	63	78	93	67
	12	69	88	97	91
	13	57	59	89	72
	15	72	78	94	-
	17	55	73	88	72
	18	60	86	91	77
	19	50	55	-	66
	20	**59 (57)**	**64 (67)**	92 (90)	**65 (68)**
Fluspirilene	0	100 (102)	100 (90)	100 (94)	100 (89)
	1	65	88	100	-
	2	48	59	71	76
	3	-	-	-	-
	4	49	-	76	69
	5	43	52	73	88
	6	49	47	90	80
	7	43	30	86	73
	8	42	26	73	66
	9	51	31	100	77
	10	**42 (35)**	**27 (27)**	88 **(79)**	**70 (72)**
	11	38	27	85	85
	12	49	29	92	-
	13	36	25	-	69
	15	37	23	83	65
	17	28	19	81	64
	18	21	24	65	64
	19	16	9	-	51
	20	**16 (26)**	**14 (20)**	**70 (65)**	**46 (55)**

		Storage Temperature			
		20°C	4°C	- 20°C	-60°C
Drug	Time (Weeks)				
Olanzapine	0	100 (83)	100 (70)	100 (160)	100 (62)
	1	23	30	34	-
	2	12	16	19	22
	3	10	13	23	22
	4	-	-	25	26
	5	11	17	32	25
	6	12	16	31	35
	7	8	12	29	20
	8	7	11	19	34
	9	9	13	23	34
	10	**5 (4)**	**12 (8)**	**2 (8)**	**28 (23)**
	11	6	12	20	30
	12	6	3	34	33
	13	5	9	25	24
	15	-	-	-	-
	17	2	6	1	17
	18	3	6	2	15
	19	1	4	1	13
	20	**0 (2)**	**3 (5)**	**1 (4)**	**17 (18)**
Thioridazine	0	100 (107)	100 (101)	100 (94)	100 (98)
	1	105	103	86	100
	2	-	88	-	-
	3	92	81	84	87
	4	103	104	92	89
	5	108	112	91	80
	6	98	-	94	99
	7	100	74	78	77
	8	73	88	74	88
	9	99	83	77	86
	10	**81** (88)	93 **(84)**	**81 (84)**	91 **(83)**
	11	82	80	98	
	12	89	85	102	93
	13	96	-	96	-
	15	85	83	90	79
	17	78	75	47	69
	18	-	-	-	-
	19	63	56	-	57
	20	**58 (68)**	**60 (67)**	**72 (74)**	**69 (67)**

		Storage Temperature			
		20°C	4°C	- 20°C	-60°C
Drug	Time (Weeks)				
Triflupromazine	0	100 (101)	100 (88)	100 (110)	100 (116)
	1	112	91	99	100
	2	89	74	101	104
	3	89	87	125	136
	4	-	-	117	123
	5	100	83	122	124
	6	83	94	113	135
	7	77	71	99	113
	8	60	60	91	100
	9	65	53	73	76
	10	**54 (69)**	**56 (68)**	**70** (91)	**76** (99)
	11	66	53	88	75
	12	73	69	81	79
	13	56	54	58	66
	15	55	65	90	100
	17	48	58	88	100
	18	52	68	93	104
	19	41	44	75	69
	20	**41 (38)**	**54 (48)**	**78 (73)**	92 **(79)**
Ziprasidone	0	100 (88)	100 (112)	100 (94)	- (96)
	1	80	122	98	100
	2	61	91	77	89
	3	52	103	80	91
	4	-	-	78	81
	5	35	111	89	87
	6	33	115	-	99
	7	25	104	87	88
	8	18	97	98	82
	9	16	94	85	99
	10	**11 (15)**	91 (90)	**66 (81)**	**77 (84)**
	11	11	90	77	96
	12	10	87	83	87
	13	8	77	70	75
	15	5	79	73	75
	17	4	74	88	64
	18	5	-	87	78
	19	3	63	57	80
	20	**2 (2)**	**57 (68)**	**58 (69)**	**72 (73)**

Figures in brackets represent the expected relative concentration defined by the best curve, '-' indicates value is outlier and therefore excluded prior to data analysis. Bold values indicate > 15 % loss at 10 and 20 weeks (highlighted in grey).

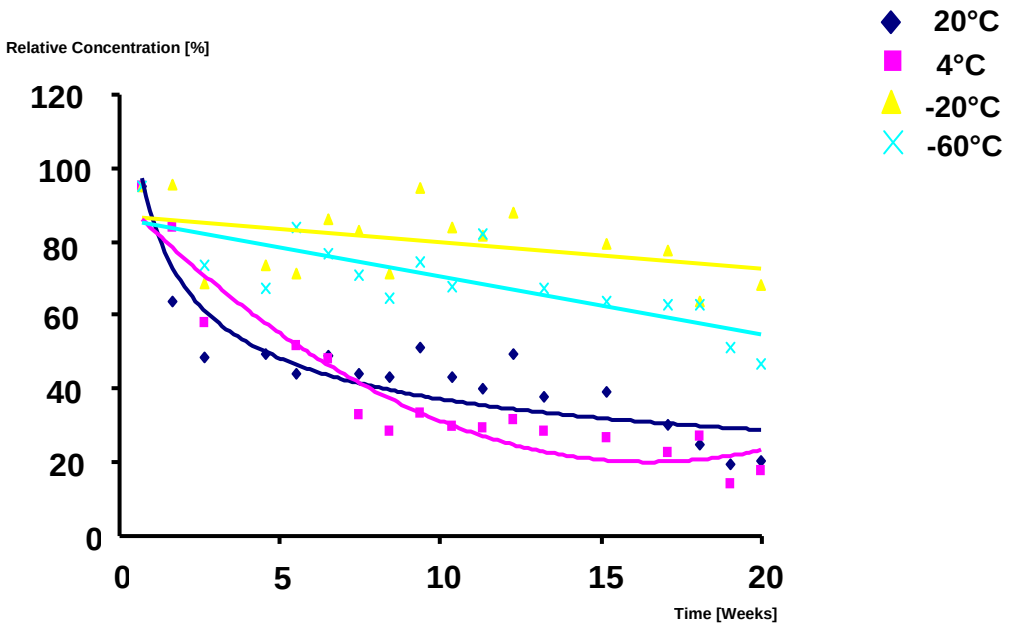

Figure 1: Relative concentration [%] of fluspirilene samples in the F experiment stored for 20 weeks at 20 ºC, 4 ºC, -20 ºC and -60 ºC

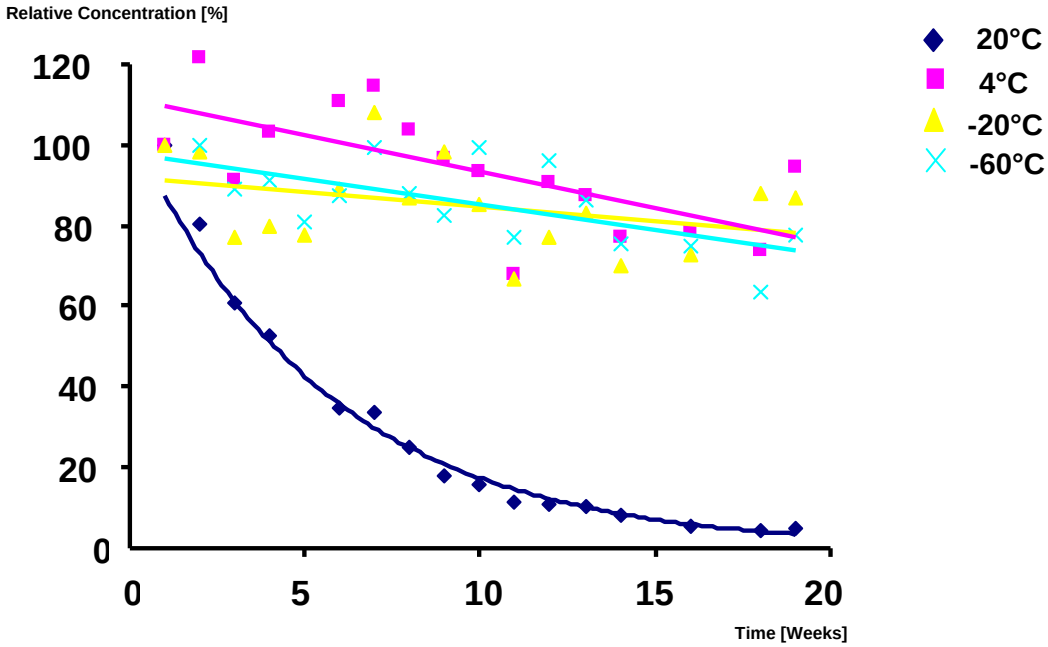

Figure 2: Relative concentration [%] of ziprasidone samples in the F experiment stored for 20 weeks at 20 ºC, 4 ºC, -20 ºC and -60 ºC

Table 3: Best fit equations for all drugs in the F experiment after 20 weeks of storage at 20°C, 4°C, -20°C, and -60°C

DRUG / STORAGE TEMPERATURE	20 °C	4 °C	- 20 °C	-60 °C
Risperidone	y = -1.3153x + 96.295	y = -1.5722x + 105.46	y = -1.9006x + 104.27	y = -1.1067x + 90.791
Haloperidol	y = -0.7531x + 91.201	y = -0.7647x + 100.34	y = -0.4103x + 99.447	y = -0.6584x + 99.723
Chlorpromazine	$y = 0.21x^2 - 6.908x + 104.82$	$y = 0.214x^2 - 6.433x + 105.27$	y = -2.8443x + 95.887	y = -0.4328x + 91.718
Chlorprothixene	y = -2.3091x + 93.077	y = -1.698x + 85.712	y = -1.8638x + 106.14	y = -0.7149x + 108.06
Droperidol	$y = 0.0525x^2 - 2.623x + 88.981$	y = -1.2579x + 93.006	y = -0.9654x + 110.64	y = -1.1841x + 93.007
Fluspirilene	y = 102.34x - 0.4499	y = 0.2749x2 - 9.549x + 99.169	y = -0.7773x + 91.083	y = -1.6946x + 90.483
Olanzapine	y = 82.925x - 1.2202	y = 69.783x-0.8876	y = 160x-1.2546	y = 62.485x - 0.4135
Thioridazine	y = -1.9333x + 109.02	y = -1.6814x + 102.5	y = -1.038x + 95.44	y = -1.5651x + 100.11
Trifluprom azine	y = -3.1384x + 103.75	y = -2.0125x + 90.267	y = -1.8487x + 111.68	y = -1.6893x + 116.27
Ziprasidone	y = 106.44e-0.1819x	y = -2.5004x + 117.04	y = -1.1653x + 93.15	y = -1.1592x + 97.221

6. Discussion

Following the unexpected death of a person, it can take days or even weeks until the individual is discovered. During this period of time, any drugs that the individual may have been exposed to prior to death remain in whole blood. The blood is therefore susceptible to the temperature conditions to which the deceased is exposed, potentially compromising the quality of the sample. PMR also becomes a concern for bodies that remain undiscovered for longer periods of time [24].

Once the body has been discovered and admitted to the mortuary, the deceased is stored at 4 °C until autopsy is performed; a period that typically averages two to three days at our Institute [25] but can vary in other organisations. The blood samples taken at autopsy for toxicological testing are stored at 4 °C, −20 °C, or -60 °C, depending on the individual laboratory policies. The testing may not occur for several weeks, during which time some drugs could degrade. Depending on the state of decomposition, it is often not possible to separate the blood cells from the sample in order to obtain plasma; therefore toxicological tests are routinely performed using whole blood [26].

In consideration of these factors, the choice of whole blood as a matrix for stability studies appears logical in order to obtain data that correlates more closely to the data obtained during analysis in many real cases. The storage temperatures of 20 °C, 4 °C, -20 °C, and -60 °C were selected as drugs in post-mortem specimens are likely to be exposed to these temperatures prior to toxicological testing.

In the P experiment, no stability issues were observed for haloperidol samples after 10 weeks under any storage condition. To the authors' knowledge, the only published study investigating the long-term stability of haloperidol in plasma (3 months at -20 ºC) was in

1987 by Haring *et al*. [27] and no stability problems were discussed. The discrepancy between the P and the F experiment regarding the storage at 20 °C for 10 weeks could possibly be explained by the inaccuracy of the method; with an expected 83% of the initial concentration still present after 10 weeks, the concentration is just outside the acceptance criteria of a 15 % loss.

The same reasoning could also explain the 10 - 20% losses of risperidone under all storage conditions after 10 weeks of storage in the F experiment, while the drug appeared to be stable in the P experiment. Chlorpromazine showed higher losses when stored at -20 °C than at 20 °C and 4 °C. This phenomenon has been seen for different drugs where lower storage temperatures seemed to be less favourable than higher temperatures [28, 29]. To the authors' knowledge, all studies to date have predominately investigated the stability of chlorpromazine in serum [30] and plasma [12], where no stability issues were discovered. A study carried out in 1984 by McKay *et al*. [31] found chlorpromazine in whole blood samples to be stable over 84 days when stored at -20 ºC; a longer storage interval has not been investigated. It seems surprising that the main degradation at -20 ºC takes place after four months of storage; further research needs to be undertaken to investigate this phenomenon.

For droperidol, the results for the stability of samples stored at -60 °C were unexpected. That the instability at -60 °C was consistent throughout the entire storage period (and similar to results at 20 °C and 4 °C) suggests that an unknown mechanism could be responsible. There have not been extensive stability studies conducted at -60 °C (as the refrigeration units required to reach these temperatures are costly and thus uncommon), and the authors have not seen this phenomenon described elsewhere in the literature. The contradictory results of

the olanzapine samples were not entirely unexpected, as several authors have reported conflicting results of stability studies involving olanzapine [7, 9, 32, 33]. A possible explanation for the differing results in P and F experiment could be the different batches of blood being used. Similar discrepancies between different blood samples have been reported by Nilsson *et al*. [21]. As it is unknown at this stage what is causing the instability of olanzapine in blood samples, different matrix influences might contribute to conflicting results. This needs always to be taken into consideration when interpreting olanzapine concentrations in blood samples in order to avoid inaccurate conclusions.

7. Conclusion

Even though the majority of the tested drugs appeared to be stable over 20 weeks of storage in whole blood, instability appears to be a serious problem for several drugs when stored at certain temperatures. Overall, -20 °C and -60 °C seem to be preferable for all drugs investigated in this study. All laboratories involved in the handling, processing and analysis of specimens, need to identify these potential risks and incorporate processes to adequately accommodate for them. As the storage conditions and turnaround times of analysis differ between laboratories, short-term stability experiments tailored to the laboratory's individual needs should be performed.

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Chapter 4

Degradation Product Analysis of Olanzapine

As the atypical antipsychotic drug olanzapine had been identified as particularly unstable in **Chapter 3**, **Chapter 4** focuses on the degradation product analysis of olanzapine. Chapter **4.1** describes the identification of 2-Hydroxymethyl-olanzapine as a novel degradation product of olanzapine in aqueous solutions. **Chapter 4.2** presents additional experiments that were carried out in order to try and determine degradation products of olanzapine formed in blood. However, these studies did not provide any conclusive results.

Chapter 4.1

Identification of 2-Hydroxymethyl-olanzapine as a Novel Degradation Product of Olanzapine

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Forensic Sci Int, 2012. **220** (1-3): p. 74-79

Monash University

Declaration for Thesis Chapter 4.1

Declaration by candidate

In the case of Chapter 4.1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conducted literature review, performed degradation studies and interpreted data. Wrote article.	85%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Gerostamoulos, D	Advised candidate regarding direction and content, reviewed and edited article	
Drummer, O. H	Advised candidate regarding direction and content, reviewed and edited article	
Beyer, J.	Advised candidate regarding direction and content, reviewed and edited article	

Candidate's Signature	[redacted]	**Date** 27/07/2012

Declaration by co-authors

The undersigned hereby certify that:

(19) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.

(20) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(21) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

(22) there are no other authors of the publication according to these criteria;

(23) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and

(24) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**Victorian Institute of Forensic Medicine**	
Signature 1	[redacted]	**Date** 27/7/12
Signature 2	[redacted]	27/7/2012
Signature 3	[redacted]	27/7/2012

ABSTRACT

Olanzapine (OLZ) is amongst the most commonly prescribed antipsychotic drugs and is associated with substantial instability. The aim of this study was to investigate the instability of OLZ and to identify the degradants formed from its breakdown. Three experiments were conducted to monitor the degradation of OLZ and the formation of degradants in blood (1), water (2), and post-extraction at 4 ºC (3). All three sample sets were analysed in duplicate and repeated in the absence (A) and presence (B) of 0.25 % ascorbic acid. One degradant was identified in sample sets 2A and 3A with m/z = 329 and confirmed as 2-hydroxymethyl-OLZ (2-OH-OLZ) using LC-MS. The addition of 0.25% ascorbic acid slowed the degradation of OLZ down in all three experiments and inhibited the formation of 2-OH-OLZ in sample sets 2A and 3A.The rate of degradation of OLZ and the rate of the formation of 2-OH-OLZ in water increased significantly following vortexing for 1 min while sonication did not affect the rate of degradation of OLZ, further suggesting the involvement of oxygen in the degradative processes. 2-OH-OLZ was only identified as a degradant of OLZ in aqueous solutions. It also degrades over time but its product is currently unknown and is under investigation.

Introduction

The atypical antipsychotic drug olanzapine (OLZ, Figure 1a) is amongst the most commonly prescribed antipsychotic drugs, not only for adults [1-5] but also for youths [6, 7]. Treatment with OLZ is associated with several health risks, including cardiovascular complications, an increased risk of sudden cardiac death (SCD) [8], diabetic complications ranging from "mild glucose intolerance to diabetic ketoacidosis" [9], a lowered seizure threshold level in epilepsy [10, 11] and fatal status epilepticus [12]. It is therefore not surprising that OLZ is commonly present in post-mortem cases [13].

A problem regularly associated with OLZ is its instability in blood. The stability in plasma and serum samples has been discussed in scientific publications for over a decade. Olesen *et al.* [14] performed several stability experiments and found that OLZ was unstable in human serum and ascorbic acid could reduce loss in stored samples [15]. In contrast, Lakso [16] found OLZ to be unstable in calf serum but stable in human serum. The stability in spiked and authentic human plasma samples without addition of an antioxidant was confirmed by Dusci *et al*. [17]. There have been several methods published, both with [18, 19] and without [20-22] antioxidants added to plasma samples prior to analysis. With contradictory results regarding the stability of OLZ in serum and plasma, it remains unclear whether anti-oxidants are necessary.

However, all studies of the stability of OLZ in whole blood have shown it to be unstable [23, 24], unless an antioxidant has been added to the blood. OLZ had to be excluded from a recently published method as the stability in post-mortem blood and processed sample stability could not be assured during validation studies [25]. Post-mortem drug testing is

most often performed in whole blood as plasma or serum is hard or impossible to obtain. While therapeutic drug monitoring (TDM) methods frequently describe the addition of an antioxidant such as ascorbic acid to samples suspected to contain OLZ, this is not common practice in post-mortem cases, where a wide range of drugs may be present. In death investigations, delays between the actual time of death, sampling and analysis, further increase the risk of significant OLZ losses.

Two degradation product studies using solid oral formulations of OLZ have been published to date and confirmed six degradation products in OLZ tablets: OLZ-lactam, OLZ-ketolactam, OLZ-ketothiolactam, OLZ-N-oxide, OLZ-keto-oxim and a dimeric compound [26, 27]. It is not known if any of these are formed in blood or plasma or whether other products are formed.

The aim of this study was to study the instability of OLZ in blood, to identify the degradants formed and if possible, study their formation and ultimate loss.

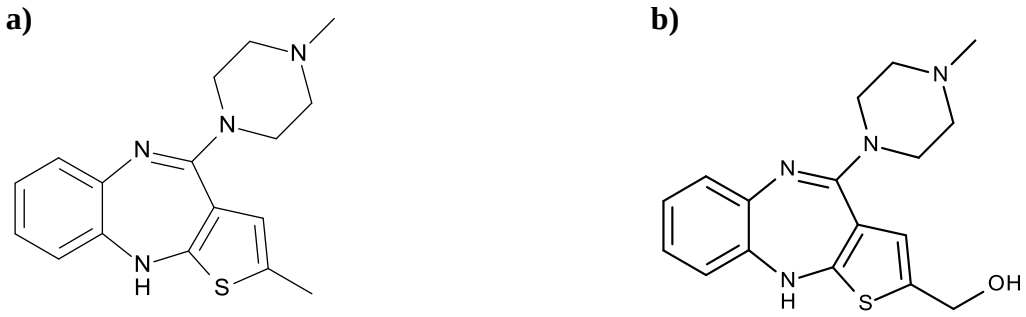


Figure 1: Chemical structure of OLZ (a) and 2-OH-OLZ (b)

Materials and methods

Chemicals and reagents

OLZ was obtained from the Division of Analytical Laboratories, (Lidcombe, NSW, Australia). 2-hydroxymethyl OLZ (2-OH-OLZ) and OLZ N-oxide was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). The isotope-labelled internal standard haloperidol-d_4 was purchased from Cerilliant (Round Rock, TX, USA). Acetonitrile (ACN), ammonium formate, 1-chlorobutane, methanol, and formic acid were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from BDH Chemicals (Kilsyth, VIC, Australia). Ascorbic acid was obtained from Crown Scientific (Minto, NSW, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia).

Specimens

Whole blood for degradation product experiments and preparation of calibration curves was obtained from the local blood bank in lithium heparin-coated plastic bags (500 mL). The blood was aliquoted into 10 mL polypropylene tubes containing 200 mg sodium fluoride and 30 mg potassium oxalate. Blood was analysed using a previously published method and was found to be "drug-free" [25]. All blood samples were stored at −20 °C prior to analysis.

Equipment

The LC-MS/MS system used for the determination of the degradation products of OLZ consisted of an AB SCIEX Q-TRAP® 5500 linear ion-trap quadrupole mass spectrometer (AB SCIEX, Melbourne, VIC, Australia), equipped with a Turbo V ion source, operated in the electron spray ionization (ESI) mode coupled with an Shimadzu Prominence high performance liquid chromatography (HPLC) system (Shimadzu, Melbourne, VIC, Australia) which consisted of a degasser, two eluent pumps, a column oven and an autosampler.

The LC-MS/MS system used for the high resolution confirmation of the identified degradation product of OLZ consisted of an AB SCIEX TripleTOF™ 5600 system (AB SCIEX, Shanghai, China) coupled with a HPLC system as described above.

Gradient elution was performed on an Agilent Zorbax Eclipse XDB-C_{18} (4.6 mm ×150 mm, 5 µm particle size; Biolab, Scoresby, VIC, Australia). The gradient elution using 50 mmol/L aqueous ammonium formate adjusted to pH = 3.5 with formic

acid (eluent A) and ACN containing 0.1 % formic acid (eluent B) has been described in a previous publication [25].

Experiments using the AB SCIEX Q-TRAP® 5500 system and the AB SCIEX TripleTOF™ 5600 system for mass spectrometric identification and confirmation of degradants used the following inlet conditions for Q1 scan and Product Ion Scan 329: gas 1 nitrogen (90 psi; 620.5 kPa); gas 2 nitrogen (90 psi; 620.5 kPa); ion-spray voltage (IS) 5500 V; ion-source temperature 750 °C; curtain gas, nitrogen (10 psi; 68.9 kPa). For the Product Ion Scan 329, the following additional settings were chosen: collision energy (CAD) was set at medium; declustering potential (DP) 140.0.

Preparation of Standards and Extracts

Stock solutions were prepared at a concentration of 1 mg/mL by separate weighings dissolved in ACN.

Working solutions of each analyte were prepared using ACN by independent dilution from each stock solution at the following concentrations: 0.1 mg/mL, 0.01 mg/mL, and 0.001 mg/mL. All solutions were stored at -60 °C.

Five calibration standards were prepared at concentrations of 0.01 mg/L, 0.05 mg/L, 0.1 mg/L, 0.25 mg/L and 0.5 mg/L using 50 uL of Eluent B and 450 uL of Eluent A and spiking solutions prepared from the working solutions as mixtures of OLZ and 2-OH-OLZ at concentrations 10 times higher than the corresponding calibration standards.

The liquid-liquid extraction using 1-chlorobutane and trizma buffer was described in a previous publication [25].

Stability experiments and identification criteria

Three experiments (Set 1-3) were set-up to examine the degradation of OLZ in blood and water in the absence (A) and presence (B) of 0.25 % ascorbic acid. All sample sets were prepared in duplicate at 0.1 mg/L and stored at 4 ºC for 21 days in order to monitor the degradation of OLZ and the formation of potential degradation products (Table 1).

Set 1 contained blood spiked with OLZ and the extraction and analysis was performed daily over three weeks.

Set 2 contained distilled water spiked with OLZ left on the autosampler.

Set 3 contained blood spiked with OLZ, extracted immediately, reconstituted into 50 mmol/L aqueous ammonium formate adjusted to pH = 3.5 with formic acid and ACN containing 0.1 % formic acid and left on the autosampler.

For sample sets 2 and 3, injection was performed daily over three weeks.

The identification of degradation products involved direct comparison with reference standards, rather than a library match. Therefore, match factors were not defined for positive identification. Accurate mass deviation limits of 5 ppm were applied and considered acceptable.

Influence of oxygen on the degradation of OLZ

To investigate the influence of oxygen on the degradation of OLZ in aqueous solutions, to replicate a real life situation post-extraction, a series of experiments were conducted in

which OLZ was present in distilled water and the solution was variously agitated to alter the exposure to air.

Set 4 consisted of three subsets A, B and C which were stored at 4 ºC for 21 days. All of these sets were prepared in triplicate and contained OLZ in distilled water at 0.2 mg/L. Set 4A had no further treatment, Set 4B was vortexed for 1 min and Set 4C was sonicated for 5 min before loading onto the autosampler. Injection and analysis was performed daily over three weeks.

Table 1: Overview over sample sets 1-4 and the concentration of OLZ

SET	A	B	C
1	blood 0.1 mg/L	blood 0.1 mg/L + ascorbic acid	-
2	water 0.1 mg/L	water 0.1 mg/L + ascorbic acid	-
3	reconstitution post-extraction 0.1 mg/L	reconstitution post-extraction 0.1 mg/L + ascorbic acid	-
4	water 0.2 mg/L 'normal'	water 0.2 mg/L vortexed	water 0.2 mg/L sonicated

Statistical analysis

Statistics were performed using IBM SPSS Statistics 19. A repeated measures analysis of variance (ANOVA) test was performed to determine the influence of different oxygen levels on the degradation of OLZ and the formation of 2-OH-OLZ. Sphericity was tested for by Mauchly's test and the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. $P < 0.05$ was considered statistically significant. The F ratio, degrees of freedom, outcome and significance values are reported.

Results and Discussion

Loss of OLZ and identification of 2-OH-OLZ as degradant

In order to investigate the loss of OLZ and formation of a degradant, a Q1 scan and product ion scan for all six m/z of previously described degradation products of OLZ in solid oral formulations [26, 27] was carried out daily on sample sets 1-3 (and sub-sets A and B).

The only significant peak in comparison with a blank sample was found for m/z 329 in sample set 2A and 3A, possibly corresponding to OLZ N-oxide as described by Baertschi *et al.* [26]. The respective peak area was plotted over time in the following experiments. The R.S.D between sample duplicates was always < 15 % unless otherwise indicated.

The degradant with m/z = 329 was compared with the commercially purchased standards of OLZ N-oxide and 2-OH-OLZ (Figure 1b), a minor *in vivo* metabolite of OLZ [28], both giving an ion at m/z 329 using the AB SCIEX TripleTOF™ 5600 system. Figure 2 shows the comparison between accurate mass and fragmentation patterns of a commercial standard of 2-OH-OLZ (a) and the degradant with m/z = 329 (b). The detected mass and the isotopic pattern of the commercial standard and the degradation product match a theoretical mass of 2-OH-OLZ within acceptance criteria.

The OLZ concentration in sample set 1A containing OLZ spiked in blood stored at 4 ºC decreased rapidly with a 100 % loss of drug after four days of storage (Figure 3). The addition of ascorbic acid slowed the loss but all OLZ was lost after ten days of storage at 4 ºC (set 1B). Despite the rapid and substantial loss of OLZ, no 2-OH-OLZ was found in the stored blood samples (set 1). This indicated that the loss of OLZ in stored blood

samples resulted in a different degradation product than 2-OH-OLZ. Comparison with a reference standard of OLZ N-oxide also did not provide a match.

However, the degradation of OLZ in water at 4 ºC (set 2A) was associated with the formation of 2-OH-OLZ. When averaging the duplicate of sample set 2A (Figure 4), the R.S.D. for degradation of OLZ and the formation of 2-OH-OLZ was > 15 %. This indicates that there were significant differences between these two single samples that formed the duplicate. The peak areas of the duplicate samples of set 2A are therefore plotted separately (Figure 4a +b). In both samples OLZ degraded over time and 2-OH-OLZ was formed. Additionally, 2-OH-OLZ appeared to be unstable, as its degradation was seen in sample one of the duplicate (Figure 4a) after five days and in sample two (Figure 4b) after 15 days. The degradation of OLZ was slower in sample two (Figure 4b), with a total loss of OLZ after 15 days of storage at 4 ºC. OLZ degradation in sample one (Figure 3a) was seen after five days at 4 ºC.

Interestingly, the time and rate of degradation of 2-OH-OLZ appears to correspond to the respective OLZ concentration. The total conversion rate from OLZ to 2-OH-OLZ was approximately 50 % in both samples.

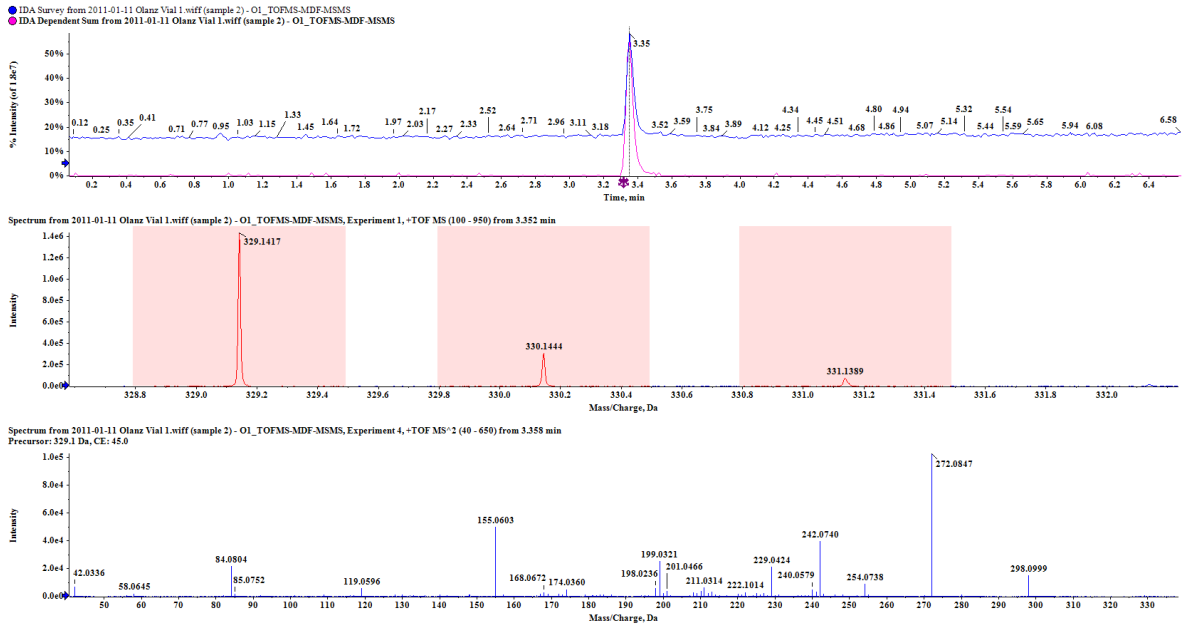
Set 2B, distinguishable to set 2A in the addition of ascorbic acid to the samples prior to storage, (Figure) showed a much slower decrease in OLZ concentration, with a ~ 30 % loss after three weeks at 4 ºC. 2-OH-OLZ was not formed in these samples. It appears that the addition of ascorbic acid to aqueous OLZ samples does not only slow the degradation of OLZ down but also completely inhibits the formation of 2-OH-OLZ.

Set 3A was used to investigate the processed sample stability of OLZ over three weeks. This set showed ~ 35 % degradation of OLZ over three weeks and a small formation of 2-OH-OLZ (Figure 5).

Sample set 3B was used to investigate the processed sample stability and formation of a degradant after addition of ascorbic acid. This set showed very little instability of OLZ and no formation of 2-OH-OLZ (Figure 5). Again, the addition of ascorbic acid slowed the rate of degradation of OLZ, similar to the previous sample sets.

The significant loss of OLZ in sample set 3A was not unexpected. Major degradation in extracted OLZ samples post-extraction was observed in a previous publication when extraction was performed using the same method [25].

a)



b)

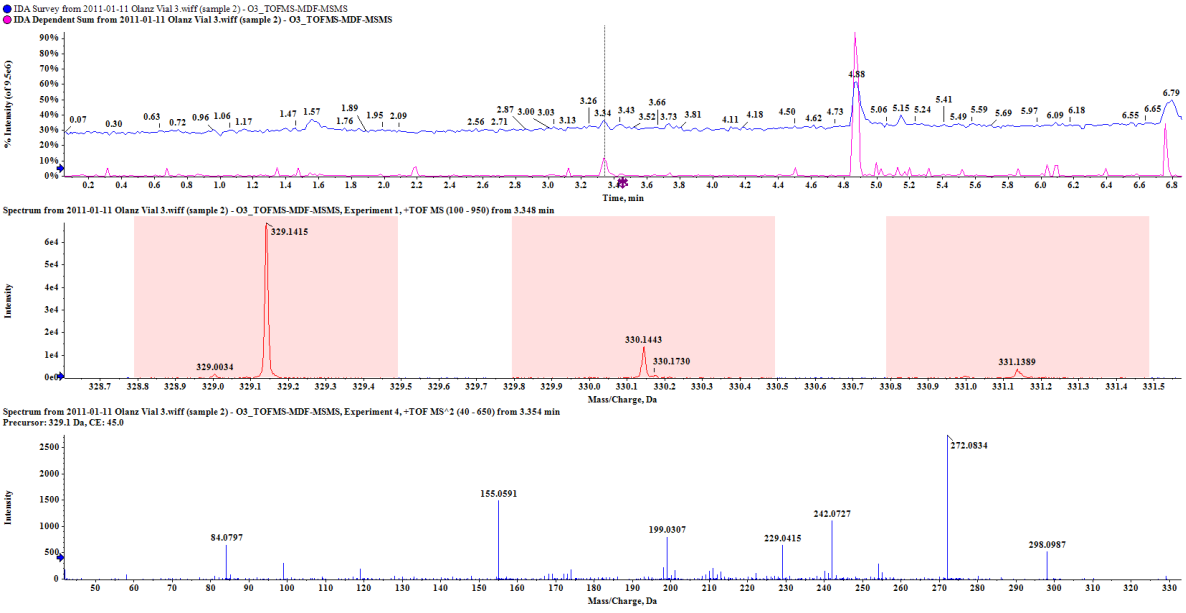


Figure 2: Chromatogram of information dependent acquisition (IDA) scan (top row), TOF scan at 3.34min (middle row), TOF-MS/MS spectra of m/z = 329 at 3.34 min (bottom row) of the synthetic standard of 2-OH-OLZ (a) and the degradation product identified in sample set 2A and 3A (b)

Influence of oxygen on the degradation of OLZ and the formation of 2-OH-OLZ

Sample sets 4A-C were prepared in order to study the effect of different oxygen concentrations on the degradation of OLZ and the formation of 2-OH-OLZ. While sample set 4A was prepared by simple addition of an OLZ spike solution to water, sets 4B and 4C were either vortexed (4B) or sonicated (4C) prior to sample analysis, in order to alter the oxygen content in the sample. The R.S.D was < 15 % from the mean for all analysed sample triplicates and therefore in an acceptable range.

Figure 6a shows the decrease in OLZ concentration over 21 days in sample sets 4A-4C.

Statistical analysis revealed that the degradation of OLZ was significantly different between the three sample sets, $F(1.416, 21.243) = 33.25$, $p<.05$. Post-hoc tests confirmed that the visual observation of the degradation of OLZ in sample set 4B (containing an additional vortexing step) was significantly different from sample set 4A and 4C, while sample set 4A and 4C were not different from each other.

Sample set 4B also showed the highest formation of 2-OH-OLZ and the most rapid increase (Figure 6b). Statistical analysis revealed that the formation of 2-OH-OLZ was significantly different between the analysed sample sets, $F(1.175, 21.41) = 29.8$, $p<.05$. Post-hoc tests revealed that sample set 4B was significantly different from sample set 4A and 4C, whereas 4A and 4C were not different from each other. It appears that even 1 min of vortexing resulted in a statistically significant loss of OLZ compared with the normal sample preparation that only included a few seconds of vortexing. 5 min of ultrasonication did not create a significant difference between sample set 4C and any

other sample set. The conversion rate of OLZ to 2-OH-OLZ appeared to be between 25 % and 40 %.

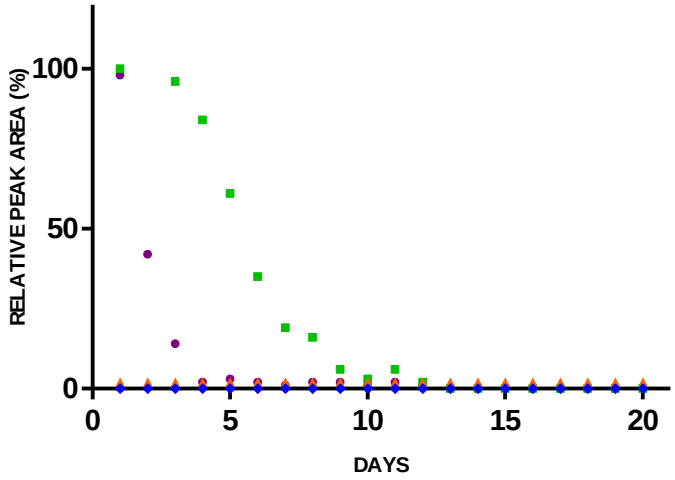


Figure 3: Degradation of OLZ in set 1A (blood) without ascorbic acid (●) and set 1B in blood samples with 0.25 % ascorbic acid (■) over three weeks at 4 ºC and the corresponding formation of a peak at m/z = 329 without (▲) and with ascorbic acid (◆)

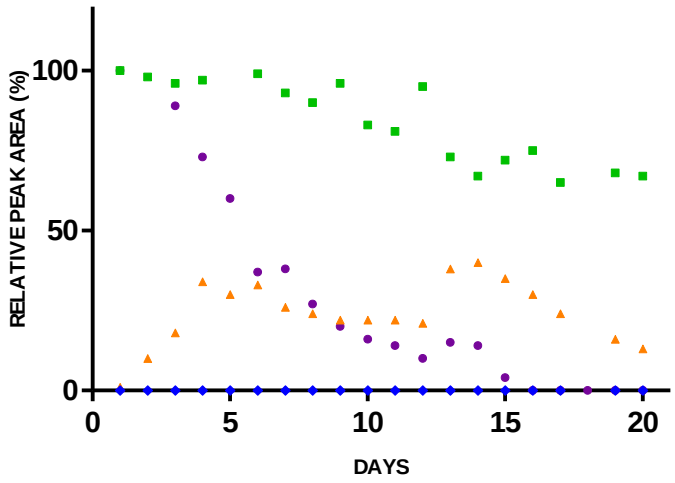


Figure 4: Degradation of OLZ in Set 2A (water) without ascorbic acid (●) and Set 2B in water samples with 0.25 % ascorbic acid (■) over three weeks at 4 ºC and the corresponding formation of a peak at m/z = 329 without (▲) and with ascorbic acid (◆)

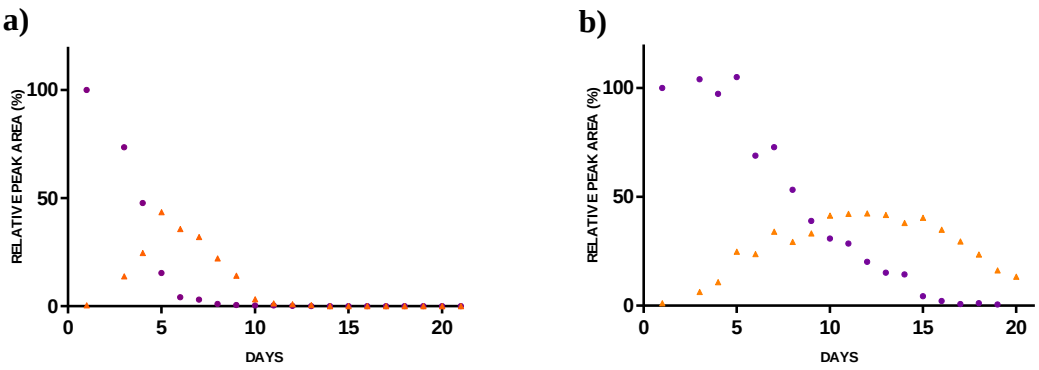


Figure 5: a) Degradation of OLZ in sample 1 of set 2A (water) without ascorbic acid (●) and the corresponding formation of a peak at m/z = 329 without ascorbic acid (▲) over three weeks at 4 ºC.

b) Degradation of OLZ in sample 2 of set 2A without ascorbic acid (●) and the corresponding formation of a peak at m/z = 329 without ascorbic acid (▲) over three weeks at 4 ºC.

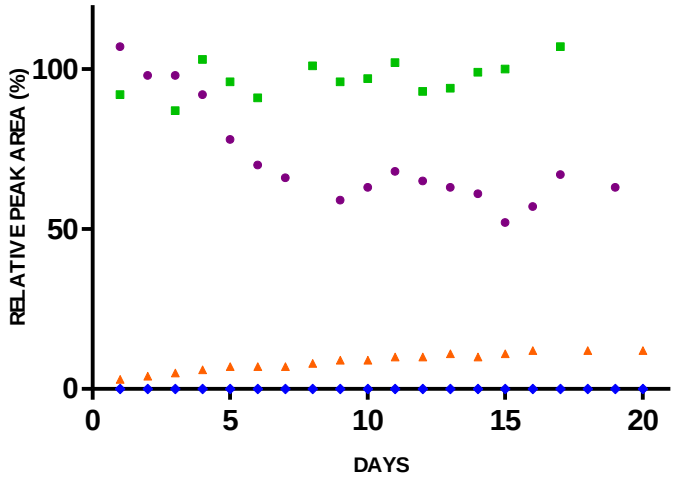


Figure 6: Degradation of OLZ in set 3A (post-extraction) without ascorbic acid (●) and set 3B post-extraction with 0.25 % ascorbic acid (■) and the corresponding formation of a peak at m/z = 329 without (▲) and with ascorbic acid (◆) over three weeks at 4 ºC

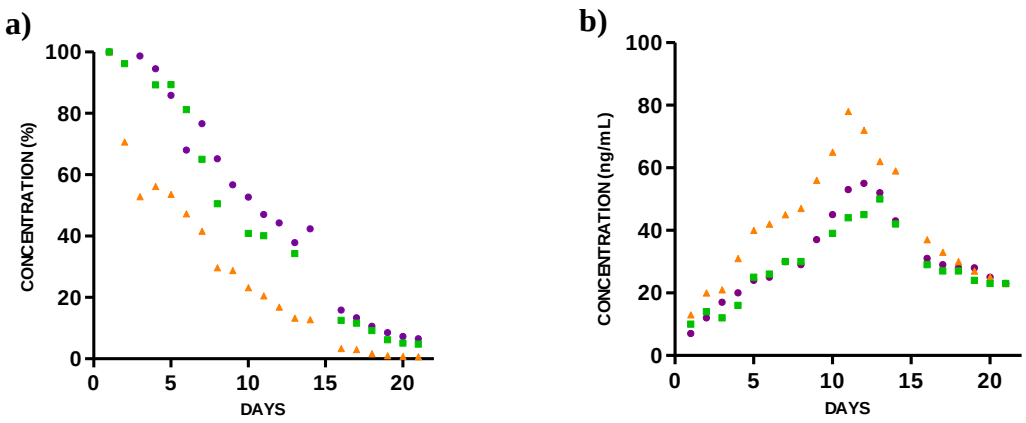


Figure 7: a) Degradation of OLZ and b) formation of 2-OH-OLZ in water kept over three weeks at 4 ºC without further preparation (set 4A ●), after 1 min of vortexing (set 4B ▲), and after 5 min of ultrasonication (set 4C ■).

Conclusions

Ascorbic acid slows down the degradation of OLZ in stored blood samples (~ 50 %) and completely inhibits instability in processed samples over three weeks.

2-OH-OLZ was identified as a degradation product of OLZ that is formed in aqueous solutions and accounted for approximately 25 – 50 % of the loss of OLZ. 2-OH-OLZ also appears to be unstable and subsequently degrades to a product that remains unknown.

The formation of 2-OH-OLZ is affected by different oxygen concentrations which might be part of the reason for the discrepancies in different reports regarding the stability of OLZ. It is recommended to keep vortexing steps consistent during sample preparation in order to preserve sample integrity. However, 2-OH-OLZ was not formed in whole blood and its formation is inhibited by the addition of ascorbic acid in aqueous solutions. As the instability of OLZ in blood samples remains a major problem in analytical toxicology, further investigations for the identification of degradation products in blood are required.

Acknowledgments:

The authors would like to thank AB SCIEX and especially Dr. Brad Patterson for the opportunity to use the AB SCIEX TripleTOF™ 5600 system in Shanghai (China) for this study and his helpful advice.

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Chapter 4.2

Derivatisation and GC-MS Analysis of Degraded Olanzapine Blood Samples

4.2.1 INTRODUCTION

As the identification of degradation products of OLZ in blood was not successful using LC-MS technology (**Chapter 4.1**) additional experiments were carried out using GC-MS. Various methods were used in an attempt to identify possible degradants of OLZ.

Spiked OLZ samples were subject to accelerated degradation when stored at room temperature for six days. The degraded samples were subjected to different extraction techniques, using basic (pH = 9.2) and acidic (pH = 4.5) in order to try and accommodate for a potential change in physicochemical properties of OLZ post-degradation. Various derivatisation techniques including acylation, trimethylsilylation and methylation were used prior to injection of the extracts into the GC-MS system. The total ion chromatograms (TIC) were compared to freshly spiked OLZ samples, subjected to the same treatment, in an attempt to identify degradation products unique to the degraded samples. As OLZ has shown to be prone to oxidation in previous studies, focus was placed on a potential mass change of multiples of 16 Da, produced by the introduction of oxygen atoms during a proposed oxidation process, in addition to the expected mass change caused by the respective derivatisation [74].

4.2.2 CHEMICAL AND REAGENTS

OLZ was obtained from the Division of Analytical Laboratories, (Lidcombe, NSW, Australia). Acetonitrile (ACN), ammonium formate, 1-chlorobutane and methanol were purchased from Merck (Darmstadt, Germany). Acetic acid and sodium acetate was purchased from BDH Chemicals (Kilsyth, VIC, Australia). Trifluoracetic anhydride (TFAA), pentafluoropropionic anhydride (PFAA) , 2,2,3,3,3,-pentafluoro-1-propanol (PFA),

heptafluorobutyric anhydride (HFBA), iodomethane containing copper as stabiliser (Reagent Plus®, 99.5 %), and N,O-Bis(trimethylsilyl)trifluoracetamide with 1% trimethylchlorsilane (BSTFA/TMCS) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) (Figure 1). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia).

4.2.3 APPARATUS

The GC-MS system used was a GC-MS-QP2010 Plus system by Shimadzu (Australia).

All separations were performed on a HP-1 GC capillary column (30 m, 0.32 mm I.D, 0.25 µm film thickness, Agilent Technologies, Australia) with the following temperature program: 70 ºC for 1 min, from 70 ºC to 300 ºC at 25 ºC / min and finally 5 min at 300 ºC. The temperature of the ion source was 200 ºC and the interface temperature 250 ºC. Helium was used as a carrier gas at a column flow of 1.88 mL/min. Injections (1 µL) were performed in splitless injector mode. Mass spectra of OLZ and its derivatives were recorded in full scan mode over a mass range of m/z 40-600.

4.2.4 METHODS AND EXTRACTION PROCEDURES

Degraded OLZ samples were prepared by spiking 500 µL of a 1 mg/mL solution of OLZ in ACN into 5 mL of whole blank blood, vortexing and separation into 50 aliquots containing 100 µL each. 50 control samples were prepared identically, without the addition of OLZ in ACN.

All samples were left to degrade at room temperature (20 ºC) for a total of six days, as a previous study had shown that significant degradation of OLZ was obtained in blood samples

under these conditions [75]. Extractions were performed at day zero, three, and six. Additionally, blood samples containing OLZ were freshly prepared on each extraction day and extracted alongside the degraded samples.

Two extraction methods were used; one at pH 9.2 for neutral and basic substances and one at pH = 4.5 for potentially acidic products. 2 M Trizma buffer was used for pH = 9.2 and 1.1 M sodium acetate buffer at pH = 4.5. 1-Chlorobutane was used as extraction solvent in accordance with a previously published method [76] (Figure 2).

Various derivatisation techniques were applied to the degraded sample extracts. Considering the structure of the degradation products was unknown, the method was designed to include a wide range of derivatising agents, which target different functional groups, such as aldehydes, ketones and carboxylic acid. Figure 1 shows the derivatising agents used and Figure 2 shows the setup of the degradation experiments.

a) Acylation

   Post basic extraction (pH = 9.2) and nitrogen dry-down, 50 µL of every derivatising agent was added to the respective sample: (a) TFAA, (b/c) PFAA/PFA, (d) HFBA

   Sample extracts were incubated for 30 min at 70 ºC, dried down under nitrogen and reconstituted in 50 µL of methanol.

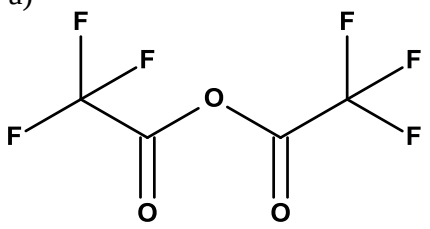
b) Trimethylsilylation

   The dry extracts following the basic extraction (pH = 9.2) were treated with 50 µL of BSTFA + 1% TMCS (e/f) and left for 30 min at 90ºC. The mixture was injected into the GC-MS system without further preparation.

c) Methylation

The dry extracts following the acidic extraction (pH = 4.5) were treated with 50 µL of iodomethane and left for 30 min at 70 ºC. Subsequently, the samples were dried down under nitrogen and reconstituted in 50 µL of methanol.

a)

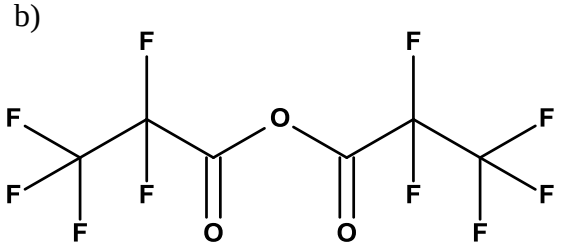


TFAA

Molecular formula: $(CF_3CO)_2O$

Formula weight: 210.03

b)

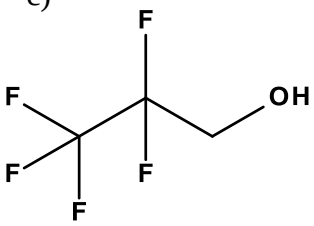


PFPA

Molecular formula: $(CF_3CF_2CO)_2O$

Formula weight: 310.03

c)

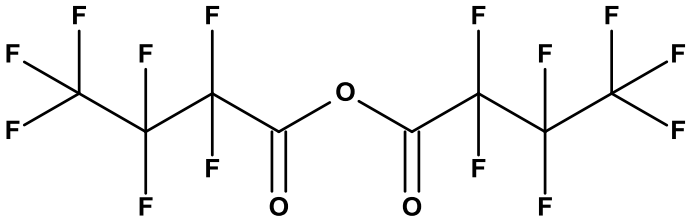


PFPOH

Molecular formula: $CF_3CF_2CH_2OH$

Formula weight: 150.01 Da

d)

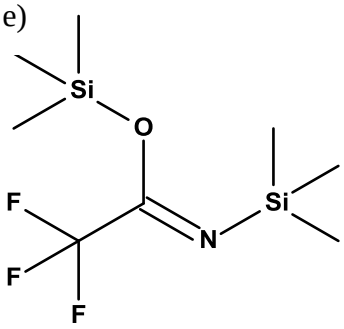


HFBA

Molecular formula: $(CF_3CF_2CF_2CO)_2O$

Formula weight: 409.96 Da

e)

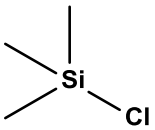


BSTFA

Molecular formula: $CF_3C[=NSi(CH_3)_3]OSi(CH_3)_3$

Formula weight: 257.08 Da

f)

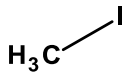


TMCS

Molecular formula: $(CH_3)_3SiCl$

Formula weight: 108.02 Da

g)



Iodomethane

Molecular formula: CH_3I

Formula weight: 141.93 Da

Figure 1: Structures, molecular formulas and formula weight [Da] of derivatising agents used in this study

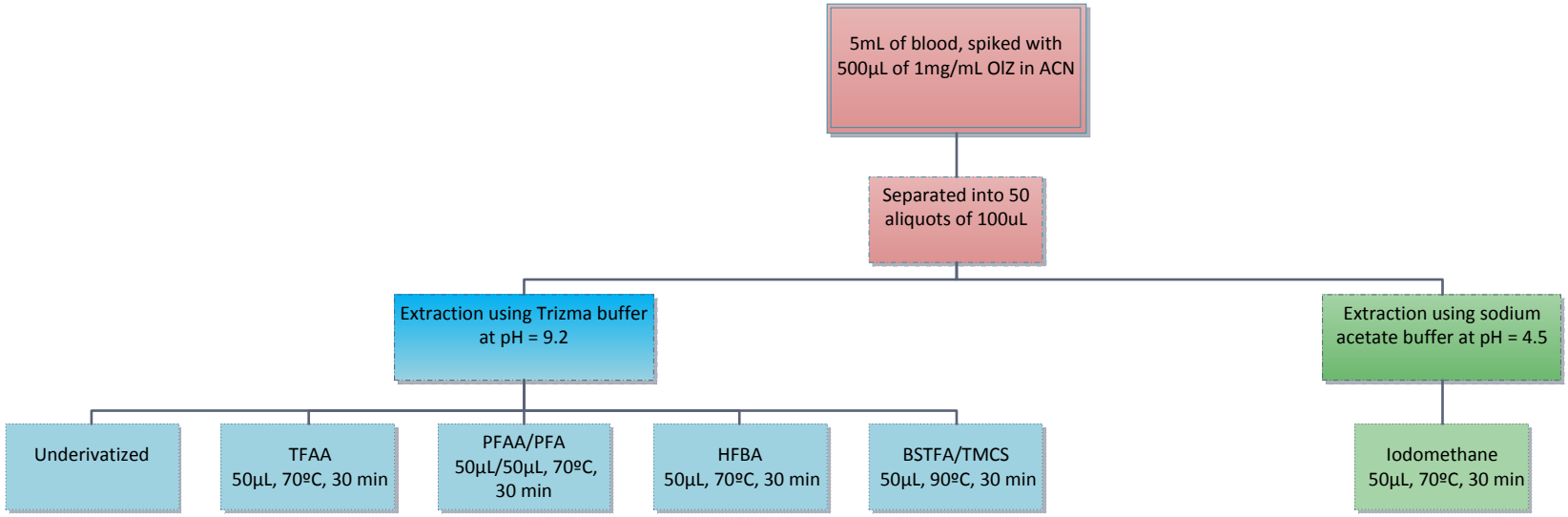


Figure 2: Setup of the degradation experiment using different derivatisation agents

Abbreviations: OLZ = olanzapine, ACN = acetonitrile, TFFA = Trifluoracetic anhydride, PFAA/PFA = pentafluoropropionic anhydride/pentafluoro-1-propanol, HFBA = heptafluorobutyric anhydride, BSTFA/TMS = N,O-Bis(trimethylsilyl)trifluoracetamide with 1 % trimethylchlorsilane

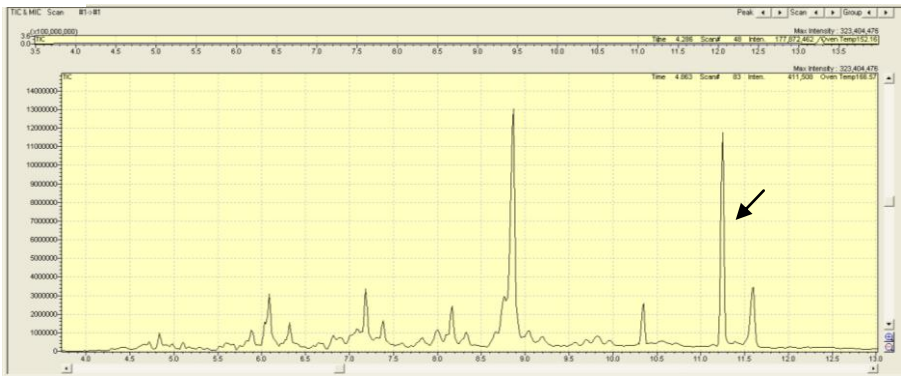
4.2.5 RESULTS AND DISCUSSION

Analysis of the underivatised blood containing 100 µg/mL OLZ extracted on Day 0 resulted in a peak at 11.25 min with base ion at m/z = 312, representing OLZ. The acylation pathway with TFAA resulted in a product with a pseudo-molecular ion of m/z = 408, eluting at 10.5 min. Derivatisation with PFAA/PFA yielded the expected product with m/z = 458 at 10.3 min; addition of HFBA resulted in a base ion at m/z = 508, eluting at 10.35 min. Trimethylsilylation with BSTFA/TMS yielded a product with m/z = 384 eluting at 10.75 min at Day 0. As expected, all OLZ derivatives eluted earlier than the parent ion, due to their increased lipophilicity in comparison with OLZ.

Acidic extraction at pH = 4.5 followed by methylation using iodomethane did not show a significant peak post-injection at Day 0, however, this was not unexpected, as no acidic product was likely to be present prior to degradation in the blood samples containing OLZ.

An example chromatogram is given in Figure 3, showing the TIC of an underivatised OLZ sample on (a) Day 0 and (b) Day 6 (b) post-degradation of six days. The peak at 11.25 min representing OLZ has decreased by approximately 90 %, which is consistent with results obtained in previous stability studies, where loss of almost 100 % was seen in blood samples containing OLZ stored at room temperature for one week [75]. Despite the substantial degradation of OLZ, no additional peaks were found in the chromatogram post-degradation at any time point.

a)



b)

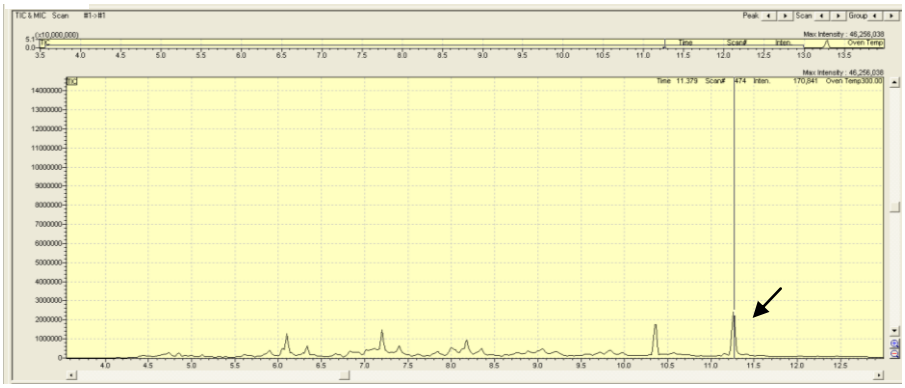


Figure 3: Total Ion Chromatogram of non-derivatised OLZ blood extract on Day 0 (a) and Day 6 (b); peak at 11.25 min is OLZ.

OLZ sample extracts which were acylated or trimethylsilylated post-basic extraction at pH = 9.2, showed similar losses of ~90 % to underivatised samples after six days of degradation. Comparison of these extracts with equally treated blank blood samples and freshly spiked OLZ samples did, however, not reveal any additional peaks. Acidic extraction (pH = 4.5) followed by methylation using iodomethane was performed on OLZ samples after six days of storage and the resulting chromatograms compared with the identical extraction on Day 0, prior to degradation. No differences were noted, suggesting that no acidic degradation product was extracted from these samples.

In a previous LC-MS studies (**Chapter 4.1**), OLZ-N-Oxide (m/z = 329) initially appeared to be the most likely degradant of OLZ in aqueous solutions, however, 2OH-methyl OLZ (m/z = 329) was identified using a reference standard with no OLZ-N-Oxide present in the degraded samples. Both compounds were targeted in this study in degraded blood samples but their presence could not be confirmed.

While no degradation products of OLZ have been determined in blood thus far, two drug substance degradation products of OLZ were identified during drug substance stress testing studies with solid oral formulations by Baertschi *et al.* in 2008 [77]. Both degradants appeared to be formed by oxidation and ring-opening of the thiophene ring of OLZ, resulting in base ion m/z = 312 and m/z = 328, respectively. Hiriyani *et al*. subjected olanzapine bulk drug to hydrolytic (acidic and alkaline), oxidising, and photolytic conditions in their study and reported three degradation products with m/z = 247, m/z = 231, and m/z = 459 [78]. However, none of these compounds could be identified in the degraded blood samples in this study.

Three possible explanations are likely. Firstly, the pKa of the resulting degradation products is currently not known, therefore it is not clear at which pH the highest extraction efficiency will be reached. Despite having included a basic (pH = 9.2) and an acidic (pH = 4.5) pH, the conditions may not be optimal to extract the compounds of interest from the sample matrix. Secondly, the degradation products may be much smaller fragments which show no similarity with OLZ and may not be seen in the profile, or alternatively, are too polar to extract under the chosen conditions. As the parent compound was extracted and analysed successfully using the extraction techniques described above, it is likely that the degradation

products formed have very different physicochemical properties to OLZ, and therefore require a different extraction or analysis technique.

Chapter 5
Post-mortem redistribution of Antipsychotic Drugs

In **Chapter 5.1**, a summary of the process of coronial death investigation at the Victorian Institute of Forensic Medicine (Victoria, Australia) was described, which formed the basis of the post-mortem redistribution study carried out in **Chapter 5.2**. The post-mortem redistribution of 10 antipsychotic drugs was investigated, using blood samples from a peripheral site. The study presented in **Chapter 5.2** revealed that most antipsychotic drugs are subject to increases and losses in the femoral vein after the death of an individual, potentially altering drug blood concentrations post-mortem.

5.1 THE PROCESS OF CORONIAL DEATH INVESTIGATION IN VICTORIA AT THE VICTORIAN INSTITUTE OF FORENSIC MEDICINE (VIFM)

In the state of Victoria, Australia, a coroner's investigation is undertaken in all unexpected, suspicious or violent deaths. The Victorian Institute of Forensic Medicine (VIFM) plays an essential role in these death investigations, providing forensic medical and associated scientific services to the coroner. This includes forensic toxicology which is conducted on most of approximately 6000 coroner's cases per year. In an attempt to streamline the death investigation process and optimise time and resources, the toxicology laboratory has adopted greater responsibility in this process in recent years. Since November 2009, upon admission of a deceased person to the mortuary, a so-called "admission blood sample" (AD sample) is taken from the femoral vein. Within 24 h, the toxicology laboratory provides a semi-quantitative result of drugs detected in this AD sample, comprising 127 of the most common drugs and drugs of abuse, including the 10 APs of interest in the study presented in **Chapter 5.2.**

Along with details of the medical history of the deceased and the circumstances surrounding the death, this result plays an integral role in determining whether or not an autopsy is required as distinct from an external examination and review of medical records and initial toxicology results. This decision is ultimately made by a coroner and the case pathologist once the analytical results and case information are available (usually the subsequent day to this initial toxicological analysis).

If an autopsy is required, an "Autopsy blood sample" (PM sample) is taken from the femoral vein during this procedure. When performing further toxicological testing and reporting results, preference is generally given to the AD specimen, as drug concentrations in the

admission sample are likely to be more closely related to drug concentrations at the time of death.

The autopsy can take days to proceed depending on legal processes between the coroner and the family which means that when post-mortem samples are collected they can be compared to the admission (AD) blood specimen. This is a unique situation that was exploited in a series of studies to understand the change sin blood concentration that might occur with anti-psychotic drugs.

Chapter 5.2

The Time-dependent Post-mortem Redistribution of Antipsychotic Drugs

Saar, E., Beyer, J., Gerostamoulos, D., & Drummer, O.H.,
Forensic Sci Int, 2012. (*ePub*)

Monash University

Declaration for Thesis Chapter 5.2

Declaration by candidate

In the case of Chapter 5.2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Conducted case searches, performed case analyses and interpreted the data. Wrote article.	85%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Beyer, J	Advised candidate regarding direction and content, reviewed and edited article	
Gerostamoulos, D	Advised candidate regarding direction and content, reviewed and edited article	
Drummer, O.H	Advised candidate regarding direction and content, reviewed and edited article	

Candidate's Signature	[redacted]	**Date** 27/07/2012

Declaration by co-authors

The undersigned hereby certify that:

(25) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(26) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(27) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(28) there are no other authors of the publication according to these criteria;
(29) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(30) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

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ABSTRACT

The post mortem redistribution of ten commonly prescribed antipsychotic drugs (APs) was investigated. Femoral blood was collected from 273 cases at admission to mortuary (AD) and at post-mortem (PM). The PM samples were collected at various times up to nine days after admission and the sample pairs analysed using LC-MS/MS. The drugs included in this study were 9OH-risperidone (paliperidone), amisulpride, chlorpromazine, clozapine, haloperidol, olanzapine, promethazine, quetiapine, risperidone, and zuclopenthixol. Haloperidol, quetiapine and risperidone showed minimal changes between AD and PM specimens, whereas the majority of drugs showed significant changes between the sample pairs collected at different time points post mortem ($p < 0.01$) in addition to an average concentration change greater than the uncertainty of measurement of the applied method. Average increases in blood concentrations after admission to the mortuary ranged up to 112 % (chlorpromazine and olanzapine) but also decreases up to -43 % (9OH-risperidone) were seen. There were large standard deviations between sample pairs and substantial day-to-day unpredictable changes that highlight the difficulty in the interpretation of drug concentrations post-mortem. Based on the presented data, we recommend that specimens for toxicological analysis should to be taken as soon as possible after admission of a deceased person to the mortuary in order to minimise the effects of the PM interval on the drug concentration in blood.

Introduction

Post mortem redistribution (PMR) is a well-recognised but under-explored phenomenon that complicates the interpretation of drug concentrations in medico-legal death investigations. It is believed to occur by diffusion of drug from tissue-bound stores at higher concentrations adjacent to blood vessels into blood after death, therefore increasing blood concentrations post-mortem [1]. The two main factors that appear to influence the PMR of a drug are sampling site and time of sampling relative to the time of death. Peripheral blood is regarded as more suitable for post-mortem drug testing because of its distance from central organs and the gastrointestinal tract [2, 3].

PMR has been most associated with a large volume of distribution (V_d) > 3 L/kg and a high degree of lipophilicity [2, 4-7]. Basic drugs are considered to be more susceptible to PMR as their ionised fraction increases with the mainly aqueous content of cells as they become more acidic post-mortem. During post-mortem lysis of cells basic drugs diffuse more easily into hydrophilic body fluids, which can potentially cause increases in drug concentrations in blood [8]. Since antipsychotic drugs (APs) are basic and generally lipophilic with a large V_d (Table 1) they are likely to be susceptible to PMR, however, this has not been studied in detail.

Currently published data on the PMR for APs has been obtained from animal studies, targeting one or a few analytes [9-11], or from human tissue distribution studies in post-mortem cases [12-19]. These studies focused predominantly on the impact of sampling site on a post-mortem drug concentration, rather than the influence of the post-mortem time interval (PMI). This is probably due to the difficulty in obtaining relevant specimens for testing and ethical restrictions on human experimentation on deceased persons.

Since an autopsy is unlikely to be carried out immediately following admission of a body to a mortuary, a PMI of a few to several days is common increasing the likelihood of substantial post-mortem changes in concentrations.

The Victorian Institute of Forensic Medicine (VIFM) is able to obtain a peripheral blood specimen on admission to the mortuary as part of its ability to conduct preliminary examinations prior to a coroners order on whether an autopsy should be conducted. The order to conduct an autopsy can take several days. This allows an opportunity to compare the blood concentrations on admission and the subsequent concentrations from blood taken at autopsy, in order to study the effect of time on the PMR.

Table 1: Volumes of distribution (V_d), Protein binding (F_b), and lipophilicity (logP) values for APs of interest

Drug	Vd	Fb	logP
Amisulpride	13 - 16	0.17	1.5
Chlorpromazine	10 - 35	0.98	5.18
Clozapine	2 - 7	0.95	3.67
Haloperidol	18 - 30	0.9	3.7
Olanzapine	10 - 20	0.93	2.65
Promethazine	13	0.93	4.52
Quetiapine	8 - 12	0.83	2.93
Risperidone	0.7 - 2.1	0.9	3.27
9OH risperidone	U/K	U/K	2.3
Zuclopenthixol	15 - 20	0.98	4.46

V_d and Fb are obtained from Baselt [20], logP values are calculated using ALGOPS 2.1. U/K: unknown

Methods

Case selection

Cases were selected in which both an admission to mortuary blood specimen (AD) and a post-mortem peripheral blood specimen (PM) taken at autopsy had been collected and showed the presence of at least one AP drug during routine toxicological testing. Only cases in which the investigation by the coroner was completed were included in this study.

Several exclusion criteria were applied. Cases that contained insufficient sample volume following routine toxicological analysis and subsequent long-term storage of 2 mL of specimen were excluded as were suspicious death cases. Additionally, all cases where the time interval between death and sampling of the AD sample was greater than 24 h were excluded from the study. Cases were also excluded where the circumstances of the death indicated significant trauma prior to death. In these instances, the integrity of the blood vessels were likely to have been compromised. Samples in this study that showed signs of decomposition (visually evaluated) were also excluded.

A total of 273 cases (546 paired specimens) were selected that showed the presence of at least one AP and matched the criteria described above. A total of ten APs were detected in these cases including 9OH-risperidone (paliperidone), amisulpride, chlorpromazine, clozapine, haloperidol, olanzapine, promethazine, quetiapine, risperidone, and zuclopenthixol.

Ethical review process

Ethics approval was granted by the Ethical Review Committee of the VIFM (Reference number: EC 5/2011).

Analysis of specimens

All specimens were analysed using a previously published validated tandem LC-MS method using three transitions per drug [21]. A matrix-matched freshly spiked seven-point calibration curve was extracted with every assay and used to calculate the respective concentrations of the drugs. Quality control (QC) samples were run after every ten samples. The assay was only accepted if all QCs were within 20 % of the target concentration. All 273 sample pairs were re-analysed despite some of them having had the AD specimen or the PM specimen tested during routine toxicological analysis. This was done in order to minimise differences in drug concentration potentially caused by different analysis times due to instability of compounds. The following formula was used to evaluate the change in concentration [%] between AD and PM sample:

$$\frac{(\text{Conc (PM)} - \text{Conc (AD)}) * 100}{\text{Conc (AD)}} = \Delta \text{ Conc [\%]}$$

where Conc = concentration

If Δ Conc [%] > 0 an increase in concentration was observed between AD and PM sample, if Δ Conc [%] < 0 a decrease in concentration was observed between AD and PM sample.

Statistical Evaluation

All AD specimens were compared with their respective PM sample using a two-tailed Wilcoxon Matched-Pairs Rank-Sum Test, with samples grouped according to the AP. This non-parametric test was chosen to evaluate the results, as normal distribution cannot be assumed for the sample set. The two-tailed approach was chosen, as concentration changes in

any direction needed to be considered. Significance values were only evaluated for individual PMI where six or more sample pairs were available, as the Wilcoxon Matched-Pairs Rank-Sum Test requires at last six matched pairs to be significantly different before assuming significant differences within a group of pairs. Subsequently, individually paired cases and their concentration change at defined time points post-mortem were combined in a group and used to evaluate a trend over time. The P-value was reported for all cases containing one drug. If there were six or more sample results for any given PMI, the significance value was provided for the individual PMI, in addition to the group value (Supplement 1). Additionally, n-values, the mean and standard deviation for each PMI are reported.

Results and Discussion

In order to evaluate the post-mortem drug concentration changes of each drug, several factors have been taken into consideration. In addition to the statistical evaluation, the average concentration change over the investigated PMI has been determined in order to make the data comparable with the outcomes of previous studies (Table 2). As inaccuracies (RSD) caused by the analytical method used in this study have proven to be under 20 % for all drugs with the exception of olanzapine (OLZ) (which was excluded from method validation due to its instability [21]), concentration changes greater than 40 % (two RSDs) were considered likely to be caused by reasons other than method inaccuracy. Additionally, the drug concentration change on every day of the PMI has been determined along with the standard deviation, giving more detailed information on the change over time (Supplement 1).

The majority of drugs showed significant changes between AD and PM specimens ($p < 0.01$) in addition to an average concentration change greater than 40 %. Average increases in blood concentrations after admission to the mortuary ranged up to 112 % (chlorpromazine and olanzapine), but also decreases up to - 43% were observed (9OH-risperidone).

9OH-risperidone was the only analyte which showed a reduction in drug concentration over all time points (35 sample pairs from cases with PMI ranging from 1 - 8 days, $p < 0.01$), with an average loss of 43 %. 9OH-risperidone is the main-metabolite of the atypical AP risperidone and is also available in some countries as paliperidone. It is formed by cytochrome (CYP) P450 enzymes, specifically CYP2D6, and is likely to contribute to the in vivo effects of risperidone [22]. In a clinical setting risperidone is rapidly metabolised and concentrations have been shown to be generally lower than 9OH-risperidone [23]. Hence, 9OH-risperidone is commonly measured in addition to risperidone, providing an indication, wherever possible, of prior risperidone ingestion in cases where risperidone can no longer be detected. With protein binding estimated at ~ 77% in human plasma and a partition coefficient (log P) of 2.3, 9OH-risperidone is less lipophilic than its parent compound, risperidone (protein binding = 90%, log P = 3.0) [24]. Consequently, 9OH-risperidone should be less likely than risperidone to distribute into organs and fatty tissue after death. However, results of this study showed losses of more than 65 % after a PMI of eight days, with a loss in concentration of approximately 40 % after four days. The significant losses of 9OH-risperidone over the time frame examined are interesting, considering a previous study investigating the stability of 9OH-risperidone in spiked whole blood samples did not reveal any significant losses over ten weeks of storage at 4 ºC, -20 ºC, and -60 ºC [21]. However,

whole blood samples in the stability study were preserved with 200 mg sodium fluoride and 30 mg potassium oxalate, which is likely to improve the stability of 9OH-risperidone.

Risperidone was the only analyte in which the concentrations decreased although a slight increase occurred at day seven (Figure 1). Risperidone showed an average loss of -15 % over the investigated PMI ($p < 0.01$). Interestingly, Rodda et *al*. reported that the heart to femoral ratio of 9OH-risperidone reflected that of risperidone [19]. This observation combined with the results of this study for the post-mortem drug concentration changes of risperidone and 9OH-risperidone, emphasise that despite sharing a similar heart to femoral ratio, drugs may undergo different patterns of post-mortem changes over time. Furthermore, risperidone shows the smallest V_d of all investigated drugs (0.7 - 2.1 L/Kg), suggesting that it is not likely to be susceptible to significant PMR in the first few days; a response that is supported by our data in the early PMI period. As the average concentration change was less than 40 %, the PMR of risperidone was considered not significant as it is unlikely to materially affect the interpretation of its likely effects.

Only two drugs (chlorpromazine and olanzapine) showed consistent increases in concentration over the nine days PMI ($p < 0.01$). These increases were generally greater than the uncertainty of measurement. Chlorpromazine showed an increase in concentration over time, with an average increase of ~ 112% over a PMI of nine days. This is consistent with reported heart/femoral blood ratios ranging from 1.57 (1.0 - 2.7; unknown sample size) up to 2.0 (0.8 - 7.2, *n=6*) and even 4.0 (1.0 - 8.0, *n=5*) [25] that have been reported in the literature, suggesting that chlorpromazine is subject to substantial PMR. This raises doubt over what can be said of blood concentrations that could be caused by redistribution since these changes could be mistaken for drug misuse and toxicity [26]. Olanzapine

concentrations increased on average ~ 112% over the investigated PMI of nine days (>100 % increase after four days) suggesting that this drug is highly susceptible to PMR. While the SDs of olanzapine were large (seven out of nine were greater than 30 %), this was not entirely unexpected due to the large case to case variation. The drug is also known to be inherently unstable [21] and it is likely that larger increases occurred but some drug was lost to degradation. While the analysis of Horak *et al*. [13] found the PMR of olanzapine to be "minimal" with a heart to femoral ratio of 1.24, this is supported by previous case studies, where the heart to femoral blood ratio of olanzapine has been reported to range from 1.1 - 1.4 [17, 27]. However, the observed variability in the detection of olanzapine highlights the limited value of single case studies as large variations in detection are likely to give misleading results. With a total of 95 sample pairs analysed in this study, olanzapine is highly likely to undergo PMR over time, however the true extent of PMR cannot be determined due to its instability.

Clozapine and promethazine showed the most significant increases in the first three days of the PMI. The largest increase in concentration occurred at four days for clozapine (> 70%) and three days for promethazine (> 170%). As the drug concentration decreased from this point onwards, drug results obtained after a longer PMI (four days onwards) intriguingly appear to be more likely to represent drug concentrations at the time of admission of a deceased person. Both drugs appear to undergo a pattern of increase in drug concentration followed by decrease, causing large inter-day concentration differences (Figure 1). Flanagan et al. investigated the PMR of clozapine in the domestic pig [9]. Two pigs were administered with a single dose of 10 mg/kg of clozapine. After death, blood was taken from a peripheral vein at different time points over a 24 h period. Interestingly, both pigs showed an increase in

blood concentration initially, followed by a decrease. Clozapine was no longer detectable in one of the pigs after 24 h. Consequently, the observed pattern of post-mortem behaviour of clozapine is supported by the results of our study.

Amisulpride and zuclopenthixol showed slower increases in concentration with the largest increase reached after four (amisulpride, > 80%), and five days (zuclopenthixol, > 145%), averaging 57 % and 62 %, respectively.

The two remaining drugs (in addition to risperidone) appeared to have undergone only minor post-mortem changes.

Quetiapine showed an average concentration change of 25 % over the investigated PMI (seven days). Following a tissue distribution study in 2000, Anderson *et al.* concluded that quetiapine was likely to undergo PMR [18]; this finding was also supported by Parker & McIntyre in 2005, who reported a heart to femoral ratio of 1.4, suggesting some propensity for PMR [28]. However, our results highlight that different conclusions may be reached depending on the time since death. With an average increase of less than 40 % over seven days of PMI, the concentration change is within the inaccuracy of the method and also would not materially affect any interpretations made.

Haloperidol was the only drug included in this study where post-mortem concentration changes were statistically not significant over the whole time frame average concentration change being only 2 % (Table 2). The only published study investigating the PMR of haloperidol is a tissue distribution study in the rat which showed an increase six hours after death [10]. No additional blood samples were collected after this time, making conclusions regarding a longer PMI difficult.

There were limitations to this study. The PM sample was taken during the autopsy process, therefore the possibility of contamination through collection of non-femoral blood, urine, faeces, serous fluid that has leaked from the chest cavity or stomach contents cannot be fully excluded. Furthermore, despite having excluded putrefied samples, a previous study has shown that even non-decomposed samples can result in altered extraction efficiencies and variable matrix effects compared with ante-mortem blood samples [29]. These outcomes suggest that variations are likely to be even higher if the sample group is not controlled. Another drawback is the unpredictability of the change in drug concentration that may have occurred in the time frame between death and taking of the AD sample.

Table 2: Number of sample pairs per drug (n), average concentration change including range [%], the investigated PMI (Time) [days] and P-value (*p=)* for the studied antipsychotic drugs.

Drug	n	Mean ΔConc [%] [Min , Max]	Time [days]	Significance (*p*=)
Amisulpride	11	**57 [43 , 84]**	2 - 8	**< 0.01**
Chlorpromazine	17	**112 [25 , 216]**	1 - 9	**< 0.01**
Clozapine	15	**41 [16 , 74]**	2 - 6	**< 0.01**
Haloperidol	18	2 [-30 , 49]	1 - 9	0.83
Olanzapine	95	**112 [17 , 234]**	1 - 9	**< 0.01**
Promethazine	22	**63 [13 , 174]**	1 - 7	**< 0.01**
Quetiapine	57	25 [16 , 38]	1 - 7	< 0.01
Risperidone	33	-15 [-36 , 12**]**	2 - 7	< 0.01
9OH-risperidone	35	**-43 [-68 , -26]**	2 - 8	**< 0.01**
Zuclopenthixol	15	**62 [28 , 146]**	1 - 7	**< 0.01**

bold: Mean ΔConc > 2 R.S.D (>40%) and p<0.01

Conclusions

In conclusion, the majority of drugs showed significant changes between AD and PM specimens ($p < 0.01$) in addition to an average concentration change greater than the uncertainty of measurement of the applied method. Haloperidol, quetiapine and risperidone did not show concentration changes greater than the extent of the uncertainty of measurement, therefore their risk to undergo significant post mortem redistribution was considered low. The outcomes of this study highlight the limitations of reporting post-mortem concentration changes. While average values as reported in this study can give an indication of whether or not a drug is subject to PMR, the analysis of samples collected over various days of the PMI has shown that individual variations between different time points of the PMI are can be significant. In addition to large standard deviations, this complicates the interpretation of post-mortem drug results, especially when a long or unknown time frame has passed between death and sampling of a specimen for toxicological analysis. Specimens for toxicological analysis need to be taken as soon as possible after admission of a deceased person to the mortuary. However, the large variations in reported results highlight that speculation concerning the magnitude of a post-mortem drug concentration change are impractical. It is more important to be aware of the variability of the change that is likely to occur.

Drug	ΔT [days]	1	2	3	4	5	6	7	8	9
9OH-Risperidone	n		5	4	**6**	**9**	**6**	4	1	
	Mean ΔConc [%]		-32.1	-26.4	-41.5	-30.7	-40.9	-61.5	-67.7	
	SD [%]		4.9	14.1	11.7	**31.8**	13.7	11.9	N/A	
	Significance if n≥6(*p=*)		N/A	N/A	0.0313*	0.0420*	0.0313*	N/A	N/A	
	Significance (*p=*)	< 0.01*								
Amisulpride	n	1	2	3	1	3	1			
	Mean ΔConc [%]	49.1	52.2	66.8	84.1	48.3	42.9			
	SD [%]	N/A	**53.0**	**29.6**	N/A	5.0	N/A			
	Significance if n≥6(*p=*)	N/A	N/A	N/A	N/A	N/A	N/A			
	Significance (*p=*)	< 0.01*								
Chlorpromazine	n	2	**6**	2	3	1		2		1
	Mean ΔConc [%]	24.7	80.3	71.1	75.7	105.0		212.6		216.2
	SD [%]	12.1	**65.6**	3.0	0.4			**225.4**		
	Significance if n≥6(*p=*)	N/A	0.0313*	N/A	N/A	N/A		N/A		N/A
	Significance (*p=*)	< 0.01*								
Clozapine	n		2	4	3	4	2			
	Mean ΔConc [%]		15.7	29.0	73.6	52.4	28.8			
	SD [%]		13.2	12	**22.3**	**53**	1.7			
	Significance if n≥6(*p=*)		N/A	N/A	N/A	N/A	N/A			
	Significance (*p=*)	< 0.01*								
Haloperidol	n	5		2	3	1	4	2		1
	Mean ΔConc [%]	32.0		48.6	3.0	1.0	-29.5	-16.8		-24.8
	SD [%]	**17.0**		**40.3**	**15.9**		4.6	9.8		
	Significance if n≥6(*p=*)	N/A		N/A	N/A	N/A	N/A	N/A		N/A
	Significance (*p=*)	0.83*								
Olanzapine	n	**6**	**17**	**14**	**18**	**16**	**13**	**6**	2	3
	Mean ΔConc [%]	17.3	87.8	65.8	112.7	94.9	127.6	161.5	110.5	233.7
	SD [%]	**69.6**	**81.7**	**160.1**	**143.2**	**167.8**	**159.5**	**193.5**	13.4	**77.0**
	Significance if n≥6(*p=*)	1.0	0.0058*	0.1070	0.0053*	0.0230*	0.0046*	0.0625	N/A	N/A
	Significance (*p=*)	< 0.01*								

Drug	ΔT [days]	1	2	3	4	5	6	7	8	9
Promethazine	n	**6**	4	2	5	3	1	1		
	Mean ΔConc [%]	34.7	115.5	174.3	69.5	18.6	14.0	12.8		
	SD [%]	**58.5**	**69.0**	**50.2**	**24.2**	8.2	N/A	N/A		
	Significance if n≥6(*p*=)	0.578	N/A	N/A	0.0313*	N/A	N/A	N/A		
	Significance (*p*=)	< 0.01*								
Quetiapine	n	4	**10**	**13**	**13**	**13**	3	1		
	Mean ΔConc [%]	20.8	24.1	37.6	27.1	29.0	19.4	15.6		
	SD [%]	**24.4**	**26.4**	**40.4**	**20.9**	**25.6**	6.5	N/A		
	Significance if n≥6(*p*=)	N/A	0.0645	0.0017*	0.0017**	0.0034*	N/A	N/A		
	Significance (*p*=)	< 0.01*								
Risperidone	n		4	4	**7**	**8**	**7**	3		
	Mean ΔConc [%]		-10.7	-36.1	-27.4	-21.5	-6.8	11.5		
	SD [%]		12.6	13.4	**16.3**	**17.2**	**32.0**	5.4		
	Significance if n≥6(*p*=)		N/A	N/A	0.0223*	0.0234*	0.0042**	N/A		
	Significance (*p*=)	0.83*								
Zuclopenthixol	n	1	2	1	1	3	3	4	1	2
	Mean ΔConc [%]	70.0	49.8	28.3	50.1	145.8	52.8	35.4	70.0	49.8
	SD [%]	N/A	**54.9**	N/A	N/A	**109.5**	**39.3**	**30.0**	N/A	**54.9**
	Significance if n≥6(*p*=)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Significance (*p*=)	< 0.01*								

Supplement 1: Percentage difference in drug concentration between AD and PM specimen over time (ΔT [days]), sample size (n), mean, standard deviation [%], significance values (*p*=) for all drugs, using a two-tailed Wilcoxon Matched-Pairs Rank-Sum Test

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Chapter 6

Discussion, Conclusions and Future Perspectives

In **Chapter 6**, the outcomes of this research are discussed and conclusions drawn from the results obtained in **Chapter 1-5** are presented, including their implications for the toxicological analysis of APs. Important factors of the analysis of these drugs are highlighted with emphasis on method validation, including recommendations for the interpretation of post-mortem drug results. Strengths and limitations of the research are emphasised, with a focus on the problems regarding the atypical antipsychotic drug olanzapine, resulting in suggestions for further research into the field of forensic toxicology of APs.

6.1 INTRODUCTION

APs are commonly prescribed for a wide range of psychotic illnesses including schizophrenia and bipolar disorder amongst adults and youth [26, 27, 79, 80]. In addition, a significant 'off-label' use of these drugs has been noted in recent years [29].A particularly large number of prescriptions outside the prescription guidelines of APs are present in nursing homes, however, no positive relationship between behavioural symptoms of patients and antipsychotic therapy were detected in several studies [81-83].

Pharmacologically, APs are divided into two main groups based on their mechanism of action. Typical APs (usually pre-1980) mainly act as dopamine receptor antagonists and show different affinities to the five subtypes (D_1 - D_5) of the receptor family. A large number of compounds in the group of typical APs have a phenothiazine structure, such as chlorpromazine, fluphenazine, perphenazine, promazine, promethazine, thioridazine, trifluoperazine, and triflupromazine. Other subgroups within the typical APs include butyrophenones (e.g. droperidol, haloperidol, trifluperidol, melperone, and pipamperone), thioxanthenes (e.g. flupentixol, zuclopenthixol, chlorprothixene, and thiothixene), diphenylbutylpiperidines (e.g. pimozide, fluspirilene, penfluridol); indoles (e.g. molindone), and others (e.g. loxapine). Due to their action on dopamine receptors, typical APs can cause severe side effects such as extrapyramidal symptoms, including parkinsonism and tardive dyskinesia [84]. Additionally, typical APs have been linked to an increased risk of cardiac arrhythmia and sudden cardiac death [10].

Due to the broad range of adverse effects associated with typical APs, a new generation of APs was introduced in the 1970s. These APs are generally referred to as "second generation" or "atypical" APs and not only act on dopamine receptors but additionally block serotonin

receptors. Structurally, this group includes indoles (e.g. ziprasidone and sertindole) benzamides (e.g. amisulpride, sulpiride), diazepines/oxazepines/thiazepines (e.g. clozapine, olanzapine, quetiapine and others (e.g. aripiprazole, risperidone, buspirone, paliperidone, zotepine).

Despite the attempt of reducing unwanted side effects of APs by introducing a newer generation of drugs, research has shown that no significant improvement was seen compared with the older generation of APs regarding the risk of sudden cardiac death and other cardiovascular events [10, 85, 86]. Several second generation APs such as olanzapine, aripiprazole, quetiapine and risperidone all showed an increased rate of death in people suffering from dementia [87]. It is therefore not surprising that these drugs are commonly present in post-mortem cases, making the interpretation of post-mortem drug concentrations critical in assessing the cause of death. While typical APs have been prescribed and researched for over 50 years, the second generation of APs (which has formed the majority of prescriptions in recent years) has not been studied in detail, largely because of the difficulty in their detection.

The administration of some APs often results in blood concentrations as low as 0.1 ng/mL, which require sensitive techniques for identification and measurement. Furthermore, there is limited information available on the stability of APs in stored whole blood specimens [88]. Stability data is often only collected as part of method validation and thus information concerning different storage conditions and data over longer periods of time is often inadequate or completely absent. These factors complicate the interpretation of drug concentrations, especially when an extended or unknown time frame has passed between sampling and analysis of the specimens.

In specimens taken from deceased persons, it has been shown that many drugs undergo changes in blood concentration. This includes distributive changes where drugs may diffuse from tissues to pooled blood resulting in sometimes large increases in blood concentration. These changes have mostly been associated with basic drugs that show a large volume of distribution (V_d) > 3 L/kg and a high degree of lipophilicity [51-55]. As APs incorporate all these characteristics, they are likely to be prone to PMR, however, this has not been studied in detail.

6.2 OVERVIEW OF RESEARCH FINDINGS

A comprehensive literature review of LC-MS/MS methods currently published for the detection and quantification of APs was performed in **Chapter 2.1**. This revealed a lack of adequately validated methods for the detection of these drugs. The most significant issues related to the evaluation of selectivity, linearity, matrix effects and stability, which are now mandatory requirements according to international guidelines accepted widely by the forensic toxicology community [72, 73, 89]. It was clearly important to accurately and reliably detect APs in biological specimens and better understand this increasingly prevalent class of drugs. Subsequently, a LC-MS/MS method was developed (**Chapter 2.2**) and fully validated according to international guidelines and was found to be satisfactory for all tested compounds. Selectivity experiments were carried out using ante-mortem and post-mortem blood samples in order to test the applicability of the method for post-mortem blood samples. During this method development, the typical APs promazine and promethazine which share the most abundant transition (285 → 86) were identified as structural isomers, as well as the isobaric compounds haloperidol and pipamperone, which also show a similar fragmentation

pattern, however, elute at different times in the chromatographic run. This observation highlighted the importance of not only monitoring a minimum of two transitions per compound, but also allowing for sufficient chromatographic run-times. The atypical AP drug OLZ had to be excluded from final method validation as it did not pass several validation criteria due to its instability (processed sample and long-term stability).

With stability having been identified as one of the critical parts of method validation, further stability studies were initiated in order to investigate if the storage of samples containing APs has an influence on the drug concentration. While specimens for drug analysis are often subject to several different storage temperatures prior to toxicological testing and for long-term storage post-testing, this is not commonly part of method validation. The significant degradation of olanzapine during method development instigated research into the influence of time and storage temperature on the stability of all APs included in the method (**Chapter 3**) with the exception of loxapine, mesoridazine and prochlorperazine. Although a large part of the tested drugs appeared to be stable over 20 weeks of storage in whole blood, especially when stored at temperatures below 0°C , instability was a serious problem for several drugs when stored at certain temperatures. Eight drugs showed instability under at least one storage temperature (20 °C, 4 °C, -20 °C, and -60 °C) after ten weeks of storage in a preliminary experiment. These drugs (chlorpromazine, chlorprothixene, droperidol, fluspirilene, OLZ, thioridazine, trifluprom azine, and ziprasidone) were included in a final experiment and spiked blood samples subjected to the same storage temperatures for 20 weeks. Overall, -20°C and -60°C appeared to be preferable for most drugs investigated in this study. The typical AP fluspirilene degraded significantly after 20 weeks under all storage conditions. Extensive degradation was seen not only when stored at 20 ºC and 4 ºC but significant losses

of up to 50% were also observed when stored at -20 ºC and -60 ºC, respectively. Ziprasidone samples showed losses of ~ 85 % after ten weeks of storage at 20 °C with a clear pattern of break-down, whereas all other storage temperatures appeared to be relatively stable up to 20 weeks. Interestingly, chlorpromazine showed the most significant losses at -20 ºC. This phenomenon where lower storage temperatures appeared less favourable than higher temperatures, has been described for different drug groups however, not for chlorpromazine to date [90, 91]. Overall, the most significant degradation was seen for the atypical AP OLZ which showed losses of almost 100% at all storage temperatures including -20 °C and -60 °C.

As the degradation of OLZ was more significant than initially anticipated, further research into this area was undertaken (**Chapter 4.1**). Three experiments were conducted to monitor the degradation of OLZ and the formation of degradants in blood, water, and post-extraction at 4 ºC. The influence of ascorbic acid on the stability of olanzapine was also investigated. 2-hydroxymethyl-OLZ was identified as the major degradation product of OLZ in water and post-extraction on the autosampler. The addition of 0.25 % ascorbic acid slowed the degradation of OLZ down in all three experiments and inhibited the formation of 2-hydroxymethyl-OLZ. However, this degradant was not found in blood specimens subjected to prolonged or accelerated storage. Hence, additional studies were undertaken using GC-MS technology. OLZ itself was successfully derivatised using various acylation and trimethylsilylation reagents and detected post-extraction using GC-MS technology. During storage at room temperature for one week, major degradation of spiked OLZ samples was seen consistent with results obtained during previous stability studies. Extraction was performed at a basic (9.2) and an acidic (4.5) pH in order to try and accommodate for the

unknown physicochemical properties of potential degradation products. However, no identifiable degradant was found in degraded samples (**Chapter 4.2).**

In addition, studies were conducted to investigate the PMR of ten of these drugs detected in actual cases (**Chapter 5.2**). Information on the PMR of APs reported in the literature to date is either obtained from animal studies [56-58] or tissue-distribution studies in single post-mortem cases [59-61]. Peripheral sites are generally considered more suitable for the collection of post-mortem specimens, as the distance from central organs and the gastrointestinal tract has shown to be less prone to undergo post-mortem drug concentration changes [51]. For this study, femoral blood was collected from a cohort of 273 cases at time of admission to mortuary and at post-mortem. The post-mortem samples were collected at various times up to nine days after admission and the sample pairs analysed using LC-MS/MS. The drugs included in this study were 9OH RIS, amisulpride, chlorpromazine, clozapine, haloperidol, OLZ, promethazine, quetiapine, RIS and zuclopenthixol. The majority of drugs showed significant changes between the sample pairs collected at different time points post-mortem. The pattern of changes was complex and generally involved a bi-phasic process with an initial increase followed by a decrease being most common. In contrast, 9OH RIS continually decreased throughout the entire time period. The most unstable drug, OLZ, increased at all time-points. Surprisingly, drugs with similar chemical structures showed different patterns of post-mortem changes. These outcomes highlight the limited use of information on PMR obtained from single case studies, without considering the time of collection. Furthermore, it was shown that samples collected from peripheral sites can potentially undergo significant post-mortem drug concentration changes despite their distance from central organs. These results highlight the difficulty in the interpretation of

post-mortem drug concentrations, especially when samples are not collected immediately after death. It is recommended to collect specimens as soon as practicable after admission to a mortuary in order to minimise the potential for changes in drug concentrations. An overview of the results obtained during the PMR studies (**Chapter 5.2**) and the stability studies (**Chapter 3.1**) is presented in Table 1.

Table 1: Overview over research findings from the post-mortem redistribution studies (**Chapter 5.2**) and stability studies (**Chapter 3.1**).

DRUG	PMR[1]	Stability 10 weeks[2]				Stability 20 weeks[3]			
		20 ºC	4 ºC	-20 ºC	-60 ºC	20 ºC	4 ºC	-20 ºC	-60 ºC
9-OH Risperidone	**Y**	2	1	1	1		-		
Amisulpride	**Y**	1	1	1	1		-		
Aripiprazole	-	1	1	1	1		-		
Bromperidol	-	1	1	1	1		-		
Buspirone	-	1	1	1	1		-		
Chlorpromazine	**Y**	**3**	2	2	1	**3**	**3**	**3**	1
Chlorprothixene	-	**3**	2	1	1	**3**	**3**	**3**	1
Clozapine	**Y**	1	1	1	1		-		
Droperidol	-	**3**	1	1	1	**3**	**3**	1	**3**
Fluphenazine	-	1	1	1	1		-		
Fluspirilene	-	**3**	**3**	1	1	**3**	**3**	**3**	**3**
Haloperidol	**N**	1	1	1	1	2	1	1	1
Levomepromazine	-	2	1	1	1		-		
Loxapine	-	-	-	-	-		-		
Melperone	-	1	1	1	1		-		
Mesoridazine	-	-	-	-	-		-		
Olanzapine	**Y**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**
Perazine	-	2	1	1	1		-		
Pericyazine	-	-	-	-	-		-		
Perphenazine	-	2	1	1	1		-		
Pimozide	-	1	1	1	1		-		
Pipamperone	-	1	1	1	1		-		
Prochlorperazine	-	-	-	-	-		-		
Promazine	-	2	1	1	1		-		
Promethazine	**Y**	2	1	1	1		-		
Quetiapine	**N**	1	1	1	1		-		
Risperidone	**N**	1	1	1	1	**3**	2	**3**	**3**

DRUG	PMR[1]	Stability 10 weeks[2]				Stability 20 weeks[3]			
		20 ºC	4 ºC	-20 ºC	-60 ºC	20 ºC	4 ºC	-20 ºC	-60 ºC
Sulpiride	-	1	1	1	1	-			
Thioridazine	-	2	2	1	1	3	3	2	3
Trifluoperazine	-	1	1	1	1	-			
Triflupromazine	-	3	2	1	1	3	3	2	1
Ziprasidone	-	3	1	1	1	3	3	3	2
Zotepine	-	1	1	1	1	-			
Zuclopenthixol	Y	1	1	1	1	-			

[1]: If a drug underwent PMR in the study in **Chapter 5.2**, it is marked '**Y**', if the drug does not undergo PMR it is marked '**N**'. If a drug was not investigated in this study, it is marked '-'.

[2,3]: Losses of each drug in the stability studies in **Chapter 3.1** are categorised as follows:
1 = < 15 %, 2 = >15 % - < 30 %, 3 = > 30 %

6.3 LIMITATIONS

During this project, a number of issues arose which must be taken into consideration when interpreting the results of the study.

Although applicability of the analytical method developed in **Chapter 2.2** was tested by analysing actual post-mortem cases, it was noted in an earlier study that matrix effects and extraction efficiencies can vary significantly when post-mortem samples are analysed [49]. The degree of decomposition of the blood samples revealed a significant influence on extraction efficiencies and matrix effects, which is hard if not impossible to account for in routine toxicological analysis. The study revealed wide ranges for both matrix effects and extraction efficiencies when blood of different "qualities" (ante-mortem to decomposed) was

analysed. This affects the interpretation of results obtained from blood samples where a degree of decomposition is present and the time of sample collection.

In **Chapter 3** the influence of different storage temperatures on the stability of APs was investigated. Several APs such as zuclopenthixol are known to be unstable when exposed to light; a contributing factor which was not examined in this study. Standard procedure at the VIFM is to store blood samples post-admission away from light. However, this may not be the case in all laboratories where samples are being processed for toxicological analysis.

OLZ had to be excluded from the method development as it did not fulfil all required validation criteria. However, the investigation of post-mortem change of OLZ formed a central part of this project (**Chapter 4, Chapter 5.2**). In this research, measures were taken in an attempt to reduce the potential for error when analysing OLZ samples (i.e., storage of samples containing OLZ for least time possible, injection of OLZ samples early in the run to minimise the potential for degradation prior to analysis, etc.). However, one must be aware that results obtained from analysis of samples containing OLZ can only be regarded as an approximate concentration, especially when sample preparation steps are not being kept consistent.

Chapter 4 focused on the investigation of degradation products of OLZ. Unfortunately it was not possible to identify a degradation product of OLZ in degrading blood samples. This may be due to the molecule being extensively broken up during the degradation process, resulting in a product that shows no or very little structural similarity with OLZ, and may be a quite small molecule, complicating the identification of a degradant.

When investigating the PMR of APs (**Chapter 5.2**), samples taken during admission of a deceased person to the mortuary were compared with samples taken during autopsy. While

the mortuary staff at the VIFM are aware of the importance of taking a peripheral sample from the femoral vein and potential damage to this site is stated in the case notes, the sample at admission of a deceased person is taken 'blindly' (i.e., without opening the body). This raises the possibility that the two samples which were compared in the study were not taken from exactly the same site. With a cohort of 273 sample pairs, the validity of the results is unlikely to be compromised to any significant degree and the standard deviations obtained during data processing are likely to account for this possible variation.

6.4 CONCLUSIONS AND FUTURE ASPECTS

This project contributed to the knowledge on the forensic toxicology of APs, particularly raising awareness regarding the difficulty in interpretation of post-mortem drug results due to the unpredictability of the post-mortem changes.

The importance of adhering to validation guidelines was emphasised during the method development and validation stages. Laboratories need to perform cross-validation experiments when adapting a previously published method for use in their laboratory.

Of the 30 APs tested in the stability studies, eight showed significant losses under at least one tested storage temperature over the ten week period. This was a novel finding based on the literature to date. As a result, appropriate storage of blood samples potentially containing APs needs to be guaranteed in order to minimise the risk of post-mortem drug concentration changes. In addition, laboratories should examine their routine procedures for sample receipt and storage, prior to and post toxicological analysis, to confirm sample integrity is assured. This includes performing stability studies whenever new compounds are included in existing

methods. As significant improvements of stability of OLZ were observed in the degradation product studies when ascorbic acid was added, laboratories which specialise in the detection of APs should consider testing the influence of this antioxidant on the stability of all compounds of interest. If beneficial results are being obtained it should be considered to add this antioxidant routinely to samples being received in order to improve stability of OLZ.

The investigation of the PMR of APs highlighted the unpredictability of the post-mortem changes of APs and raised more questions regarding the interpretation of post-mortem drug concentrations. As the PMR of APs generally involved a bi-phasic process with an initial increase followed by a decrease, the time at which a sample is taken appeared to play a more central role than initially anticipated. Taking into consideration the results of this study, one needs to question the use of tissue-distribution studies for the interpretation of post-mortem drug results, when the time of sampling is not considered. Significant changes in drug concentrations were observed in post-mortem samples from deceased individuals in the mortuary, despite controlled storage conditions at a temperature of 4 ºC. This suggests that in cases exposed to even higher temperatures, such as delayed discoveries in suicide, changes in post-mortem drug concentrations will be even more significant. Samples for toxicological analysis should be taken immediately after admission of a deceased person to the mortuary in order to try and minimise the potential for drug blood concentration changes.

There are a number of future research prospects resulting from the outcomes of this project. The identification of degradation products of OLZ in blood as a marker for the degradation of this inherently unstable drug did not succeed despite applying a variety of analytic techniques. Finding a marker would be highly desirable, as well as establishment of a

conversion rate. This could be a useful tool when interpreting post-mortem OLZ concentrations.

Additionally, the unexpected results obtained during the PMR studies raised further questions regarding the post-mortem changes that APs undergo. In order to try and explain the bi-phasic drug concentration change, it might be useful to measure drug concentrations in tissue surrounding the femoral vein.

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General Introduction and Integrated Discussion

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Appendices

Appendix 1

Papers published during Ph.D candidature

2.1 Review: The Analysis of Antipsychotic Drugs in Human Matrices using LC-MS(/MS)
Drug Testing and Analysis **6** (2012) 376-394.

2.2 Identification and Quantification of 30 Antipsychotics in Blood using LC-MS/MS
J Mass Spectrom, 2010. **45**(8): p. 915-25

1.1 Assessment of the Stability of 30 Antipsychotic Drugs in Stored Blood Specimens
Forensic Sci Int, 2012. **215**(1-3): p. 152-8

4.1 Identification of 2-Hydroxymethyl-olanzapine as a Novel Degradation Product of Olanzapine
Forensic Sci Int, 2012. **220** (1-3): p. 74-79

1.2 The Time-dependent Post-mortem Redistribution of Antipsychotic Drugs
Forensic Sci Int, 2012. *(ePub)*

Appendix 1.1

Review: The Analysis of Antipsychotic Drugs in Human Matrices using LC-MS(/MS)

Saar, E., Gerostamoulos, D., Beyer, J., Drummer, O. H
Drug Testing and Analysis **6** (2012) 376-394.

Review

Drug Testing and Analysis

Received: 24 January 2012 Revised: 17 February 2012 Accepted: 17 February 2012 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/dta.1337

The analysis of antipsychotic drugs in human matrices using LC-MS(/MS)

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Antipsychotic drugs (APs) are prescribed for a wide range of psychotic illnesses. With more than 35 APs currently available worldwide, this drug class has rapidly gained importance in both clinical and forensic settings. On account of their chemical properties, many APs are present in human specimens at very low concentrations, which complicate their detection using standard gas chromatography–mass spectrometry (GC-MS) procedures that often cannot provide the required sensitivity. Recent advances in liquid chromatography-(tandem) mass spectrometry LC-MS(/MS) technology have enabled accurate detection and quantification of these compounds in various human specimens, indicated by the increasing number of published methods. Method validation has been a particular focus of analytical chemistry in recent times. Recommendations set by several guidance documents are now widely accepted by the toxicology community, as reflected by the guidelines drafted by leading toxicological societies. This review provides a critical review of single-stage and tandem LC-MS procedures for the detection and quantification of APs, with a particular emphasis on appropriate method validation.

The quality of published methods is inconsistent throughout the literature. While the majority of authors incorporate some validation experiments in their respective method development, a large number of published methods lack essential components of method validation, which are considered mandatory according to the guidelines.

If adapting a method for the detection of APs for use in a laboratory, analysts should ensure successful validation experiments for appropriateness and completeness have been conducted, and perform additional experiments when indicated.

Keywords: antipsychotic drugs; LC-MS(/MS); method validation

Introduction

In the 1950s, the phenothiazine derivative chlorpromazine was the first drug introduced for the treatment of psychotic illnesses, largely replacing electroconvulsive therapy and psychosurgery. Subsequent to the success of chlorpromazine, a large number of compounds were introduced for the treatment of patients suffering from mental illnesses. The main category of neuroleptic drugs is the phenothiazine derivatives, butyrophenones, and thioxanthenes, known as 'typical' antipsychotics (APs). While these drugs show significant improvement in the symptoms of psychotic illness, they are also associated with unwanted extrapyramidal side-effects resulting from their activity at dopamine receptors. A new generation of APs introduced around 1995 largely overcame these side-effects via decreased activity at dopamine receptors compared with their traditional counterparts. These 'second generation' or 'atypical' APs now account for the vast majority of AP prescriptions. Reports in the USA indicate a steady increase from 1.0 M prescriptions in 1995 to 13.3 M in 2008, while typical agents decreased significantly over the same timeframe.[1] However, studies in recent years have shown that atypical APs are not free from side-effects. An increased risk of mortality in addition to cardiovascular complications have been reported in patients suffering from dementia when treated with atypical APs.[2] Furthermore, second-generation APs do not only increase the risk of diabetes[3] compared with typical agents, but also show a similar risk of sudden cardiac death to their typical counterparts.[4] With more than 35 APs currently available worldwide, this drug class has rapidly gained importance in both a clinical and forensic setting, which makes the ability to reliably detect APs in human biological specimens a necessity.

In a clinical environment, the analysis of APs in blood is necessary in order to monitor patient compliance and to maintain drug concentrations within the recommended therapeutic range of the respective drug. The absence of prescribed APs in a clinical case may also indicate non-compliance, a common issue among patients suffering from mental illness. In a forensic setting, the detection of APs is crucial in determining whether these drugs played a role in the cause of death. A sub-therapeutic concentration of an AP in forensic cases may be particularly relevant in cases where mental disturbances have contributed to the death of a person by another, for example, homicides. Analytically, APs have been traditionally measured using gas chromatography (GC) with mass spectrometry (MS).

Zhang *et al.*[5] presented an overview of bioanalytical methods for the determination of APs up until 2007. The authors focused primarily on GC and liquid-chromatography (LC) methods with

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various detectors such as ultraviolet (UV), nitrogen phosphorus, fluorescence, and electrochemical detection (EC), concluding that LC was the most suitable separation technique for these mostly involatile compounds. MS/MS in combination with LC now dominates the analytical field, providing a particularly convenient tool in the analysis of APs. The high sensitivity of LC-MS/MS methods often allows analysis times to be substantially reduced compared with traditional UV and EC methods, which is particularly useful for a large sample throughput or when fast turn-around times are required.

Method validation has been a particular focus in recent times, in order to ensure true performance of methods and provide an objective tool to establish whether a method works as intended. The reproducibility of an analytical method is mandatory in preventing serious legal consequences that can result from discrepancies in forensic investigations. Specific guidelines for method validation were published two decades ago[6,7] and have since been revisited by the authors[8,9] to produce contemporary guidelines specifying the minimum requirements for method validation. These guidelines are now widely accepted in the toxicology community, reflected in guidelines drafted by leading toxicology societies such as The International Association of Forensic Toxicologists (TIAFT), the Society of Forensic Toxicologists (SOFT) and the Society of Toxicological and Forensic Chemistry (GTFCh). However, a large number of methods still exist that either lack crucial parts of validation, or that have not adequately performed the obligatory validation experiments.

This paper provides a critical review of single-stage and tandem LC-MS procedures for the detection and quantification of APs with a particular emphasis on appropriate method validation.

Methods

Papers for this review were selected following a comprehensive PubMed search for English articles using LC-MS or LC-MS/MS methods for the detection of one or more APs in various human specimens (blood, plasma, serum, urine, hair, saliva, and cerebrospinal fluid). Selected papers were reviewed for analytical details and assessed with regard to the extent of validation studies against current guidelines.[6–9]

Choice of biosamples

Blood is the preferred specimen for AP analysis as it provides the most accurate representation of the relevant pharmacological effects. In a clinical setting, plasma and serum are matrices of choice for drug analysis, as they are the most common specimens used in diagnostic medicine. Therapeutic drugs monitoring (TDM) methods are common and are more likely to focus on one or very few analytes. Whole blood is the most common specimen used in forensic cases since lysis is common in death investigations, and centrifugation shortly after collection is not always possible.[10]

Urine is a useful specimen for general unknown screening (GUS) procedures, particularly when overdose is suspected and qualitative results are required. APs are included in most published non-targeted screening procedures as part of big libraries. However, since these methods lack the ability to produce quantitative results, they are less relevant for the detection of APs and will not be discussed in this review.[11–13] Targeted published methods for detection of APs in urine using LC-MS(/MS) are rare and usually include an additional matrix.[14–17]

Hair has become an increasingly popular alternative specimen to blood, as drugs and their metabolites are likely to remain in hair samples long after the compounds have been eliminated from the body. Segmental hair analysis in particular can provide an indication of the long-term history of drug use in an individual. While hair analysis is frequently used as a tool in the analysis of drugs of abuse, only a limited number of methods targeting APs in hair using LC-(MS/)MS technology have been published to date.[16,18–22]

Oral fluid is used as an alternative to blood, which has increasingly gained importance due to the relatively short drug detection windows in addition to non-invasive collection of specimens. These factors make oral fluid a useful specimen in circumstances where trained medical staff is not available, such as roadside and workplace drug testing. APs are known to reduce salivary flow rate[23] and may therefore not be ideal for detection in oral fluid. This is reflected in the limited number of published methods for APs[24] to date using this specimen.

Cerebrospinal fluid (CSF) is commonly analyzed in order to help diagnose various diseases and conditions affecting the central nervous system (CNS), such as meningitis and encephalitis. It is also useful in diagnosing bleeding of the brain or tumours within the CNS. CSF is most commonly obtained by lumbar puncture, a complex and invasive procedure that requires specialized medical staff. While it is likely that drug concentrations in CSF are more closely related to pharmacological effects than blood concentrations, the complicated process of sample collection makes it a less favourable specimen in drug analysis, with only one published method for the detection of APs.[25]

General considerations

Sample volume and LLOQ

In published analytical methods, sample volumes below 0.1 ml are rare,[24,26–28] whereas volumes closer to 1 ml are frequently used. When selecting a sample volume for an analytical method targeting APs, several factors must be considered. Using a small sample volume in an analytical method provides several advantages, including easier handling during sample extraction and the ability to conduct analysis in cases where only limited specimens are available – for example, post-mortem cases. However, APs are mostly lipid-soluble weak bases, which are quickly absorbed into body fat and organs following administration, signifying a large volume of distribution (V_D). Despite their high V_D, most common APs also significantly bind to plasma proteins (Fb). Both the large V_D and high Fb significantly reduce the amount of unbound drug available in the blood for detection. Analytical requirements dictate that the lowest therapeutic blood concentration of a drug must be quantified. This equates to determining the lower limit of quantification (LLOQ), usually involving two different approaches: a signal-to-noise ratio (S/N) of 10 is considered satisfactory[29] and so is a precision and accuracy of $<$20% at the desired LLOQ.[6,8] Huang *et al.* [30] reported an S/N of 3 at the LLOQ, which is generally acceptable for a limit of detection (LOD), but not for the LLOQ. However, they conducted validation experiments which confirmed the precision and accuracy at the LLOQ to be within 20%, and therefore meet acceptance criteria. It needs to be guaranteed that a method is sufficiently sensitive to fulfill at least one of these two criteria when selecting the sample volume. Table 1 shows pharmacokinetic parameters of common APs.

Table 1. Pharmacokinetic parameters of common APs.

Drug	Common daily oral dose range in adults (mg)[1]	Blood concentrations expected following therapeutic use (ng/ml)[2]	$t_{1/2}$ (h)[3]	V_D (L/Kg)[4]
9OH-Risperidone*	3–12	10–100	23	N/A
Amisulpride	400–1200	50–400	11–27	13–16
Aripiprazole	10–30	50–350	60–90	4.9
Bromperidol	1–15	1–20	15–35	N/A
Buspirone	20–30	1–10	3–12	5–6
Chlorpromazine	200–600	30–300	7–119	10–35
Chlorprothixene	40–80	20–200	8–12	11–23
Clozapine	300–450	200–800	6–17	2–7
Flupentixol	3–6	1–15	19–39	14.1
Fluphenazine	1–5	2–20	13–58	220
Fluspirilene¥	2–5 (i.m)	N/A	21 days (decanoate)	N/A
Haloperidol	1–15	5–50	18	18–30
Levomepromazine	25–50	15–60	15–30	30
Loxapine	20–100	10–100	3–4	N/A
Melperone	100–400	5–40	2–4	7–10
Mesoridazine	100–400	15–100	2–9	3–6
Molindone	50–100	~500	1.2–2.8	3–6
Olanzapine	5–20	10–100	21–54	10–20
Penfluridol	20–60 (once per week)	4–25	70	N/A
Perazine	50–600	100–230	8–15	N/A
Pericyazine	15–60	5–60	N/A	N/A
Perphenazine	12–24	0.6–2.4	8–12	10–35
Pimozide	7–10	15–20	28–214	11–62
Pipamperone	80–120	100–400	12–30	N/A
Prochlorperazine	15–40	10–500	14–27	13–32
Promazine	200–800	10–400	7–17	27–42
Quetiapine	300–450	70–170	6–7	8–12
Risperidone	2–6	10–100	3–20	0.7–2.1
Sertindole	12–20	50–500	N/A	20–40
Sulpiride	400–600	50–400	4–11	2.7
Thioridazine	150–300	200–2000	26–36	18
Thiothixene	6–30	N/A	12–36	N/A
Trifluoperazine	15–20	1–50	7–18	N/A
Triflupromazine	165–375	30–100	N/A	N/A
Ziprasidone	40–160	50–120	2–8	1.5–2.3
Zotepine	75–300	5–300	12–30	50–168
Zuclopenthixol	20–50	5–100	12–28	15–20

[1]: Common daily oral dose data for the treatment of schizophrenia, psychoses or bipolar disorder from Drugdex® Evaluations in the Micromedex® Internet database.[96] Where the drug is indicated for other disorders (e.g. depressive disorders obtained), dosages may vary.

[2]: Blood concentrations expected following therapeutic use obtained from TIAFT guidelines. [97]

[3]: Terminal elimination half–life and; [4]: Volume of distribution obtained from Baselt. [98]

* : Also referred to as 'Paliperidone'.

¥ : Only available as i.m. injection

Single-analyte methods *vs* multi-analyte methods

Single-analyte methods are mostly used in a TDM-setting, where only specific compounds are the target of drug monitoring. Methods targeting the atypical AP risperidone (RIS) should always include its major metabolite 9OH-risperidone (9OH RIS), also referred to as paliperidone. 9OH RIS is formed by cytochrome (CYP) P450 enzymes, specifically CYP2D6, and is likely to contribute to the in vivo effects of RIS.[31] Whilst plasma concentrations of RIS and 9OH RIS show a large variation between individuals,[32–34] RIS levels are generally lower than 9OH RIS levels. In fact, a study measuring plasma concentration of RIS and 9OH RIS after oral administration of RIS in steady-state found RIS was not detectable at a LLOQ of 0.1 ng/ml in ~18% of all tested individuals, whereas 9OH RIS was detected in all cases.[32] Measuring only the parent compound, especially in TDM methods, can therefore lead to inaccurate conclusions regarding patient compliance.

While the same risk of interferences exists for single-analyte and multi-analyte procedures, chances are higher that they will be identified during method development when a greater number of analytes are included in the method. Generally, multi-analyte procedures are preferred over single-analyte approaches, as the inclusion of a number of analytes in one method saves time and resources.

Sample preparation

Extraction of APs from blood, plasma, and serum

Table 2 shows an overview of currently published single-analyte LC-MS(/MS) methods using blood, plasma, or serum. Table 3 contains all published multi-analyte studies.

Due to the high specificity of LC-MS methods, it was initially thought that the sample preparation step may not be as crucial as with other analytical methods, particularly for MS/MS methods since transitions greatly reduce the risk of interference from other drugs. However, this view was soon revised. While endogenous components might no longer be detected using LC-MS methods, they can still significantly interfere with the quantification of a drug.[35,36]

Therefore, liquid-liquid extraction (LLE)[25,30,37–51] and solid-phase extraction (SPE)[15,16,52–59] are still most commonly used as a sample treatment prior to injection into the LC-MS system, as they provide the most thorough sample clean-up. Saar *et al.*[60] systematically evaluated nine different combinations of extraction solvents and buffers in order to find the most suitable LLE method for the extraction of 19 APs.[60] The method showing the best results overall for extraction recoveries and matrix effects used trizma buffer and 1-chlorobutane (BuCl) and was subsequently compared with a standard SPE method. While extraction efficiencies were comparable between LLE and SPE methods, blockages of SPE cartridges were a common problem, especially when dealing with post-mortem samples. Nirogi *et al.*[46] applied a similar approach when comparing six organic solvents and their combinations in order to optimize extraction recovery for their method targeting olanzapine (OLZ) in plasma. A mixture of diethylether and dichloromethane (7:3, *v/v*) yielded the highest recovery of OLZ and was therefore used in their detection method. Gutteck *et al.*[48] stated that due to the different 'extraction coefficients. . . and different concentration ranges in human serum', four different extraction procedures had to be applied for determination of thirteen antidepressants and five APs. Minor variations in organic solvents used for the LLE, differences in the volumes of the mobiles phases and varying internal standards mark the differences between the four methods. A more practical approach would have been to have one extraction method and chromatographic conditions that allowed the analysis of all drugs in a single cost-effective method, especially since it is not clear which factors resulted in the development of the four different methods.

Simple protein-precipitation (PP) may be used for 'cleaner' matrices such as serum or plasma. [26,27,61,62] It needs to be noted, however, that matrix effects must be investigated closely as PP might fail to remove phospholipids from plasma or serum which might cause interferences.[63,64] Interestingly, Klose Nielsen *et al.* [65] compared LLE methods with different combinations of organic solvents and SPE techniques prior to the development of their method for the determination of OLZ in whole blood, and found none of them to be functional. However, a simple PP appeared to produce sound results. Few methods employed direct-injection,[14,28] while one published method used direct injection in combination with column switching [24] in order to decrease matrix influences. One published approach uses solid-phase micro-extraction (SPME) as a solvent-free and concentrating extraction technique.[17] While traditionally combined with GC, employing heat assisted desorption from the fibre, a simple interface coupling SPME with LC makes it functional for non-volatile substances. Online-SPE has been applied in order to reduce human error and increase time-efficiency.[57] Upscaling of the extraction is achieved by work-up in the 96-well format.[27,53]

Extraction of APs from hair

Table 4 shows an overview of methods published for the detection of APs in hair, using LC-MS(/MS).

The Society of Hair Testing recommends that hair be washed prior to analysis (e.g. in methanol (MeOH)) and the wash solution be subsequently analyzed for drug content.[66] A high concentration of the drug of interest in the wash solution may indicate external contamination of the hair sample. To date, however, a conclusion has not been reached concerning the best decontamination strategy.[67–73]

Among the most commonly used extraction procedures for hair analysis are alkaline hydrolysis using NaOH followed by SPE, or extraction with MeOH and aqueous buffer using an ultrasonicator.[74] Whilst both techniques are used for analysis of APs in hair, methods using NaOH appear to be preferable for alkaline-stable drugs such as APs. Josefsson *et al.* [16] did not attempt a full validation of their LC-MS/MS method for the identification of 19 APs and their major metabolites in hair. Incubation with NaOH was performed prior to extraction with BuCl and back extraction into formic acid. Two SRM transitions were chosen per AP (and where possible per metabolite) for identification of the drugs of interest. The authors highlighted the importance of including metabolites of drugs of interest in hair methods. In hair analysis, the issue of incorporation of a drug into the hair from external sources rather than ingestion is a frequent point of discussion, especially in court cases where an accused person denies the use of a drug. For some drugs, the presence of metabolites in a certain ratio to the parent drug can be an additional indication that ingestion of the drug has occurred and facilitate interpretation of results of hair analyses.[75]

Nielsen *et al.* [20] tested different combinations and ratios of organic and aqueous solvents prior to the development of their detection and quantification method. This involved 52 common pharmaceuticals and drugs of abuse in hair, including five APs. This 'mixed' approach was fully validated in accordance with international guidelines.[9] When extracting basic compounds such as APs from hair, the use of a neutral or slightly acidic aqueous buffer is recommended in order to facilitate ionization of the compounds prior to transition into the aqueous phase.[74] Mueller *et al.*[19] and Weinmann *et al.*[22] performed ultrasonication with MeOH prior to mixed-mode SPE. Thieme *et al.*[21] divided the initial 50 mg segment of hair into individual hairs prior to analysis; 30 fg on column was sufficient to detect clozapine in single hairs. The authors, however, acknowledge the uncertainty associated with hair analysis, mainly resulting from the unknown recovery of drug from hair combined with the uncertainty of the exact length of single hair segments.

Extraction of APs from cerebrospinal fluid, oral fluid, and urine

Table 5 shows an overview of published methods for the detection of APs in CSF, oral fluid, and urine using LC-MS(/MS).

Several authors have attempted to validate previously developed methods for the detection of APs in plasma or blood for urine[14,15,17]. Bogusz *et al.*[76] applied full-scan mode to urine samples of patients treated with OLZ in order to find proposed metabolites. A large number of OLZ metabolites in urine have been confirmed by Kassahun *et al.* in their comprehensive study

Table 2. Summary of single–analyte methods for the detection of APs in blood (a), plasma (b), and serum (c) using LC-MS/MS.

a)

Author (Year)	Volume [ml]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Klose Nielsen *et al.*[65] (2009)	0.19	olanzapine	dibenzepine	acidic MeOH–induced PP	Zorbax Extend C_{18} (50 x 2.1 mm, 5 μm)	gradient with 5 mM ammonium hydroxide in ACN and ACN	ESI, positive mode, SRM, MS/MS	linearity, selecticity, matrix effects, recovery, LLOQ, precision, accuracy, PS stability, LT stability
Kollroser *et al.*[43]◇ (2001)	1	zuclopenthixol	flupentixol	LLE (ammonia solution and ethylacetate)	Symmetry C_{18} Waters (3.0 x 150 mm, 5 μm)	gradient with ACN and 0.1% formic acid	ESI, positive mode, SRM, MS/MS	linearity, accuracy, precision, LOD

b)

Author (Year)	Volume [ml]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Aravagiri *et al.*[37] (2001)	0.5	clozapine, norclozapine, clozapine–N–oxide	"a derivative of risperidone"	LLE (ethyl acetate, methylene chloride, pentane)	Phenomenex C_{18} (50 x 4.6 mm, 5 μm)	isocratic with 60 mM ammonium acetate MeOH and ACN	ESI, positive mode, SRM	precision, accuracy
Aravagiri *et al.*[38] (2000)	0.5	risperidone, 9OH–risperidone	R68808	LLE (0.5 ml sat solution of sodium carbonate (pH = 10.5) 15% methylene chloride in pentane	Phenomenex phenyl hexyl column (5 μm, 50 x 4.6 mm)	isocratic with 0.15 mM ammonium acetate, MeOH, and ACN	ESI, positive mode, SRM	precision, accuracy,
Arinobu *et al.*[14] ## (2002)	1	haloperidol, reduced haloperidol, 4–(4–chlorophenyl)–4–hydroxypiperidine	4–[4–(4–chlorophenyl)–4–hydroxy–1–piperidinyl]–(4–chlorophenyl–1–butanone	addition of 3 ml of dH_2O with 0.09% formic acid and 20 mM ammonium acetate, freezing, thawing, centrifugation, injection of 20μL of supernatant	Mspak GF–310 4B (50 x 4.6 mm)	gradient with formic acid and 20 mM ammonium acetate in dH_2O and ACN	SSI, positive mode, MS	linearity, LOD, precision, accuracy
Barret *et al.*[52] (2007)	0.5	quetiapine	clozapine	SPE	Atlantis dC_{18} (100 mm x 3 mm, 3 μm)	isocratic with ACN–MeOH–0.01 M ammonium acetate	ESI, positive mode, SRM, MS/MS	selectivity, LOD, LLOQ, recovery, matrix effects, linearity, precision, F/T and LT stability, PS stability

Table 2. *(Continued)*

Author (Year)	Volume [ml]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Bhatt *et al.*[62] (2006)	0.1	risperidone, 9OH–risperidone	methyl risperidone	PP (ACN)	Betasil C_{18} column (3 μm, 100 x 3 mm)	isocratic with ammonium acetate and ACN	ESI, positive mode, SRM MS/MS	selectivity, linearity, LLOQ, precision, accuracy, recovery, F/T and LT stability, PS stability
De Meulder *et al.*[15] ## (2006)	0.2	risperidone, 9OH–risperidone	$^{2}H_{2}$–$^{13}C_{2}$–risperidone and $^{2}H_{2}$–$^{13}C_{2}$–9OH–risperidone	SPE (mixed mode)	Chiralcel OJ column (50 mm x 4.6, 10 μm)	gradient with hexane, 0.01 mM ammonium acetate in isopropanol, 0.01 mM ammonium acetate in ethanol	ESI, positive mode, SRM, MS/MS	selectivity, precision, accuracy, recovery, F/T and LT stability, PS stability
Flarakos *et al.*[24] ## (2004)	0.025	risperidone, 9OH–risperidone	R068808	online cleanup, column switching	Zorbax SB_{18} (30 x 2.1 mm, 3.5 μm)	isocratic with 10 mM ammonium acetate/ACN	SRM, MS/MS	linearity, selectivity, precision, accuracy, recovery, matrix effects, F/T and LT stability
Gschwend *et al.*[42] (2006)	0.25	amisulpride	sulpiride	LLE (diisopropylether: dichloromethane, 1:1)	Phenomenex Synergi Polar–RP analytical column (75 mm x 4.6 mm, 4 μm)	isocratic with 5 mM ammonium formate/ ACN	ESI, positive mode, SRM, MS/MS	linearity, selectivity, recovery, precision, accuracy, F/T and LT stability, PS stability
Kubo *et al.*[44] (2005)	0.4	aripiprazole, OPC–14857	OPC–14714	LLE (diethylether)	RP Chemcobond ODS–W (150 x 2.1 mm, 5 μm)	isocratic with dH_2O /ACN	ESI, positive mode, SRM, MS/MS	selectivity, linearity, accuracy, precision, recovery, F/T and LT stability, PS stability
Moody *et al.*[45] (2004)	1	risperidone, 9OH–risperidone	RO68808	LLE (pentane/ methylene chloride)	Intersil 5 ODS3 (150 x 2.1 mm)	gradient with dH_2O and ACN	APCI, positive mode, SRM, MS/MS	selectivity, extraction efficiency, accuracy, precision, F/T stability, LT stability
Nirogi *et al.*[46] (2006)	0.5	olanzapine	loratadine	LLE (diethylether: dichloromethane)	Inertsil ODS column (3 μm, 100 x 3 mm)	isocratic with 10 mM ammonium acetate:ACN	ESI, positive mode, SRM, MS/MS	linearity, precision, accuracy, LLOQ, recovery, F/T and LT stability, PS stability
Remmerie *et al.*[54] (2003)	0.5	risperidone, 9OH-risperidone	Method A: R068809 Method B: $^{2}H_{2}$-$^{13}C_{2}$-risperidone and	SPE (10cc/130mg Bond Elut Certify)	3–μm C18 BDS–Hypersil	gradient with 0.01 M ammonium formate and ACN	ESI, positive mode, SRM, MS/MS,	linearity, selectivity, accuracy, precision, recovery, PS stability,

			$^{2}H_{2}$-$^{13}C_{2}$-9OH-risperidone		column (100 × 4.6mm)			LT stability, F/T stability, matrix effects
Swart *et al.*[47] (1998)	1	fluspirilene	?	LLE (4% isoamyl alcohol in hexane)	Phenomenex Luna C_{18} 5μm, 150 × 2.1mm)	isocratic with MeOH and dH_2O	ESI, positive mode, scanning product ion spectrum from m/z 130 - 500	selectivity, recovery, LLOQ, accuracy, precision, PS stability, LT stability

c)

Author (Year)	Volume [ml]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Huang *et al.*[30] (2008)	0.3	risperidone	paroxetine	LLE (ACN)	Alltima–C_{18} (2.1 mm × 100 mm, 3 μm)	isocratic with formic acid/ACN	ESI, positive mode, SRM, MS/MS	selectivity, linearity, precision, accuracy, recovery, PS stability, F/T stability
Josefsson *et al.*[25]## (2010)	0.2	olanzapine, N–desmethylo-lanzapine	olanzapine–d_3	LLE (tert–butyl–methyl–ether)	Synergi Hydro–RP (50mm × 2mm, 2.5μm)	gradient with 10mM ammonium formate with formic acid and MeOH with formic acid	ESI, positive mode, SRM, MS/MS	linearity, LLOQ, precision, accuracy, recovery, matrix effects, F/T stability, LT stability
Nozaki *et al.*[26] (2009)	0.03	zotepine	imipramine	PP (ACN)	Tosoh ODS–100 V (50 mm × 2 mm, 5 μm)	gradient with 10 mM ammonium formate containing ACN and 10 mM ammonium formate containing 90% ACN	ESI, positive mode, EC–MS/MS	linearity, LLOQ, accuracy, precision, recovery, matrix effects

Abbreviations: ACN: acetonitrile, APCI: atmospheric pressure chemical ionization, dH_2O: deionized water, EC: electrochemistry, ESI: electrospray ionization, F/T: freeze/thaw, LLOQ: lower limit of quantification, LOD: limit of detection, LT: long term, m/z: mass over charge ratio, MeOH: methanol, SRM: selected reaction monitoring, MS/MS: tandem mass spectrometry, PP: protein precipitation, PS: processed sample, SPE: solid phase extraction, SSI: sonic spray ionization

[#] : More drugs are included in this method but do not belong to the group of APs.

[##] : This method was applied to more than one matrix.

[◇] : Post–mortem specimens were analyzed in this method.

[?] : An IS appears to have been used but is not specified.

Table 3. Summary of multi–analyte methods for the detection of APs in blood (a), plasma (b), and serum (c) using LC–MS/MS

a)

Author (Year)	Sample [ml]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Josefsson *et al.*[16] ◇ ## (2003)	1	buspirone, chlorpromazine, chlorprothixene, clozapine, dixyrazine, flupentixol, fluphenazine, haloperidol, hydroxyzine, levomepromazine, melperone, olanzapine, perphenazine, pimozide, prochlorperazine, risperidone, thioridazine, ziprasidone, zuclopenthixol#	N/A	SPE	Zorbax Stable Bond Cyano column (50 x 2.1 mm, 3.5 μm)	gradient with different ratios of MeOH:ACN: 20 mM ammonium formiate	ESI, positive mode, SRM, MS/MS	N/A
Kumazawa *et al.*[17] ## (2000)	1	perazine, thioridazine, prochlorperazine, perphenazine, trifluoperazine, flupentixol, fluphenazine, thioproperazine#	propericiazine	SPME (polyacrylate–coated fiber)	Capcell Pak C_{18} UG120, S–5 μm, 2.0 x 150 (Shiseido)	gradient with 10 mM ammonium acetate and ACN	ESI, full scan m/z 50–500, SRM, MS/MS	linearity, precision, accuracy,
Roman *et al.*[49] ◇ (2008)	1	buspirone, fluphenazine, flupentixol, perphenazine, risperidone, 9OH–risperidone, ziprasidone, zuclopenthixol	haloperidol–d_4	LLE (trizma buffer, methyl t–butyl ether)	Zorbax Stable Bond Cyano column (50 x 2.1 mm, 3.5 μm)	gradient with different ratios MeOH, ACN, 20 mM ammonium formate	ESI, positive mode, SRM, MS/MS	selectivity, linearity, LLOQ, precision, recovery, matrix effects
Saar *et al.*[50] (2010)	0.1	9OH-risperidone, amisulpride, aripiprazole, bromperidol, buspirone, chlorpromazine, chlorprothixene, clozapine, droperidol, fluphenazine, fluspirilene, haloperidol, levomepromazine, loxapine, melperone, mesoridazine, olanzapine, perazine, pericyazine, perphenazine, pimozide, pipamperone, prochlorperazine, promazine, promethazine, quetiapine, risperidone, sulpiride, thioridazine, trifluoperazine, triflupromazine, ziprasidone, zotepine, zuclopenthixol	haloperidol–d_4	LLE (trizma buffer, 1–chlororbutane)	Zorbax Eclipse XCB–C_{18} (4.6 x 150, 5 μm)	gradient with ammonium formate and ACN	ESI, positive mode, SRM, MS/MS	selectivity, linearity, accuracy, precision, PS stability, LT stability, LLOQ, extraction efficiencies, matrix effects, process efficiencies, F/T stability
Seno *et al.*[95] (1999)	1	flupentixol, perazine, prochlorperazine, trifluoperazine, thioproperazine, perphenazine, fluphenazine, propericiazine, thioridazine#	?	SPE	Capcell Pak C_{18} UG80, S–5 μm, 1.0 x 250 mm (Shiseido)	gradient with 10 mM ammonium acetate and ACN	ESI, positive mode, SRM, MS/MS (for Flupentixol)	linearity, recovery,
Verweij *et al.*[59] (1994)	1	chlorprothixene, flupentixol, thiothixene, zuclopenthixol	N/A	SPE (Bond Certify 3 cc column (Varian)	HP 5 μm Asahipak ODP–50, 4.0 x 125 mm	isocratic with ACN and 50 mM ammonium acetate in dH_2O (85:15)	ESI, comparison of fullscan and SRM	selectivity, linearity

b)

Author (Year)	Sample [ml]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Choong *et al.*[55] (2009)	0.5	aripiprazole, clozapine, olanzapine, sertindole, dehydroaripiprazole, norclozapine, dehydrosertindole#	remoxipride	SPE (mixed mode support)	Xbridge C_{18} column (2.1 mm x 100, 3.5 μm)	gradient with ammonium acetate 20 mM and ACN	ESI, positive mode, MS, SIM	selectivity, repeatability, precision, trueness, accuracy, matrix effects, F/T and LT stability, PS stability
Kollroser *et al.*[28] (2002)	0.05	clozapine, desmethylclozapine, olanzapine	dibenzepine	direct injection procedure, HPLC–integrated sample clean–up with Oasis® HLB extraction column (50 mm x 1.3, 5 μm)	Symmetry C_{18} Waters (3.0 x 150 mm, 5 μm)	isocratic with ACN/ formic acid	ESI, positive mode, SRM, MS/MS	selectivity, linearity, recovery, LLOQ, accuracy, precision,
Kratzsch *et al.*[56] (2003)	0.5	amisulpride, bromperidol, clozapine, droperidol, flupentixol, fluphenazine, haloperidol, melperone, olanzapine, perazine, pimozide, risperidone, sulpiride, zotepine, zuclopenthixol, norclozapine, clozapine–N–oxide, 9OH-risperidone	trimipramine–d3	SPE	Merck LiChroCART column (125 x 2 mm)	gradient with 5 mM aqueous ammonium formate and ACN	APCI, positive mode, MS/MS, SRM	selectivity, linearity, accuracy, precision, F/T stability, LT stability, PS stability, recovery
Remane *et al.*[82] (2011)	0.5	9OH-risperidone, amisulpride, aripiprazole, benperidol, bromperidol, chlorpromazine, clozapine, clozapine–N–oxide, droperidol, flupentixol, fluphenazine, fluspirilene, haloperidol, levomepromazine, melperone, norclozapine, perazine, perphenazine, pimozide, pipamperone, promazine, prothipendyl, quetiapine, risperidone, sulpiride, thioridazine, ziprasidone, zotepine#	citalopram–d_6, norclozapine–d_8, nordazepam–d_5, trimipramine–d3, zolpidem–d_6	LLE (butyl acetate/ ethyl acetate)	TF Hypersil GOLD Phenyl column (100 x 2.1 mm, 1.9 μm)	Gradient with 10 mM aqueous ammonium formate plus 0.1% formic acid (pH = 3.4) and ACN plus 0.1% formic acid	APCI, positive mode, MS/ MS, SRM	selectivity, linearity, accuracy, precision, ion suppression/ enhancement of co–eluting analytes, PS stability, LT stability, LLOQ, extraction efficiencies, matrix effects, process efficiencies, "crosstalk", F/T stability
Zhou *et al.*[51] (2004)	0.5	clozapine, olanzapine, risperidone, quetiapine	diazepam	LLE (ether)	Macherey–Nagel C18 (2 mm x 125 mm, 3 μm)	isocratic with dH_20 (formic acid: 2.7 mmol/l, ammonium acetate: 10 mmol	ESI, SRM,	accuracy, precision, LT stability, F/T stability

Table 3. *(Continued)*

c)

Author (Year)	Sample [ml]]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Gutteck *et al.*[48] (2003)	1	flupentixol, fluphenazine, pipamperone, thioridazine, zuclopenthixol	imipramine–d3, doxepine–d3, chlorohaloperidol	LLE (n–hexane/ dichloromethane 4:1) or dichloromethane	Silice Uptisphere column RP C_{18} (12.5 cm x 2 mm, 5 μm)	isocratic with four different combinations of 50 mM acetate buffer and ACN	ESI, positive mode, MS, SIM	linearity, selectivity, precision, accuracy, recovery, LLOQ
Hasselstrom *et al.*[27] (2011)	0.06	clozapine, quetiapine, ziprasidone	clozapine–d3, quetiapine–d8, ziprasidone–d8	Zinc sulphate, MeOH, 96–well plate	Zorbax SB–C_8 (2.0 x 50 mm, 1.8 μm)	gradient with formic acid in dH_2O and formic acid in MeOH	ESI, positive mode, SRM MS/MS,	selectivity, recovery, matrix effects, LLOQ, precision, trueness, LT stability
Kirchherr *et al.*[61] (2006)	0.1	amisulpride, aripiprazole, benperidol, chlorpromazine, chlorprothixene, olanzapine, flupentixol, fluphenazine, haloperidol, 9OH-risperidone, levomepromazine, olanzapine, perazine, perphenazine, pimozide, pipamperone, quetiapine, risperidone, sulpiride, thioridazine, ziprasidone, zotepine, zuclopenthixol	clonidine, methylrisperidone, MBHZ	PP (ACN:MeOH)	Chromolith Speed ROD C_{18} (50 mm x 4.6 mm, 5 μm)	gradient with MeOH and acetic acid	ESI, positive mode, SRM, MS/MS	linearity, accuracy, precision, LLOQ, recovery, matrix effects
Niederlaender *et al.*[57] (2006)	N/A	clozapine, desmethylclozapine, clozapine–N–oxide	mirtazapine	SPE (online)	Zorbax Eclipse XDB–C_{18} (4.6 x 150 mm, 5 μm)	isocratic with MeOH–aqueous ammonium acetate buffer (25 mM)	ESI, positive mode, MS, SIM	linearity, recovery, accuracy, precision. LLOQ
Rittner *et al.*[58] (2001)	1	clozapine, haloperidol, levomepromazine, perazine, pimozide, sulpiride[#]	flunitrazepam–d3	SPE	Symmetry WAT C_{18} (1.0 x 150 mm, 3.5 μm)	gradient with ACN, dH_2O , MeOH	ESI, positive mode, MS fullscan mode (m/z = 100–650)	N/A

Abbreviations: ACN: acetonitrile, APCI: atmospheric pressure chemical ionization, dH_2O: deionized water, ESI: electrospray ionization, F/T: freeze/thaw, LLOQ: lower limit of quantification, LOD: limit of detection, LT: long term, m/z: mass over charge ratio, MeOH: methanol, SRM: selected reaction monitoring, MS: single stage mass spectrometry, MS/MS: tandem mass spectrometry, PP: protein precipitation, PS: processed sample, SIM: single ion monitoring, SPE: solid phase ectraction, SPME: solid–phase micro–extraction

[#] : More drugs are included in this method but do not belong to the group of APs.

[##] : This method was applied to more than one matrix.

[◇] : Post–mortem specimens were analyzed in this method.

[?] : An IS appears to have been used but is not specified.

Table 4. Summary of methods for the detection of APs in hair.

Author (Year)	Sample [g]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Josefsson *et al.*[16] ## (2003)	0.01–0.02	buspirone, chlorpromazine, chlorprothixene, clozapine, dixyrazine, flupentixol, fluphenazine, haloperidol, hydroxyzine, levomepromazine, melperone, olanzapine, perphenazine, pimozide, prochlorperazine, risperidone, thioridazine, ziprasidone, zuclopenthixol#	N/A	Incubation for 15 min in 1 M NaOH, 25 mM trizma buffer, extraction with BuCl, back extraction into formic acid	Zorbax Stable Bond Cyano column (50 x 2.1 mm, 3.5 μm)	Gradient with MeOH–ACN–20 mM ammonium formate and MeOH– ACN–20 mM ammonium formate	ESI, positive mode, MS/MS, SRM	N/A
McClean *et al.*[18] (2000)	0.5	chlorpromazine, flupentixol, trifluoperazine, risperidone	trimipramine	MeOH, NaOH, 4 M hydrochloric acid, final extraction with hexane	Phenomenex Luna C_{18} (150 x 4.6 mm)	Isocratic with 0.02 mol/L ammonium acetate/0.1% acetic acid in dH_2O and ACN	ESI, positive mode, MS/MS, SRM	linearity, LOD, recovery
Mueller *et al.*[19] (2000)	0.05	pipamperone#	doxepine–d_3	MeOH, SPE (mixed mode)	RP–C_8–select B (2 mm x 125 mm, 5 μm)	Gradient with ACN 25% aqueous ammonia and formic acid	ESI/CID–MS, ProdI scan, positive mode, MS/MS, SRM	N/A
Nielsen *et al.*[20] (2010)	0.01	chlorprothixene, clozapine, levomepromazine, promethazine, quetiapine#	mianserin– d_3	Incubation with MeOH:ACN: ammonium formate (2 mM, 8% ACN, pH = 5.3) at 37 °C for 18 hrs, Mini–Uniprep vials (PTFE filter)	Waters 100 mm x 2.1 mm ACQUITY HSS T3 1.8 μm C_{18}	Gradient with 0.05% formic acid and MeOH	ESI, positive mode, TOF–MS	LOD, LLOQ, matrix effects, selectivity, carry–over, linearity, trueness, precision
Thieme *et al.*[21] (2007)	0.05 (divided into single hairs for segmentation)	clozapine, norclozapine	5–(4–methylphenyl)–5–phenyl hydantoine	Decontamination with 5 ml petroleum benzene,] Ultrasonication with 3 ml MeOH for 3 hrs, reduce to single hairs, segmentation, 3 hrs ultrasonication in 30uL dH_2O / MeOH (50/50)	Synergy Polar–RP (Phenomenex, 75 mm x 2.0 mm, 4 μm)	Isocratic with ammonium acetate buffer in (50:50) water and ACN	ESI, ProdI, MS/MS, SRM	N/A

Table 4. *(Continued)*

Author (Year)	Sample [g]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
Weinmann *et al.*[22] (2002)	0.02–0.05	clozapine, norclozapine, haloperidol, penfluridol, thioridazine, northioridazine, flupentixol, zuclopenthixol, de–(hydroxyethyl)–zuclopenthixol	doxepine–d_3	Ultrasonication with 4 ml MeOH for 2 hrs, SPE (mixed mode)	RP–C_8–select B (2 mm x 125 mm, 5 μm)	Gradient with 1 mM ammonium formate/ 0.1% formic acid, and ACN/0.1% formic acid	ESI, ProdI, MS/MS, SRM	linearity, LOD, LLOQ, recovery, precision

Abbreviations: ACN: acetonitrile, BuCL: 1–chlorobutane, dH_2O: deionized water, CID: collision induced dissociation ESI: electrospray ionization, LLOQ: lower limit of quantification, LOD: limit of detection, MeOH: methanol, SRM: selected reaction monitoring, MS: single stage mass spectrometry, MS/MS: tandem mass spectrometry, NaOH: sodium hydroxide, ProdI: Product Ion Scan, SPE: solid phase extraction, TOF: time of flight

[#] : More drugs are included in this method but do not belong to the group of APs.

[##] : This method was applied to more than one matrix.

Table 5. Summary of methods for the detection of APs in CSF, saliva, and urine using LC–MS/MS.

Author (Year)	Matrix	Sample [g]	Drugs	IS	Extraction	Stationary Phase	Mobile Phase	Detection mode	Validation data
Arinobu *et al.*[14] ## (2002)	urine	1	haloperidol, reduced haloperidol, 4–(4–chlorophenyl)–4–hydroxypiperidine	4–[4–(4–chlorophenyl)–4–hydroxy–1–piperidinyl]–(4–chlorophenyl–1–butanone	addition of 3 ml of dH_2O with 0.09% formic acid and 20 mM ammonium acetate, freezing, thawing, centrifugation, injection of 20μL of supernatant	Mspak GF–310 4B (50 x 4.6 mm)	gradient with formic acid and 20 mM ammonium acetate in dH_2O (A) and ACN (B)	SSI, positive mode, MS	LOD, precision, accuracy
De Meulder *et al.*[15] ## (2008)	urine	0.2	risperidone, 9OH-risperidone	$^2H_2$–$^{13}C_2$–risperidone and $^2H_2$–$^{13}C_2$–9OH–risperidone	SPE (mixed mode)	Chiralcel OJ column (50 mm x 4.6, 10 μm)	gradient with hexane, 0.01 mM ammonium acetate in isopropanol, 0.01 mM ammonium acetate in ethanol	ESI, positive mode, SRM, MS/MS	selectivity, precision, accuracy, recovery, F/T and LT stability, PS stability
Flarakos *et al.*[24] ## (2004)	saliva	0.025	risperidone, 9OH-risperidone	R068808	online cleanup, column switching	Zorbax SB_{18} (30 x 2.1 mm, 3.5 μm)	isocratic with 10 mM ammonium acetate/ACN	N/A	linearity, selectivity, precision, accuracy, recovery, matrix effects, F/T and LT stability

Table 5. *(Continued)*

Author (Year)	Matrix	Sample [g]	Drugs	IS	Extraction	Stationary Phase	Mobile Phase	Detection mode	Validation data
Kumazawa *et al.*[17] ## (2000)	urine	1	perazine, thioridazine, prochlorperazine, perphenazine, trifluoperazine, flupentixol, fluphenazine, thioproperazine#	propericiazine	SPME (polyacrylate–coated fiber)	Capcell Pak C_{18} UG120, S–5 μm, 2.0 x 150 (Shiseido)	gradient with 10 mM ammonium acetate and ACN	ESI, full scan m/z 50–500, SRM, MS/MS	linearity, precision, accuracy,
Josefsson *et al.*[25] ## (2010)	CSF	0.2	olanzapine, N–desmethylolanzapine	olanzapine–d_3	LLE (tert–butyl–methyl–ether)	Synergi Hydro–RP (50 mm x 2 mm, 2.5 μm)	gradient with 10 mM ammonium formate with formic acid and MeOH with formic acid	ESI, positive mode, SRM, MS/MS	linearity, LLOQ, precision, accuracy, recovery, matrix effects, F/T stability, LT stability
Bogusz *et al.*[76] ## (1999)	urine	1	olanzapine	LY170222	SPE	Super Spher RP $_{18}$ (125 x 3 mm; 4 μm) (Merck)	isocratic with ACN/ammonium formate, OLZ metabolites with Gradient	APCI, positive mode, MS	recovery, LLOQ, precision, linearity, selectivity, F/T and LT stability
Josefsson *et al.* [16] ## (2003)	urine	0.5	buspirone, chlorpromazine, chlorprothixene, clozapine, dixyrazine, flupentixol, fluphenazine, haloperidol, hydroxyzine, levomepromazine, melperone, olanzapine, perphenazine, pimozide, prochlorperazine, risperidone, thioridazine, ziprasidone, zuclopenthixol	N/A	SPE	Zorbax Stable Bond Cyano column (50 x 2.1 mm, 3.5 μm)	Gradient with different ratios of MeOH:ACN:20 mM ammonium formiate	ESI, positive mode, SRM, MS/MS	N/A

Legend: ACN: acetonitrile, APCI: atmospheric pressure chemical ionization, dH_2O: deionized water, ESI: electrospray ionization, F/T: freeze/thaw, LLOQ: lower limit of quantification, LOD: limit of detection, LT: long term, m/z: mass over charge ratio, MeOH: methanol, SRM: selected reaction monitoring, MS: single stage mass spectrometry, MS/MS: tandem mass spectrometry, PS: processed sample, SIM: single ion monitoring, SPE: solid phase extraction, SPME: solid–phase micro–extraction, SSI: sonic spray ionization

: More drugs are included in this method but do not belong to the group of APs.

: This method was applied to more than one matrix.

of the metabolism of OLZ in humans.[77] It was hypothesized that OLZ-10-N-glucuronide and N-desmethyl-OLZ would be present in urine samples following OLZ ingestion. However, the compounds were not unequivocally identified as a valid reference standard was not available.

To the authors' knowledge, the only method for the detection of APs in oral fluid was published by Flarakos *et al.* in 2004.[24] Their fully validated method applied online clean-up with column switching for the detection of RIS and 9OH RIS in 25 μl saliva and plasma, aiming to establish a salivary/plasma (S/P) ratio. A wide range of S/P ratios obtained from 13 plasma and saliva samples (seven adults and six children) confirmed that saliva analysis only provided a qualitative tool for the presence of RIS and 9OH RIS but did not allow a conclusion regarding plasma concentrations at the time of sampling.

Josefsson *et al.* applied their detection method for OLZ and N-desmethyl OLZ not only to serum but also to CSF.[25] The authors postulated that the pharmacological effects of OLZ are likely to be more closely related to its concentration in the CFS than in serum. With a LLOQ of 0.2 ng/ml in plasma, the method showed sufficient sensitivity for the expected low concentrations in CSF. The authors postulated a linear correlation between serum and CSF OLZ concentrations ($r^2 = 0.77$). While there were only six individuals included in this study, the developed method was successfully applied to a cohort of 37 individuals. The authors also considered the influence of gender, age, smoking, and pharmacogenetics, when investigating the ratio between OLZ and metabolite concentrations in serum and CSF.[78]

LC separation

All APs possess hydrophobic properties and as such, all currently published methods for the detection and quantification of APs in biological matrices have employed reversed phase (RP) stationary phases, with mostly silica-based packings containing C_8 and C_{18} chains. Cabovska *et al.*[40] and de Meulder *et al.*[15] used chiral columns in order to separate the (+) and (−) enantiomers of 9OH RIS. 9OH RIS is the main metabolite of the atypical AP RIS and has shown to be almost equipotent to risperidone in animal studies.[79] Due to its efficacy, racemic 9OH RIS (paliperidone) is also marketed as a drug in its own right.[80] The separation of the two enantiomers is useful for kinetic studies, as the formation of the (+)-form appears to be catalyzed by CYP2D6, whereas CYP3A4 and CYP3A5 are essential for the formation of the (−)-form.[81] The separation of these enantiomers is usually not essential in routine drug analysis.

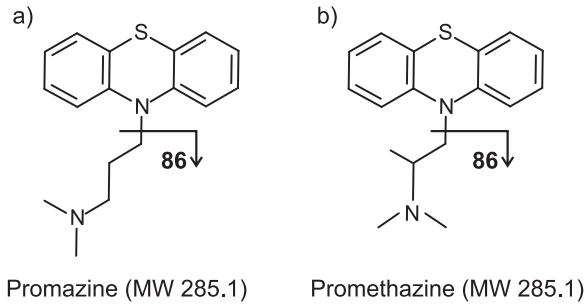
Columns packed with <2 μm particles are referred to as ultra high pressure LC (UHPLC) columns and are said to reduce analytical run times due to improved compound separation. This is desirable in a TDM environment where a large number of samples are tested for very few compounds. To the authors' knowledge, there are only two methods using UHPLC published to date. Hasselstrom *et al.*[27] used a Zorbax SB-C_8 column with a particle size of 1.8 μm, resulting in the detection and quantification of 13 antidepressants and APs, in addition to 13 deuterated IS over a total analytical run time of 4 min. Remane *et al.*[82] covered a total of 62 compounds including 31 APs over a total run time of 26 min, employing a TF Hypersil GOLD Phenyl column with a particle size of 1.9 μm. A recent review, however, compared the separation power of columns with particle sizes of 1.8 μm and 5 μm at a 'fast' (1 ml/l) and a 'slow' (0.3 ml/l) flow rate, and concluded that the particle size was less significant than initially proposed. The column particle size appeared to make only a modest difference in the peak height, peak width, or resolution, with the difference for each parameter being less than a factor of 2. Higher flow rates distinctively increased peak height by 6–7-fold and the peak width decreased by about 3-fold when using the faster flow rate.[64] In a post-mortem environment, larger particle sizes (3–5 μm) have proven to be favourable due to the higher robustness which is required for more complex matrices such as whole blood.[50] The presented methods show a wide range of isocratic and gradient elutions, including various aqueous and organic elution solvents. Details are shown in the column 'Mobile Phase' in Tables 3 and 4.

MS detection

Ionization of compounds in LC-MS technology is usually achieved with either electrospray ionization (ESI) or atmospheric-pressure chemical ionization (APCI). The reason ESI is used in the majority of presented methods for the detection of APs is likely to be associated with the higher sensitivity achieved by ESI. Bhatt *et al.* compared ESI with APCI, prior to development of their method for the detection of RIS and 9OH RIS in plasma. They found APCI to be less favourable when compared with ESI.[62] In a comprehensive study investigating the influence of anticoagulant and lipemia on matrix effects when analyzing OLZ, Chin *et al.* reported that the analyte response with APCI was five times less than with ESI.[83] Therefore, the required LLOQ of 0.05 ng/ml for OLZ was not achieved in APCI mode. The higher sensitivity achieved by ESI, however, was at the expense of lower selectivity. Many authors have found matrix effects to be more prominent when applying ESI.[84,85] Ionization efficient neutral compounds including matrix particles, co-eluting compounds, or additives such as salts in biological samples, can compete with analytes during the evaporation process. This is likely to lower the ionization rate of the compounds of interest. It is further suggested that during the evaporation process, the analyte of interest may precipitate from solution by itself or as a co-precipitate with non-volatile sample components.[84] This highlights the need for thorough sample clean-up prior to MS analysis and the assessment of matrix-effects as a crucial part of method validation. This is discussed later in this paper.

Due to the predominantly basic properties of APs, ionization takes place in the positive mode. The vast majority of published methods apply selected reaction monitoring (SRM) as an easy way for the detection and quantification of APs. International guidelines[86–88] require a minimum of two SRM transitions for reliable identification of an analyte – unfortunately a large component of SRM methods do not comply with this rule. The best example of possible misidentification of a compound due to monitoring a single SRM transition is the structurally similar O-desmethyl metabolite of the antidepressant venlafaxine and the synthetic opioid tramadol. Due to their almost identical chemical structure, they do not only elute at the same time but also share the most abundant transition (m/z 264.2 → 58.2).[89] Less common examples in the field of APs include the structural isomers promazine and promethazine (Figures 1a and 1b). These drugs share the most abundant transition (m/z 285 → 86), representing the cleavage of the side chain[50] and also elute at the same time.

The isobaric compounds pipamperone and haloperidol (Figures 2a and 2b) share the two most abundant transitions



Promazine (MW 285.1) Promethazine (MW 285.1)

Figure 1. Structures of promazine (a) and promethazine (b), their molecular weights and the side-chain fragmen-tation resulting in the most abundant fragment for both compounds (m/z=86).

(m/z 376.2 → 123 and m/z 376.0 → 165).[50] If sensitivity can still be maintained, it is recommended to pick a transition with a smaller abundance for one of the two analytes or, alternatively, add a third transition in order to guarantee reliable differentiation.

While MS in the SRM mode certainly provides an efficient tool for compound identification, these examples highlight the need to critically evaluate parameters (such as most abundant transitions) provided by the instrument during compound optimization. Few authors use screening procedures that allow subsequent quantification of APs of interest.[17,47,58]

Validation issues

Tables 2 and 3 present an overview of single-analyte and multi-analyte published methods, respectively, for the detection of APs in blood, plasma and serum using LC-MS(/MS). It is generally accepted that all methods must be validated using internationally accepted guidelines. Specific validation criteria must be met to satisfy the following minimum requirements:[7–9] selectivity, matrix effects, extraction efficiency, process efficiency, processed sample stability, linearity, accuracy, precision, and freeze-thaw stability. Although some authors claim to have conducted all/specific components of the method validation experiments, the quality and reputability of these experiments is not consistent across all papers. Parameters which are frequently associated with inconsistencies will be discussed below.

Internal standard

A variety of internal standards (IS) have been used in the reviewed methods. Preferred internal standards are deuterated compounds of the drug class of interest, such as clozapine-d3,[27] haloperidol-d4, [49,50] olanzapine-d3,[25] quetiapine-d8,[27] and ziprasidone-d8.[27] If these IS are unavailable to a laboratory, it is recommended to use a deuterated IS from a different drug-class rather than an AP that is in therapeutic use.[90] To the contrary, it has been suggested that high concentrations of a drug can influence the peak areas of their co-injected deuterated analogues when using APCI mode with isotope peaks (M + 1 to M + 3) of analytes contributing to the peak area of the IS. This can lead to miscalculation of the IS concentration and subsequently underestimation of the drugs of interest. However, for masses (M + 5) and higher, no isotopic contribution was observed.[91]

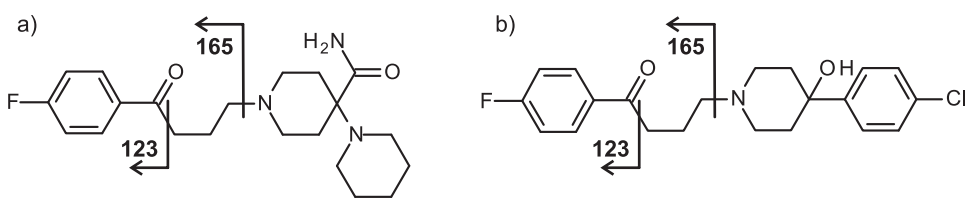
As co-medication and therapeutic use of a compound can never be fully excluded, overestimation of an IS is likely to result in underestimation of a drug concentration. Swart *et al.*[47] did not achieve good results in their detection method for fluspirilene in human plasma when using dimethothiazine as an IS. Their decision not to use an IS at all defies the guidelines of acceptable analytical practice. Particularly in cases where only few analytes are included in a method, a suitable deuterated IS is preferred in all instances. Unfortunately, this is not an isolated event. A large number of analytical methods still use therapeutic drugs as IS.[17,26,28,30,41,42,46,51,52,55,57,61,65,92]

Selectivity

In order to guarantee selectivity of an analytical method, it would be ideal that all possible interferences arising from matrix compounds, other drugs, and IS, are excluded. As this is impractical, the analysis of six blank specimens from different sources is widely considered acceptable[6] and is applied by most authors. The testing of 10 blank specimens, however, has been employed by some authors[50,56] and is encouraged for improved selectivity.[93] Josefsson *et al.*[25] performed method validation in accordance with international guidelines in their method for the detection of OLZ and N-desmethyl OLZ in CSF; however, selectivity of the method was not investigated. This is surprising, as despite the more invasive nature of sample collection compared with taking blood, the authors obtained drug-free CSF samples from six different patients. Several authors do not state clearly how many different sources of blank specimens were tested for interferences.[30,48] Klose Nielsen *et al.*[65] examined the interferences from other possible drugs in forensic samples by spiking blank blood samples with 66 common drugs such as benzodiazepines, analgesics, antidepressants, APs, β-blockers, narcotics and stimulants. Two 'zero' samples (blank sample containing IS) should be included in validation-experiments in order to exclude possible interferences of the IS on the selectivity of the method.

Calibration

Linearity is an important part of method validation whenever quantification of analytes via a standard curve is carried out,



Pipamperone (MW 376.2) Haloperidol (MW 376.0)

Figure 2. Structures of pipamperone (a) and haloperidol (b), their molecular weights and the fragmentations resulting in the two most abundant fragment for both compounds (m/z=123 and m/z=165).

which is the case in the vast majority of all published methods. An alternative is presented by Rittner *et al.* in their method for the detection of 70 psychoactive drugs, where they semi-quantify several analytes using the method of standard addition.[58]

Peters *et al.*[7] comprehensively summarized the requirements for an adequate calibration model in their review (which is beyond the scope of this paper). The calibration range should cover at least the therapeutic range of the drug of interest; however, as long as linearity can be assured, a greater range can be included.

Arinobu *et al.*[14] include 14 calibrators in order to cover the wide calibration range of 1 ng/ml–800 ng/ml for the detection of haloperidol and its metabolites in plasma and urine, measuring 10 replicates per calibrator. Moody *et al.*[45] could not guarantee linearity of calibration curves in their method targeting RIS and 9OH RIS when using ESI. As the calibration curves started to plateau above 10 ng/ml when using ESI, APCI was used to continue the method validation. The plateau could be caused by saturation of the detector. This is, however, unlikely as the concentrations injected are not very high with the highest calibrator at 25 ng/ml. Furthermore, the problem of the plateau does not exist in APCI mode, confirming that detector saturation is not the reason. A more likely cause is a saturation of the droplets during the ionization process; a problem not occurring in APCI mode as the ionization of compounds takes place in the gas-phase.

Matrix effects

The investigation of matrix effects is considered to be an essential part of method development. As discussed earlier, ESI appears to cause greater matrix effects than APCI; however, no new method should be accepted without appropriate investigation of matrix effects. Two approaches for the evaluation of matrix effects have been accepted by the analytical community: the post-column infusion approach presented by Bonfiglio *et al.*[94] and the post-extraction spike method by Matuszewski *et al.*[85]

While the evaluation process of matrix effects using these methods is considered to be common knowledge, there is some inconsistency throughout the literature when it comes to interpreting the details. When Matuszewski *et al.*[85] stated that an appropriate IS can compensate for matrix effects 'assuming the relative matrix effect exhibits the same pattern for the drug and the internal standard in all lots studied', some authors[40] unfortunately misinterpret this observation by stating that a deuterated IS can compensate for matrix effects. First, it must be confirmed that the matrix effects are equivalent for a drug and the respective IS, which is more likely if they show a similar chromatography and elute close to each other. Secondly, when it comes to low drug concentrations, ion suppression may lower the concentration of a drug below the LOD, in which case a positive case may be missed despite the concentration of the IS being lowered by the same percentage.

Berna *et al.* report to have investigated matrix effects in both their methods for the detection of OLZ in plasma and serum[53] and whole blood;[39] however, they do not report any outcomes. Swart *et al.*[47] conclude it is 'doubtful' that matrix effects are present in their method for the detection of fluspirilene in plasma as their calibration curves appear to be 'fairly linear'. There is no evidence to suggest that linear calibration curves give an indication of possible matrix effects, this assumption is therefore unjustified.

Stability

Processed sample stability

Prior to progressing to further validation experiments, the stability of the drugs of interest in processed samples must be verified. Extracted samples should not be stored longer than the stability in processed samples has been tested and assured; 24 h[39,40,52,53,62] is the most commonly investigated timeframe as runtimes are unlikely to exceed one day. Nevertheless, it can be useful to obtain stability information for a longer period of time in cases where instrument issues may cause samples to be re-run on the next day.[7,8]

There are three ways the result can be reported. Either as a percentage loss over a defined timeframe (given as the mean with SD);[42] a comparison between the initial drug concentration and the concentration after storage using a paired *t*-test; [40] or as more frequent injections over the investigated timeframe, a curve is generated and (after regression analysis) a negative slope significantly different from zero ($p < 0.05$) indicates instability.[56] Kratzsch *et al.* accurately plotted absolute peak areas as opposed to relative peak areas against the time of injection, in order to prevent the IS from correcting for eventual losses.[56] Some authors followed the recommendations of testing two concentrations (one low and one high of the calibration range),[42,44,47,50,55,56] whereas others improved on this by including an additional concentration.[15,30,54] Josefsson *et al.* investigated processed sample stability and found sample extracts to be unstable over 24 h, with significant losses for both OLZ and N-desmethyl OLZ.[25] This outcome is not surprising as significant stability issues in processed samples containing OLZ have been reported in other matrices such as whole blood.[50] If processed sample stability is not guaranteed over 24 h, it is recommended that analysis is completed prior to degradation of OLZ taking place.

Freeze-thaw stability

Assuring that multiple cycles of freezing and thawing do not compromise the integrity of tested samples is crucial in routine toxicological analysis. A blood sample is likely to be tested for different groups of analytes and therefore be thawed and frozen again several times. Experimental factors should be selected based on the conditions that are intended to be used on real cases, i.e. the temperature at which routine samples are being stored should be the temperature applied in the freeze-thaw (F/T) experiments. Shah *et al.* recommended the testing of at least three F/T cycles and two concentration levels in triplicate.[6,8] While there are variations in the number of concentration levels and F/T cycles tested by some authors, it is most concerning that there is still a large number of methods where no F/T stability experiments were conducted at all.[14,16,17,26–28,37,38,40,43,46–49,57–59,61,65,92,95]

Conclusions

Currently, there are more than 35 different APs available worldwide for the treatment of a range of psychotic illnesses. Over the past 15 years, recent advances in LC-MS(/MS) technology has enabled the detection and quantification of these drugs in exceptionally low concentrations; the newer generation APs in particular. This has led to the development of numerous LC-MS (/MS) methods for the analysis of APs in human biological specimens. A requirement for the success of such detection

methods is that they are suitably sensitive to cover the low therapeutic range in which APs are usually present. Proficiency with LC-MS(/MS) technology has increase dramatically over the past decade. Aspects of method development that require particular attention in order to guarantee reproducible results are identified and summarized in various method validation guidelines.[7–9] However, the quality of published methods with regard to validation criteria is not always consistent. The most significant issues relate to the evaluation of selectivity, linearity, matrix effects and stability. Addressing these issues in future analytical studies is mandatory to accurately detect APs in biological specimens and, consequently, to better understand this increasingly prevalent class of drugs.

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Appendix 1.2

Identification and Quantification of 30 Antipsychotics in Blood using LC-MS/MS

Saar, E., Gerostamoulos, D., Drummer, O.H., Beyer, J
J Mass Spectrom, 2010. **45**(8): p. 915-25

Research Article

Journal of MASS SPECTROMETRY

Received: 22 March 2010 Accepted: 14 June 2010 Published online in Wiley Interscience:

(www.interscience.com) DOI 10.1002/jms.1783

Identification and quantification of 30 antipsychotics in blood using LC-MS/MS

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Over the last decade, the prescription rates of antipsychotic (AP) drugs have increased worldwide. Studies have shown that the risk of sudden cardiac death is threefold higher among patients treated with APs. To investigate the presence of APs in postmortem cases, a liquid chromatography (LC)-MS/MS method was developed using only 0.1 ml of blood sample with 10 μl of internal standard (IS) (haloperidol-d_4, 1 μg/ml). After the addition of 0.2 ml of Trizma buffer, the blood sample was extracted using liquid–liquid extraction (LLE) with 1 ml of 1-chlorobutane for 5 min on a shaker at 1500 rpm. After centrifugation at 12 000 rpm for 1 min, the separated solvent layer was transferred to an autosampler vial and evaporated to dryness under N_2. The residue was reconstituted in 0.05 ml acetonitrile containing 0.1% formic acid, vortexed for 30 s and an additional 0.45 ml of 50 mmol/l ammonium formate pH 3.5 was added and the sample vortexed; 0.1 ml of the final extract was injected into a Shimadzu Prominence HPLC system, with detection of drugs achieved using an Applied Biosystems 3200 Q-TRAP® LC-MS/MS system equipped with a Turbo V ion source [electron spray ionization (ESI), multiple reaction monitoring (MRM) mode]. The method has been validated according to international guidelines and was found to be selective for all tested compounds. Calibration was satisfactory for all drugs, except olanzapine, from subtherapeutic to toxic concentrations. The lower limits of quantifications (LLOQs) corresponded to the lowest concentrations used for the calibration curves. With the exception of the lowest concentrations of bromperidol, buspirone and perphenazine, accuracy data were within the acceptance interval of ±15% (±20% at LLOQ) of the nominal values for all drugs. The method has been proven to be useful for the routine analysis of APs in postmortem blood samples.

Supporting information may be found in the online version of this article.

Keywords: antipsychotics; quantification; detection; LC-MS/MS; blood

Introduction

Antipsychotic (AP) drugs are widely prescribed for the treatment of schizophrenia and psychosis. The so-called first generation or typical APs were developed in the 1950s and show severe side-effects such as extrapyramidal symptoms due to their pharmacological action on d_2 and d_4 receptors. As a measure of reducing these severe side-effects, a range of 'second generation' APs were developed. These newer generation drugs act considerably less on d_2 and d_4 receptors and therefore exhibit less extrapyramidal side-effects.

Over the last decade, the prescription rate of APs (more notably second generation) has increased worldwide,[1–3] especially among young adults and children.[1–4] However, second generation APs are not entirely free of side-effects. Irrespective of their generation, it has been shown that these drugs can increase the risk of sudden cardiac death with studies showing that the risk of sudden cardiac death is increased threefold among patients treated with APs.[5–7]

There are several published multi-analyte procedures for the detection of APs in human blood.[8–15] Methods using high-performance liquid chromatography coupled with UV detection (HPLC-UV)[8–10] and gas chromatography coupled with nitrogen phosphorous detection (GC-NPD)[11] show that these techniques do not provide the required sensitivity or selectivity for the detection of low-dose APs in postmortem blood. Currently, the use of liquid chromatography coupled with mass spectrometry (LC-MS) has replaced some of the more traditional GC-MS assays showing superior selectivity and sensitivity.[12–15] Validated methods have been published by Kirchherr *et al*.[12] and Kratzsch *et al*.[13] covering a wide range of APs; however, neither method is suitable for postmortem blood, which is subject to decomposition and change in matrix effects. In addition, the method published by Kirchherr *et al*.[12] used only one transition for compound identification, despite contrary international recommendations. Roman *et al*.[15] described the detection of seven low-dose APs in postmortem blood, however, this study did not allow the simultaneous detection of other common APs. Although a study published by Josefsson *et al*.[14] covers the 19 most common APs in postmortem samples, this method was not validated.

Our unit is monitoring the presence of APs in forensic cases and we are not only interested in the detection but also the relative safety of these drugs either alone and/or in combination with other therapeutic agents. Therefore, a reliable, sensitive and validated quantitation method for a range of APs in blood samples has been developed. This article describes the detection and validated quantification of 30 APs in postmortem blood samples. The method has been validated according to internationally accepted criteria and guidelines.[16,17]

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Materials and Methods

Chemicals and reagents

Bromperidol, chlorpromazine, fluspirilene, haloperidol, pipamperone, prochlorperazine, thioridazine, trifluoperazine, triflupromazine and Trizma base were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Buspirone, chlorprothixene, mesoridazine, olanzapine, promazine, promethazine, risperidone and zuclopenthixol were obtained from the Division of Analytical Laboratories (Lidcombe, NSW, Australia). Droperidol, fluphenazine, loxapine, pericyazine, perphenazine, pimozide and sulpiride were provided by Australian Government Analytical Laboratories (Pymble, NSW, Australia). Levomepromazine, melperone, perazine and zotepine were obtained from Phast GmbH (Homburg/Saar, Germany). Amisulpride, aripiprazole, quetiapine and ziprasidone were purchased from National Institute of Forensic Science (Melbourne, VIC, Australia). Clozapine was provided by Sandoz (Pyrmont, NSW, Australia). 9-OH-risperidone was obtained from Janssen–Cilag (North Ryde, NSW, Australia). The isotope-labeled internal standard (IS) haloperidol-d_4 was purchased from Cerilliant (Round Rock, TX, USA). Acetonitrile, ammonium formate, 1-chlorobutane, methanol and formic acid were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from BDH Chemicals (Kilsyth, VIC, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia).

Trizma buffer (pH 9.2) was prepared by dissolving 242 g of Trizma base in 1 l water.

Specimens

Blood sample for calibration purposes was obtained from drug-free volunteers. Samples were collected into spray-coated K_2EDTA preserved plastic tubes (BD Australia, North Ryde, NSW). Postmortem blood samples were submitted to the authors' laboratory for routine toxicological analysis. The postmortem blood samples were regarded as drug free if none of the existing tests showed the presence of the studied drugs in any specimen (including blood, liver and urine). All postmortem blood samples were collected into plastic tubes containing 1% fluoride–oxalate. This is the standard collection tube in the laboratory. All blood samples were stored at −20 °C prior to analysis.

Apparatus

The LC-MS/MS system consisted of an Applied Biosystems 3200 Q-TRAP® linear ion-trap quadrupole mass spectrometer (Applied Biosystems, Melbourne, VIC, Australia) equipped with a Turbo V ion source, operated in the electron spray ionization (ESI) mode, and an Agilent Technologies (AT) 1200 Series HPLC system (Agilent, Melbourne, VIC, Australia) which consisted of a degasser, a binary pump and an autosampler.

HPLC conditions

Gradient elution was performed on an Agilent Zorbax Eclipse XDB-C_{18} (4.6 × 150 mm, 5 µm particle size; Biolab, Scoresby, VIC, Australia). The mobile phase consisted of 50 mmol/l aqueous ammonium formate adjusted to pH 3.5 with formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). During use, the mobile phase was degassed by the integrated Agilent 1200 degasser. Before starting the analysis, the HPLC system was equilibrated for 10 min with a mixture of 90% eluent A and 10% eluent B. The HPLC system was additionally equilibrated for 4 min prior to each run. The flow rate and gradient were programmed as follows: equilibration time (−4.00 to 0.00 min) 10% eluent B, flow rate of 1.4 ml/min; 0.00–1.00 min: 10% eluent B, flow rate of 1.4 ml/min; 1.01–18.00 min: gradient increase to 100% eluent B, flow rate increase to 2.2 ml/min; 18.01–20.00 min: 100% eluent B, flow rate of 2.2 ml/min.

The column oven was set at 60 °C. The autosampler was operated at room temperature; the autosampler needle was rinsed using a wash vial filled with a mixture of eluent A and eluent B (90 : 10).

MS/MS conditions

For detection and quantification, the following ESI inlet conditions were applied: gas 1, nitrogen (90 psi; 620.5 kPa); gas 2, nitrogen (90 psi; 620.5 kPa); ion spray voltage, 5500 V; ion source temperature, 750 °C; curtain gas, nitrogen (10 psi; 68.9 kPa). The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with the collision gas set at medium. The dwell times were optimized using scheduled MRM algorithm incorporated in Analyst® software 1.5. The MRM detection window was set at 60 s, the target scan time was 1.5 s. All other settings were analyte specific and were determined using Analyst® software in the quantitative optimization mode (Table 1). The most abundant MRM transition for each analyte was considered as quantifier ion.

Preparation of stock solutions, calibration standards and control samples

Stock solutions of amisulpride, bromperidol, buspirone, chlorpromazine, chlorprothixene, fluphenazine, fluspirilene, haloperidol, levomepromazine, loxapine, melperone, mesoridazine, perazine, pericyazine, perphenazine, pipamperone, promethazine, quetiapine, sulpiride, thioridazine, trifluperazine, ziprasidone, zotepine and zuclopenthixol were prepared at a concentration of 1 mg/ml by separate weighings using methanol. Stock solutions of 1 mg/ml of 9-OH-risperidone, aripiprazole, clozapine, droperidol, olanzapine, pimozide, prochlorperazine, promazine, risperidone and trifluoperazine were prepared using eluent B. The preparation of stock solutions in acetonitrile with formic acid was necessary due to the lack of solubility of these drugs in methanol.

Working solutions of each analyte were prepared using methanol by independent dilution from each stock solution at the following concentrations: 0.1 mg/ml, 0.01 mg/ml and 0.001 mg/ml. All solutions were stored at −60 °C.

The calibration standards were prepared using pooled blank blood and spiking solutions prepared from the working solutions as mixtures of the 34 APs at concentrations ten times higher than the corresponding calibration standards. The quality control (QC) samples were prepared using pooled blank blood and independently prepared mixtures of the 34 APs at concentrations 100 times higher than the concentrations of the corresponding QC samples and stored at −60 °C.

The final blood concentrations of the calibration standards and QC sample are given in Table 2.

Extraction procedure

In a 2-ml Eppendorf tube (Eppendorf Australia, North Ryde, NSW), 0.1 ml of blood sample was mixed with 10 µl of IS haloperidol-d_4

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Table 1. Analytes, multiple reaction monitoring (MRM) transitions and parameter settings including declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision cell energy (CE) and collision cell exit potential (CXP) used in LC-ESI-MS/MS

ID	Q1 Mass (Da)	Q3 Mass (Da)	Retention time (min)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
9-OH-Risperidone (Quant)	427.0	207.2	6.6	61	4.5	18	39	4
9-OH-Risperidone (Qual)		110.2		61	4.5	18	59	4
9-OH-Risperidone (Qual)		69.1		61	4.5	18	75	4
Amisulpride (Quant)	370.1	242.2	5.0	61	8	32	41	4
Amisulpride (Qual)		195.9		61	8	32	55	4
Amisulpride (Qual)		112.1		61	8	32	39	4
Aripiprazole (Quant)	448.0	285.2	8.9	71	9.5	20	33	4
Aripiprazole (Qual)		176.1		71	9.5	20	43	4
Aripiprazole (Qual)		98.2		71	9.5	20	51	4
Bromperidol (Quant)	422.0	123.1	8.5	1	12	50	59	4
Bromperidol (Qual)		165.1		1	12	50	37	4
Bromperidol (Qual)		95		1	12	50	103	4
Buspirone (Quant)	386.1	122.2	7.2	71	10	32	43	4
Buspirone (Qual)		79		71	10	32	105	4
Buspirone (Qual)		95.2		71	10	32	75	4
Chlorpromazine (Quant)	319.1	86.1	9.5	46	5	14	31	4
Chlorpromazine (Qual)		58.2		46	5	14	55	4
Chlorpromazine (Qual)		246.1		46	5	14	33	4
Chlorprothixene (Quant)	316.0	271.1	9.6	51	3.5	18	23	4
Chlorprothixene (Qual)		231		51	3.5	18	39	4
Chlorprothixene (Qual)		221.2		51	3.5	18	49	4
Clozapine (Quant)	327.1	270.2	7.8	51	4.5	30	29	4
Clozapine (Qual)		192.2		51	4.5	30	59	4
Clozapine (Qual)		164.1		51	4.5	30	95	4
Droperidol (Quant)	380.1	123.1	7.3	41	5.5	16	63	4
Droperidol (Qual)		194.2		41	5.5	16	21	4
Droperidol (Qual)		165.1		41	5.5	16	39	4
Fluphenazine (Quant)	438.1	171	10.1	61	6.5	60	25	4
Fluphenazine (Qual)		100		61	6.5	60	63	4
Fluphenazine (Qual)		143.1		61	6.5	60	57	4
Fluspirilene (Quant)	476.1	98.2	10.4	61	7.5	24	47	4
Fluspirilene (Qual)		371.3		61	7.5	24	25	6
Fluspirilene (Qual)		55.1		61	7.5	24	85	4
Haloperidol (Quant)	376.0	123.1	8.3	56	4.5	26	57	4
Haloperidol (Qual)		165.2		56	4.5	26	35	4
Haloperidol (Qual)		95		56	4.5	26	93	4
Levomepromazine (Quant)	329.1	58.1	9.1	41	6	34	59	4
Levomepromazine (Qual)		100.2		41	6	34	25	4
Levomepromazine (Qual)		242.1		41	6	34	29	4
Loxapine (Quant)	328.1	271.1	8.5	41	3.5	30	33	50
Loxapine (Qual)		84.2		41	3.5	30	33	4
Loxapine (Qual)		164		41	3.5	30	85	4
Melperone (Quant)	264.0	123.1	6.9	116	4	42	43	4
Melperone (Qual)		165.2		116	4	42	19	4
Melperone (Qual)		95.3		116	4	42	63	4
Mesoridazine (Quant)	387.7	98.2	7.6	51	8	56	51	4
Mesoridazine (Qual)		126.1		51	8	56	35	4
Mesoridazine (Qual)		70		51	8	56	87	4
Olanzapine (Quant)	313.1	256.2	4.9	56	4.5	14	31	4
Olanzapine (Qual)		198.1		56	4.5	14	53	4
Olanzapine (Qual)		84.2		56	4.5	14	33	4
Perazine (Quant)	340.0	141.2	9.0	56	9	32	27	4
Perazine (Qual)		113.1		56	9	32	39	4
Perazine (Qual)		70		56	9	32	57	4

Table 1. (*Continued*)

ID	Q1 Mass (Da)	Q3 Mass (Da)	Retention time (min)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Pericyazine (Quant)	365.8	114.2	8.2	56	5.5	34	43	4
Pericyazine (Qual)		142.1		56	5.5	34	33	8
Pericyazine (Qual)		44.1		56	5.5	34	77	4
Perphenazine (Quant)	404.0	171.1	9.6	56	10.5	18	31	4
Perphenazine (Qual)		143.2		56	10.5	18	39	4
Perphenazine (Qual)		100.2		56	10.5	18	57	4
Pimozide (Quant)	462.1	109.1	9.9	396	10.5	56	71	4
Pimozide (Qual)		328.3		396	10.5	56	33	4
Pimozide (Qual)		147.1		396	10.5	56	55	4
Pipamperone (Quant)	376.2	123.2	6.2	51	12	16	65	4
Pipamperone (Qual)		165.2		51	12	16	37	4
Pipamperone (Qual)		98.2		51	12	16	39	4
Prochlorperazine (Quant)	374.1	141.4	10.1	46	7.5	40	27	4
Prochlorperazine (Qual)		113.1		46	7.5	40	35	4
Prochlorperazine (Qual)		70.2		46	7.5	40	63	4
Promazine (Quant)	285.1	86.2	8.4	46	4.5	34	27	4
Promazine (Qual)		58.1		46	4.5	34	53	4
Promazine (Qual)		180.1		46	4.5	34	51	4
Promethazine (Quant)	285.1	86.1	8.5	36	4.5	32	27	4
Promethazine (Qual)		198.1		36	4.5	32	35	4
Promethazine (Qual)		71.2		36	4.5	32	57	4
Quetiapine (Quant)	384.1	253.2	7.9	61	5	18	29	4
Quetiapine (Qual)		221.3		61	5	18	53	4
Quetiapine (Qual)		279.2		61	5	18	33	4
Risperidone (Quant)	411.1	191.2	7.1	56	9	18	41	4
Risperidone (Qual)		110.2		56	9	18	69	4
Risperidone (Qual)		82.2		56	9	18	81	4
Sulpiride (Quant)	342.0	112.2	3.0	66	4.5	40	37	4
Sulpiride (Qual)		214.1		66	4.5	40	45	4
Sulpiride (Qual)		84.1		66	4.5	40	57	4
Thioridazine (Quant)	371.1	126.2	10.2	51	8.5	16	33	4
Thioridazine (Qual)		98.3		51	8.5	16	47	4
Thioridazine (Qual)		70		51	8.5	16	87	4
Trifluoperazine (Quant)	408.0	70	10.6	61	5	34	67	4
Trifluoperazine (Qual)		113.2		61	5	34	39	4
Trifluoperazine (Qual)		141.3		61	5	34	31	4
Triflupromazine (Quant)	353.0	58.1	10.0	56	1	12	55	4
Triflupromazine (Qual)		86.3		56	1	12	33	4
Triflupromazine (Qual)		280.2		56	1	12	31	4
Ziprasidone (Quant)	413.0	194	7.7	66	8.5	22	41	4
Ziprasidone (Qual)		130		66	8.5	22	91	4
Ziprasidone (Qual)		159.2		66	8.5	22	55	4
Zotepine (Quant)	332.1	72.1	9.7	16	5.5	14	39	4
Zotepine (Qual)		42.2		16	5.5	14	109	6
Zotepine (Qual)		72.6		16	5.5	14	31	58
Zuclopenthixol (Quant)	401.0	231.2	9.7	66	4.5	38	55	4
Zuclopenthixol (Qual)		221.1		66	4.5	38	69	4
Zuclopenthixol (Qual)		271		66	4.5	38	37	4
Haloperidol-d_4 (Quant)	380.1	169.2	8.3	41	5	18	33	4
Haloperidol-d_4 (Qual)		127.1		41	5	18	57	4

at a concentration of 1 µg/ml. To the blood sample, 0.2 ml of Trizma buffer and 1 ml of 1-chlorobutane were added and mixed thoroughly. The sample was extracted for 5 min on a VXR basic IKA® Vibrax shaker at 1500 rpm. After a brief centrifugation to separate layers, the solvent layer was transferred to an autosampler vial and evaporated to dryness using a Ratek dry block heater DBH10 operated at room temperature.

The residue was reconstituted in 50 µl of eluent B, and diluted with 450 µl of eluent A; 0.1 ml of the final extract was injected into the LC-MS/MS system.

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Table 2. Concentrations of calibration standards and quality control samples of all studied analytes as well as respective described therapeutic blood concentrations

Drug	S[a]1	S2	S3	S4	S5	S6	S7	QC LOW	QC MED	QC HIGH	Therapeutic level (µg/l)
Bromperidol	0.1	0.5	7.5	15	22.5	30	40	0.3	20	35	1–20[13]
Buspirone	0.1	0.5	7.5	15	22.5	30	40	0.3	20	35	1–10[18]
Perphenazine	0.1	0.5	7.5	15	22.5	30	40	0.3	20	35	0.6–2.4[12]
Droperidol	1	5	20	40	60	80	100	3	50	90	5–50[13]
Fluphenazine	1	5	20	40	60	80	100	3	50	90	2–20[13]
Fluspirilene	1	5	20	40	60	80	100	3	50	90	N/A[b]
Haloperidol	1	5	20	40	60	80	100	3	50	90	5–50[13]
Levomepromazine	1	5	20	40	60	80	100	3	50	90	15–60[12]
Pericyazine	1	5	20	40	60	80	100	3	50	90	5–60[18]
Pimozide	1	5	20	40	60	80	100	3	50	90	15–20[12]
Trifluoperazine	1	5	20	40	60	80	100	3	50	90	1–50[18]
9-OH-Risperidone	1	5	40	80	120	150	200	3	100	175	10–100[13]
Loxapine	1	5	40	80	120	150	200	3	100	175	10–100[12]
Olanzapine	1	5	40	80	120	150	200	3	100	175	10–100[13]
Risperidone	1	5	40	80	120	150	200	3	100	175	10–100[13]
Zuclopenthixol	1	5	40	80	120	150	200	3	100	175	5–100[13]
Aripiprazole	10	50	150	250	350	450	600	30	300	500	50–350[12]
Chlorpromazine	10	50	150	250	350	450	600	30	300	500	30–300[12]
Chlorprothixene	10	50	150	250	350	450	600	30	300	500	20–200[12]
Perazine	10	50	150	250	350	450	600	30	300	500	100–230[12]
Quetiapine	10	50	150	250	350	450	600	30	300	500	70–170[12]
Triflupromazine	10	50	150	250	350	450	600	30	300	500	30–100[18]
Ziprasidone	10	50	150	250	350	450	600	30	300	500	50–120[12]
Amisulpride	10	50	200	350	500	650	800	30	400	700	50–400[13]
Melperone	10	50	200	350	500	650	800	30	400	700	50–400[13]
Pipamperone	10	50	200	350	500	650	800	30	400	700	100–400[18]
Promethazine	10	50	200	350	500	650	800	30	400	700	50–400[18]
Sulpiride	10	50	200	350	500	650	800	30	400	700	50–400[13]
Clozapine	10	50	250	500	800	1200	1600	30	650	1400	200–800[13]
Mesoridazine	10	50	300	700	1100	1600	2000	30	900	1800	150–1000[18]
Prochlorperazine	1	50	200	350	500	750	1000	3	450	850	10–500[18]
Promazine	1	5	150	300	450	650	800	3	400	700	10–400[18]
Thioridazine	10	600	1100	1800	2500	3200	4000	30	2150	3600	200–2000[12]
Zotepine	1	5	150	300	400	500	600	3	350	550	5–300[13]

All concentrations are given in µg/l.
[a] S, standard, all concentrations given in µg/l.
[b] No therapeutic concentration available.

Validation experiments

Selectivity

Selectivity experiments were carried out using postmortem and antemortem blood samples sent to the authors' laboratory for toxicological analysis. Ten postmortem and 5 antemortem samples were extracted as described previously without the addition of IS. The samples were analyzed to exclude any interference with endogenous peaks. In addition, two zero samples (blank sample + IS) were analyzed to check for the absence of analyte ions in the respective peaks of IS.

Linearity

Aliquots of blank blood samples were spiked at concentrations given in Table 2 and extracted as described previously to obtain calibration standards. The chosen concentrations ranged from half the lowest described therapeutic concentration of each AP to double the highest described therapeutic concentration.

Replicates ($n = 6$) at each of the 7 concentration levels were analyzed. Daily calibration curves using the same concentrations (single measurements per level) were prepared with each batch of validation and authentic samples.

Accuracy and precision

QC samples 'QC LOW', 'QC MEDIUM' and 'QC HIGH' were prepared at concentrations described in Table 2. Two samples of each QC concentration were measured over a period of eight consecutive days. Daily calibration curves were used to calculate the concentration of the QCs. Accuracy was calculated for each analyte as bias determined by calculating the percent deviation of the mean of all calculated concentration values at a specific level from the respective nominal concentration. Precision data [given as relative standard deviations (RSDs)] for within-day (repeatability) and time-different intermediate precision (combination of within- and between-day effects) of

Table 3. Mean values and ranges of recoveries and matrix effects using different sources of blank blood ($n = 5$) spiked at 'QC LOW' and 'QC HIGH' concentrations

Drug	Recovery		Matrix effects	
	LOW Mean (range)	HIGH Mean (range)	LOW Mean (range)	HIGH Mean (range)
9-OH-Risperidone	105 (102–108)	95 (82–103)	53 (46–62)	76 (69–88)
Amisulpride	68 (57–82)	72 (66–77)	91 (77–97)	104 (93–117)
Aripiprazole	105 (101–112)	97 (87–108)	90 (75–102)	107 (93–115)
Bromperidol	***96 (61–116)***	110 (100–124)	***245 (166–337)***	104 (92–113)
Buspirone	***120 (98–171)***	100 (89–110)	91 (65–115)	105 (89–124)
Chlorpromazine	91 (84–97)	86 (80–97)	96 (81–104)	106 (89–118)
Chlorprothixene	100 (91–108)	93 (84–105)	100 (86–112)	108 (96–112)
Clozapine	108 (99–115)	98 (91–109)	104 (84–115)	105 (93–117)
Droperidol	101 (85.6–112)	94 (79–105)	93 (83–104)	108 (92–122)
Fluphenazine	100 (92–107)	94 (83–110)	98 (83–110)	98 (83–114)
Fluspirilene	98 (87–110)	85 (73–93)	92 (85–100)	102 (94–109)
Haloperidol	110 (90–127)	99 (91–110)	112 (105–119)	108 (90–116)
Levomepromazine	91 (76–99)	85 (78–97)	99 (82–111)	107 (94–120)
Loxapine	107 (95–123)	89 (87–92)	88 (81–97)	109 (95–116)
Melperone	104 (88–116)	90 (85–100)	108 (95–121)	***97 (66–117)***
Mesoridazine	101 (93–112)	98 (95–101)	93 (83–105)	102 (93–110)
Olanzapine	79 (64–91)	83 (70–92)	***237 (172–292)***	140 (122–157)
Perazine	104 (100–110)	92 (90–94)	101 (91–109)	106 (91–121)
Pericyazine	84 (81–86)	88 (81–93)	116 (100–135)	102 (92–115)
Perphenazine	***132 (91–199)***	89 (80–96)	79 (56–95)	102 (83–118)
Pimozide	93 (92–93)	89 (83–96)	87 (72–117)	105 (95–122)
Pipamperone	104 (95–118)	94 (89–99)	85 (76–97)	100 (90–107)
Prochlorperazine	97 (90–103)	95 (89–101)	97 (86–106)	97 (87–108)
Promazine	93 (78–103)	93 (91–94)	101 (92–116)	106 (92–119)
Promethazine	98 (91–103)	93 (86–100)	100 (84–112)	105 (96–114)
Quetiapine	107 (102–114)	102 (88–111)	95 (82–105)	105 (88–122)
Risperidone	108 (99–119)	103 (94–114)	97 (85–108)	101 (81–115)
Sulpiride	6 (5–7)	8 (7–8)	103 (91–119)	103 (90–118)
Thioridazine	96 (89–102)	93 (88–102)	96 (85–104)	100 (87–110)
Trifluoperazine	96 (89–102)	84 (82–86)	93 (92–96)	102 (88–120)
Triflupromazine	88 (85–93)	81 (78–86)	96 (82–110)	105 (94–120)
Ziprasidone	101 (95–106)	88 (81–97)	93 (87–98)	105 (97–115)
Zotepine	94 (90–99)	89 (80–98)	109 (107–111)	102 (88–111)
Zuclopenthixol	111 (93–126)	91 (90–92)	104 (101–108)	***95 (77–115)***

Data sets where the range is more than ±20% difference of the mean value (not acceptable) are marked in bold and italics.

the method were calculated according to Beyer *et al.*,[19,20] using one-way analysis of variance (ANOVA) with the grouping-variable 'day'. The acceptance intervals of within-day (repeatability) and intermediate precision were ≤15% RSD (≤20% RSD at 'QC LOW') and ±15% for bias (±20% at 'QC LOW') of the nominal values.[21]

Processed sample stability

For estimation of stability of the processed samples, under the conditions of LC-MS/MS analysis, 'QC LOW' and 'QC HIGH' samples ($n = 8$ each) were extracted as described earlier. The resulting extracts at each concentration level were pooled. Aliquots of these pooled extracts at each concentration level were transferred to autosampler vials and injected into the LC-MS/MS system and analyzed under conditions given previously. The time intervals between the analyses of the QC samples were extended to 2 h by the injection of five blank samples. Stability of the extracted analytes was tested by regression analysis plotting absolute peak areas of each analyte at each concentration *versus* injection time. The instability of the processed samples was indicated by a negative slope, significantly different from zero ($p \leq 0.05$).[16]

Freeze/thaw and bench-top stability

Combined freeze/thaw and bench-top stability were evaluated by analysis of QC samples (6 replicates at each concentration) prior to (control samples) and after four freeze/thaw cycles (stability samples). For each cycle, the samples were kept at −60 °C for 22.5 h. The thawed samples were kept at room temperature for 1 h prior to the next freeze cycle to incorporate bench-top stability. The experiments were carried out together with the accuracy and precision experiments and the concentrations of the control and stability samples were calculated via daily calibration curves. Stability was tested against an acceptance interval of 90–110% for the ratio of the means (stability samples *vs* control samples)

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and an acceptance interval of 80–120% from the control samples' mean for the 90% CI of stability samples.[16]

Long-term stability

Experimental design for the study of long-term stability was similar to the freeze/thaw stability. Analyte stability for long-term storage was evaluated by analysis of QC samples ($n = 6$ at each concentration) before (control samples) and after storage for 6 weeks at −20 °C (stability samples). Stability was measured against an acceptance interval of 90–110% for the ratio of the means (stability samples *vs* control samples) and an acceptance interval of 80–120% from the control samples' mean for the 90% CI of stability samples.[16]

Lower limits of quantification

The lower limits of quantification (LLOQ) in the MRM mode was defined as the lowest point of the calibration curve (see Table 2 for concentrations) and fulfilled the requirement of LLOQ signal-to-noise ratio of 10 : 1.[16,17] The limit of detection (LOD) was not systematically evaluated.

Extraction efficiencies, matrix effects and process efficiencies

Extraction efficiencies, matrix effects and process efficiencies were estimated in a previously published study using 500 µl of blank blood for extraction.[22] The experiments were repeated under the conditions described earlier using 0.1 ml of blank blood and compared with the previously published results.

Application to authentic samples

Applicability experiments were carried using postmortem blood samples sent to the authors' laboratory for toxicological analysis. A total of 183 samples have been analyzed using the described method.

Results and Discussion

Extraction procedure

In a previous study,[22] different extraction procedures for APs were compared in terms of the extraction efficiencies and matrix effects. Based on the results of this study, a liquid–liquid extraction (LLE) procedure using Trizma buffer and 1-chlorobutane was chosen. Although this extraction procedure showed considerably lower extraction efficiencies for sulpiride, the method gave overall the best results in terms of extraction efficiencies and matrix effects.

Table 3 shows the mean values of recoveries and matrix effects including ranges for this method. Data sets where the range is greater than ±20% difference of the mean value (not acceptable) are marked in bold. The extraction recoveries of most analytes (other than sulpiride and to a lesser extent amisulpride) exceed 80%, which is acceptable for this method. Other than melperone and zuclopenthixol at high concentrations, as well as bromperidol and olanzapine at low concentrations, the variations of matrix effects over five different blood samples were acceptable. Overall, this method has shown less matrix effects than the previously published study,[22] most likely because of lower blood sample volume used for analysis.

In toxicological analysis, a lack of sample volume provided may reduce the possible number of tests able to be conducted. In this study, a small sample volume of 0.1 ml provided the required sensitivity. This sensitivity is evident in the ability to detect the concentrations of APs in the lowest calibrator which was prepared at least half of the lowest described therapeutic concentration.

Preliminary experiments showed that the reconstitution of samples was critical for the performance of the assay. The composition of the reconstitution solvent usually matches the start conditions of the mobile phase in order to avoid chromatographic changes for early eluting drugs. A pre-mixed solution of eluent A and eluent B (90 : 10) did not offer the lipophilic properties to re-dissolve some compounds. Therefore, 50 µl of eluent B was used to dissolve the APs. This solution was diluted using 450 µl of eluent A to provide the constitution required for the chromatographic conditions.

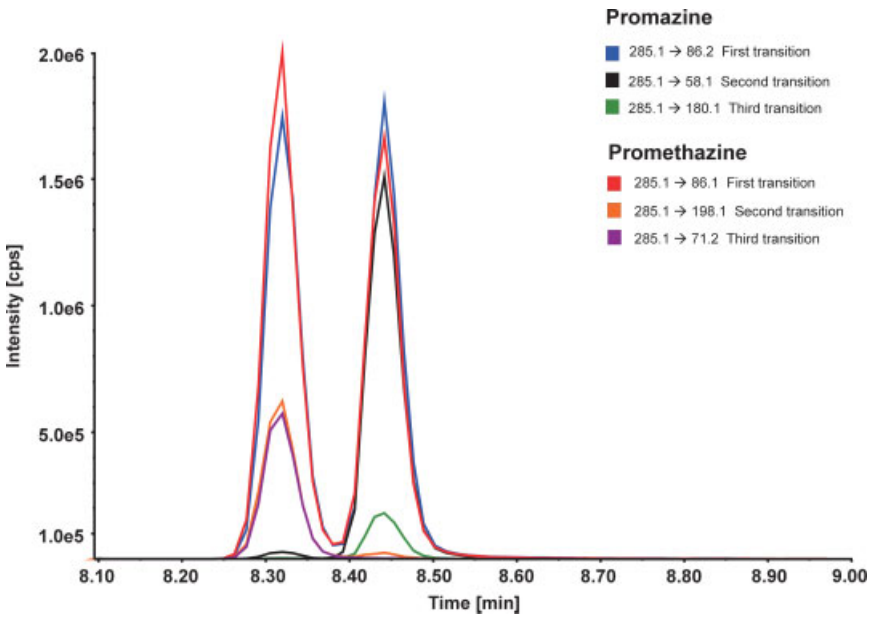

Figure 1. Chromatogram of promazine and promethazine recorded in the scheduled MRM mode showing three MRM transitions of each analyte.

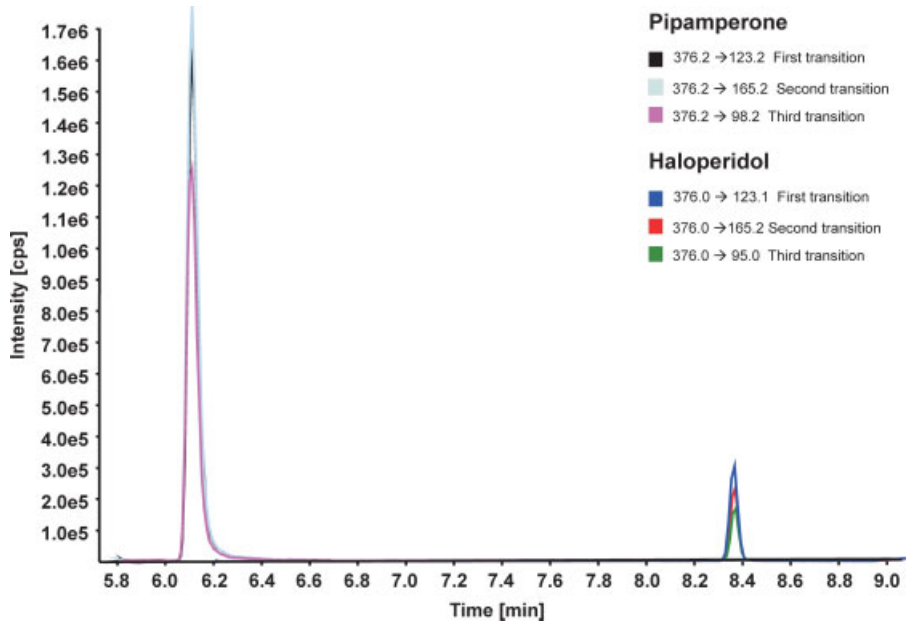

Figure 2. Chromatogram of haloperidol and pipamperone recorded in the scheduled MRM mode showing three MRM transitions of each analyte.

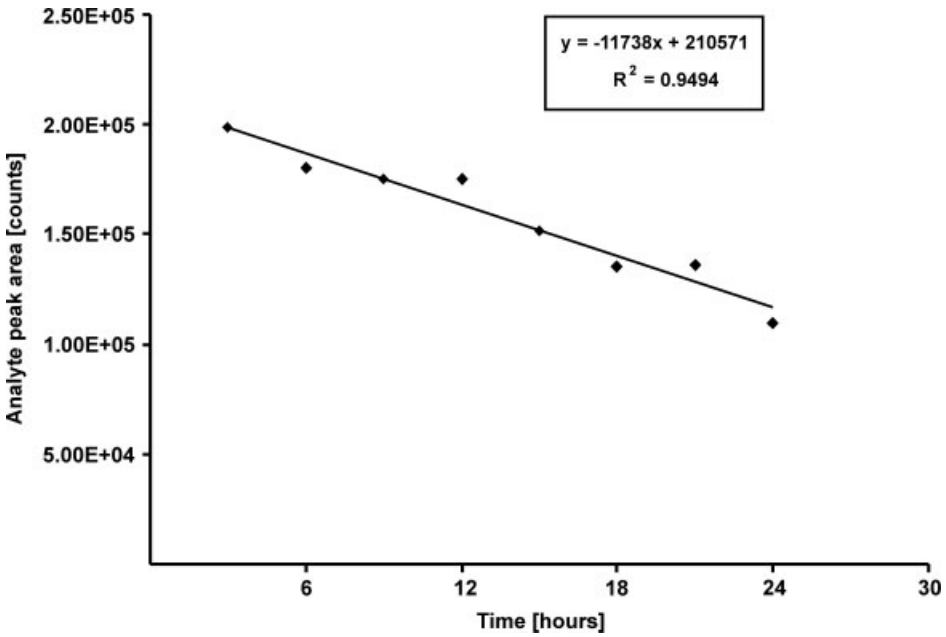

Figure 3. Peak area plot over time in autosampler stability of olanzapine.

Antemortem blood was chosen as the matrix for the calibration standards rather than postmortem blood due to a number of reasons. First, excess 'blank' postmortem blood from deceased persons is difficult to obtain ethically for assay calibration purposes, whereas antemortem blood is readily available through blood banks. Second, postmortem blood is often of variable quality and, depending upon postmortem change, can lead to unknown matrix effects and variable recovery, which could affect assay results if used as a calibration matrix. The validation data have clearly shown that postmortem blood does not seriously affect background signals and matrix effects until it is quite decomposed. Unfortunately, the degree of putrefaction cannot be quantified in individual cases.

Detection

After extraction from blood, the drugs were separated using gradient elution on an XBD C_{18} column. Preliminary experiments showed increased chromatographic robustness using a large size column and considerably high flow rates (data not shown). The increase of flow rate over the run improved the separation and peak shape of lipophilic compounds. For the detection of the APs, three MRM transitions were used for each analyte; their use and their respective peak area ratios enabled unambiguous identification of all APs included in the assay and showed no inference in a number of drug-free samples.

The potentials described in Table 1 were chosen using Analyst software and additionally critically reviewed. Extreme values such

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Table 4. Accuracy, intermediate precision and repeatability data of the LC-MS/MS assay for the studied analytes

		QC LOW	QC MED	QC HIGH
9-OH-Risperidone	Repeatability	**12.8**	**4.4**	**6.4**
	Precision	13.7	12.2	10.5
	Accuracy	8.7	−0.5	−4.2
Amisulpride	Repeatability	8.7	4.6	10.6
	Precision	18.1	14.6	11.9
	Accuracy	7.9	12.8	−4.1
Aripiprazole	Repeatability	13.7	5.6	7.2
	Precision	13.7	10.8	11.3
	Accuracy	−2.5	0.3	−5.0
Bromperidol	Repeatability	**57.1**	8.5	8.8
	Precision	**57.1**	10.9	11.4
	Accuracy	6.5	12.0	0.0
Buspirone	Repeatability	**29.0**	5.3	5.3
	Precision	**29.0**	12.4	12.1
	Accuracy	10.6	−7.7	−5.4
Chlorpromazine	Repeatability	8.4	4.6	7.2
	Precision	10.2	12.0	9.9
	Accuracy	7.3	7.0	5.5
Chlorprothixene	Repeatability	9.1	3.1	4.1
	Precision	10.0	10.1	10.8
	Accuracy	19.1	13.9	14.0
Clozapine	Repeatability	13.9	4.3	6.0
	Precision	13.9	12.5	11.0
	Accuracy	13.9	0.7	−2.3
Droperidol	Repeatability	14.5	11.2	11.7
	Precision	16.3	11.2	13.3
	Accuracy	−6.6	−6.6	−7.0
Fluphenazine	Repeatability	15.6	3.7	8.2
	Precision	16.6	8.5	9.7
	Accuracy	6.6	11.1	9.8
Fluspirilene	Repeatability	17.2	6.6	8.9
	Precision	18.5	14.1	13.3
	Accuracy	−7.7	−2.9	3.0
Haloperidol	Repeatability	14.6	4.1	7.6
	Precision	14.6	10.3	11.1
	Accuracy	2.9	0.6	−1.1
Levomepromazine	Repeatability	13.4	6.6	6.9
	Precision	14.8	14.1	11.4
	Accuracy	−9.8	−3.9	−0.7
Loxapine	Repeatability	10.6	6.2	6.7
	Precision	13.8	12.0	11.1
	Accuracy	18.5	3.4	2.7
Melperone	Repeatability	10.0	10.3	12.6
	Precision	11.6	14.7	14.5
	Accuracy	8.3	−4.6	4.0
Mesoridazine	Repeatability	8.4	3.1	7.8
	Precision	12.9	12.5	14.6
	Accuracy	−7.1	−5.0	−11.0
Olanzapine	Repeatability	17.8	7.8	7.5
	Precision	18.2	11.8	11.3
	Accuracy	−16.7	−0.9	−2.2
Perazine	Repeatability	8.0	4.5	5.2
	Precision	10.0	10.0	7.5
	Accuracy	1.1	8.5	6.4
Pericyazine	Repeatability	12.6	6.7	7.9
	Precision	15.4	12.1	9.9
	Accuracy	−7.0	−7.5	−4.1
Perphenazine	Repeatability	**54.0**	5.2	7.0
	Precision	**54.0**	13.0	9.5
	Accuracy	3.8	9.6	3.3
Pimozide	Repeatability	11.2	11.5	11.4
	Precision	18.5	14.7	14.2
	Accuracy	13.3	−5.5	−0.2
Pipamperone	Repeatability	5.0	4.4	7.1
	Precision	12.1	9.6	12.3
	Accuracy	5.0	−0.7	−1.4
Prochlorperazine	Repeatability	9.5	3.6	7.6
	Precision	14.6	11.5	9.8
	Accuracy	6.6	1.2	1.0
Promazine	Repeatability	6.0	4.6	8.0
	Precision	14.0	12.4	10.7
	Accuracy	5.9	−0.9	−1.3
Promethazine	Repeatability	9.9	4.9	6.1
	Precision	12.5	12.2	11.4
	Accuracy	−0.9	2.2	0.9
Quetiapine	Repeatability	7.3	4.9	6.3
	Precision	14.9	9.7	9.1
	Accuracy	−0.4	2.2	−5.3
Risperidone	Repeatability	6.6	4.4	7.0
	Precision	10.3	9.9	9.9
	Accuracy	−3.9	−4.6	−5.2
Sulpiride	Repeatability	8.9	6.4	11.6
	Precision	14.5	12.7	12.0
	Accuracy	−10.1	−6.1	−13.9
Thioridazine	Repeatability	6.7	6.5	8.2
	Precision	7.8	14.2	13.4
	Accuracy	−7.2	11.4	6.0
Trifluoperazine	Repeatability	14.0	4.5	5.5
	Precision	14.6	8.8	8.3
	Accuracy	−18.4	−7.9	−8.0
Triflupromazine	Repeatability	9.4	6.5	5.6
	Precision	16.0	12.6	12.8
	Accuracy	8.9	3.9	5.0
Ziprasidone	Repeatability	9.8	4.6	7.7
	Precision	11.4	10.2	11.1
	Accuracy	13.7	2.4	1.5
Zotepine	Repeatability	7.0	5.3	8.8
	Precision	8.4	13.0	9.6
	Accuracy	−2.7	−1.1	−13.6
Zuclopenthixol	Repeatability	11.7	3.2	6.3
	Precision	15.8	13.0	12.0
	Accuracy	−6.1	−3.8	−3.7

Values greater than international acceptance criteria are highlighted in bold. Quality control samples were prepared at concentrations given in Table 2.

as the declustering potential (DP) of bromperidol (DP = 1) and pimozide (DP = 396) and the entrance potential (EP) of triflupromazine (EP = 1) were identified as the best given option.

The structural isomers promazine and promethazine (formula and product ion spectra shown in Supporting information, Fig. S1) show both the presence of a most abundant transition 285 → 86, which represents the cleavage of the side chain. This cleavage

can occur despite the different side chain structures. However, the cleavage of the side chain in α position of the side chain nitrogen results in a fragment *m/z* 58 in case of promazine, and a fragment *m/z* 71 in case of promethazine. The resulting transition using this fragmentation allows the differentiation of the two structural isomers. A sample chromatogram showing the different MRM transitions for promazine and promethazine is given in Fig. 1.

The isobaric compounds pipamperone and haloperidol (structures and product ion spectra shown in Supporting information, Fig. S1) also show a similar fragmentation pattern. Owing to the significantly different retention times and the use of scheduled MRM, transitions of the structurally related compound are not monitored in the respective expected retention time range. An example chromatogram of a sample including both compounds can be seen in Fig. 2.

The fragmentation pattern of zotepine did not provide three fragments with sufficient sensitivity. The software-assisted optimization process selected two transitions (332.1 → 72.1 and 332.1 → 72.6), which are likely to reflect the same fragment. This needs to be considered when applying the method to routine casework. It is however still possible to identify the presence of zotepine, according to internationally accepted guidelines, as the requirement of two transitions and their ratio is fulfilled.

Table 5. Number of positive cases, range and median of concentrations (µg/l) detected in the applicability study

Drug	Number of positive cases	Range (µg/l)	Median (µg/l)
Quetiapine	49	13–4 330	468
Olanzapine	33	1–37 200	155
Risperidone	24	1–305	10
9-OH-Risperidone	23	3–112	12
Zuclopenthixol	14	4–153	31
Clozapine	12	13–15 900	677
Haloperidol	6	2–38	12
Amisulpride	5	701–25 900	1 460
Aripiprazole	4	105–264	175
Chlorpromazine	3	119–393	158
Fluphenazine	3	1–4	2
Promethazine	2	12–1 560	–
Buspirone	1	524	–
Fluspirilene	1	16	–
Perphenazine	1	178	–
Thioridazine	1	91	–
Trifluoperazine	1	35	–

Validation experiments

The described procedure was validated according to internationally accepted recommendations.[16,17,23] The assay was found to be selective for all tested compounds, no interfering peaks were observed in the extracts of the different postmortem and antemortem blank blood samples. A comparison of sample chromatograms of a blank blood sample, a zero sample and a lowest calibrator are shown in Fig. S2a–c. The MRM transition signals observed in the blank blood are non-significant as their intensities are considerably lower than the intensities in the lowest calibrator. The MRM transitions of IS are of similar intensity; therefore, only one peak is visible in the zero sample (Fig. S2b).

Calibration curves were linear in the range given in Table 2. All analytes were visually checked for a linear fit, a weighted second-order model fit and a quadratic fit. A linear fit was used for 9-OH-risperidone, haloperidol, levomepromazine, loxapine, olanzapine, perphenazine and zuclopenthixol. Linear regression ($1/x^2$ weighting) was used for aripiprazole, bromperidol, buspirone, chlorpromazine, chlorprothixene, droperidol, fluphenazine, fluspirilene, melperone, perazine, pericyazine, pimozide, pipamperone, prochlorperazine, sulpiride, triflupromazine and ziprasidone. A quadratic fit was used for amisulpride, clozapine, mesoridazine, promazine, promethazine, quetiapine, risperidone, thioridazine, trifluoperazine and zotepine. The calibration fit showed a coefficient of determination of $r^2 > 0.99$ for all drugs.

With the exception of olanzapine, all drugs appeared to be stable for up to 24 h when stored in the autosampler. Figure 3 shows the autosampler degradation rate of olanzapine with time. As previously described, olanzapine is unstable in blood samples.[24] This instability was also confirmed in our processed sample stability experiments. In freeze/thaw stability experiments, all drugs appeared to be stable, however, low concentrations of buspirone, bromperidol and perphenazine (Table 4) showed variability in detection at low concentrations. As already mentioned previously, due to the lack of processed sample stability, olanzapine could not be quantified reliably, hence the inability to determine freeze/thaw stability.

All drugs appeared to be stable over a period of 6 weeks when stored at −20 °C, with the exception of olanzapine, which showed losses of approximately 80% compared with control samples at all three concentrations. The LLOQs corresponded to the lowest concentrations used for the calibration curves with a signal-to-noise ratio of at least 10. Accuracy data were within the acceptance interval of ±15% (±20% at LLOQ) of the nominal values for all drugs. Within-day (repeatability) and intermediate precision data were within the required limits of 15% RSD (20% RSD at LLOQ), with the exception of low concentrations of buspirone, bromperidol and perphenazine (Table 4).

As olanzapine showed instability in several validation experiments and its detection could therefore not be reliably performed, it was excluded from this method.

Applicability of the previously described method was shown by the analysis of postmortem blood samples. The results obtained from the analysis of 183 postmortem blood samples including the mean concentrations are given in Table 5. Cases that were above the standard curve were diluted appropriately to provide accurate results. These concentrations vary depending on the type of death, which range from therapeutic use to suicidal ingestion (high concentration). The use of this method has importantly enabled the detection of a large range of APs simultaneously and accurately. The minimal use of blood (0.1 ml) is also advantageous and combined with the use of LC-MS/MS has led to significant progress toward a single assay for detection of numerous typical and atypical APs. In summary, this method is robust, reliable, sensitive and validated for the measurement of APs in blood samples.

Conclusions

The LC-MS/MS assay presented is a suitable procedure for separation, detection and quantification of 30 APs in blood samples. It has proven to be selective, linear, accurate and precise for all studied drugs. However, olanzapine must be analyzed promptly as it can degrade quite rapidly after extraction. The

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presented LC-MS/MS assay has been found to be applicable for clinical and forensic toxicological casework.

Supporting information

Supporting information may be found in the online version of this article.

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Appendix 1.3

Assessment of the Stability of 30 Antipsychotic Drugs in Stored Blood Specimens

Saar, E., Gerostamoulos, D., Drummer, O.H., Beyer, J
Forensic Sci Int, 2012. **215**(1-3): p. 152-8

Forensic Science International 215 (2012) 152–158

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Assessment of the stability of 30 antipsychotic drugs in stored blood specimens☆

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ARTICLE INFO

Article history:
Received 15 October 2010
Received in revised form 18 February 2011
Accepted 23 February 2011
Available online 26 March 2011

Keywords:
Antipsychotic drugs
LC–MS/MS
Stability
Whole blood
Toxicology

ABSTRACT

The stability of 30 common antipsychotics (APs) in spiked whole blood was investigated over ten weeks in a preliminary experiment (designated "P experiment"). Pools of blank blood spiked with drugs at two different therapeutic levels were stored at four different temperatures: 20 °C, 4 °C, −20 °C, and −60 °C and extracted once weekly in duplicate, using a previously published method. A loss of >15% of the initial drug concentration was considered to indicate possible instability and the respective drugs were selected for further investigation in a final experiment (designated "F experiment").

Eight APs (chlorpromazine, chlorprothixene, fluspirilene, droperidol, olanzapine, thioridazine, triflupromazine, and ziprasidone) were incorporated into the F experiment. The same conditions were used in both experiments, however only a high therapeutic drug concentration was chosen for the F experiment and the storage time was extended to 20 weeks.

All drugs of interest in the F experiment showed significant losses after 20 weeks of storage under at least one storage condition. The most notable results involved olanzapine, where losses of almost 100% in all storage temperatures were observed. Drug degradation in fluspirilene samples was significant after 20 weeks under all storage conditions. Overall, extensive degradation was seen with approximately 80% drug loss when stored at 20 °C and 4 °C with samples also seriously affected by degradation of up to 50% when stored at −20 °C and −60 °C, respectively. Ziprasidone remained stable when stored at 4 °C, −20 °C, and −60 °C over 9 weeks, however significant degradation was observed when stored at 20 °C, with a loss of almost 100% after 20 weeks of storage.

The time period and temperature of storage of biological samples can have a significant influence on the stability of several APs. It is therefore important to be aware of potential changes in drug concentrations during storage when interpreting analytical results.

1. Introduction

An increase in the number of prescriptions of antipsychotics drugs (APs) in recent years [1,2] in addition to mounting evidence suggesting that these drugs can increase the risk of sudden cardiac death [3–5] may present an explanation for the high prevalence of APs in forensic cases. The advent of liquid chromatography with tandem mass spectrometry (LC–MS/MS) has greatly facilitated the identification of these drugs, however the interpretation of their concentrations in biological specimens still requires further research.

In forensic toxicology, potential post-mortem changes such as post-mortem redistribution (PMR) or instability of the target drugs provide an additional challenge. The atypical AP olanzapine has been shown previously to be unstable in blood [6–9]. Various conclusions have been drawn from these studies but the extent to which this drug degrades remains uncertain. There is little information published on any other AP.

Evaluation of stability of drugs in bioanalytical methods is often performed using four different experiments: long-term stability in the sample matrix, freeze/thaw stability, bench-top stability, and stability in the prepared samples under conditions of analysis [10]. Stability studies have targeted either single or a few drugs and are mainly carried out using a plasma matrix [11–14]. In contrast, there is little information available on the stability of APs in whole blood [15]. Furthermore, stability data is typically only collected as part of method validation and thus information concerning different storage conditions and data over longer periods of time is often inadequate or completely absent.

Unfortunately, the definition and evaluation of 'stability' is not very consistent throughout the literature. In 1998 Hartmann et al. defined 'stability' as the "absence of an influence of time on the

☆ "48th Annual Meeting of the International Association of Forensic Toxicologists (TIAFT). Joint meeting with the society of Toxicological and Forensic Chemistry (GTFCh)". August 29th to September 2nd, 2010, Bonn, Germany, Guest edited by Thomas Kraemer, Hans. H. Maurer and Frank Musshoff.

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0379-0738/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.forsciint.2011.02.022

concentration of the analyte in a sample'' [16]. Despite the various definitions and wide-ranging requirements to determine the long term stability of an analyte in a sample matrix [16–19], most of the approaches show a similar experimental setup. In order to determine long term stability of drugs in the sample matrix, it is frequently suggested to analyze a set of samples (''control samples'') at the beginning of the stability study and an additional set of samples (''stability samples'') after a certain time of storage. Various statistical tests have been used to determine if drug concentrations differ significantly between control samples and stability samples, consequently providing an indication of stability problems. Unfortunately these approaches do not provide any information about the pattern of break-down. A similar experiment design has been used in two very recent publications by Nilsson et al. [20,21] looking at the hypnotic drug zopiclone and providing useful information regarding storage requirements of this particular drug.

The aim of this study is to determine the stability of 30 APs in stored blood samples at different temperatures at a number of time intervals over a 10–20 week period.

2. Materials and methods

2.1. Chemicals and reagents

Bromperidol, chlorpromazine, fluspirilene, haloperidol, pipamperone, trifluoperazine, triflupromazine and Trizma base were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Buspirone, chlorprothixene, olanzapine, promazine, promethazine, risperidone and zuclopenthixol were obtained from the Division of Analytical Laboratories (Lidcombe, NSW, Australia). Droperidol, fluphenazine, perphenazine, pimozide and sulpiride were provided by Australian Government Analytical Laboratories (Pymble, NSW, Australia). Levomepromazine, melperone, perazine and zotepine were obtained from Phast GmbH (Homburg/Saar, Germany). Amisulpride, aripiprazole, quetiapine, and ziprasidone were purchased from the National Institute of Forensic Science (Melbourne, VIC, Australia). Clozapine was provided by Sandoz (Pyrmont, NSW, Australia) and 9-OH Risperidone (paliperidone) was obtained from Janssen–Cilag (North Ryde, NSW, Australia). The isotope-labelled internal standard haloperidol-d_4 was purchased from Cerilliant (Round Rock, TX, USA). Acetonitrile, ammonium formate, 1-chlorobutane, methanol and formic acid were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from BDH Chemicals (Kilsyth, VIC, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia).

Eluent A consisted of 50 mmol/L aqueous ammonium formate adjusted to pH 3.5 with formic acid. 2 M Trizma buffer (pH 9.2) was prepared by dissolving 242 g Trizma base in 1 L water.

2.2. Specimens

Whole blood for stability experiments was obtained from the local blood bank in lithium heparin-coated plastic bags (500 mL). The blood was aliquoted into 10 mL polypropylene tubes containing 200 mg sodium fluoride and 30 mg potassium oxalate. Blood was analysed using a previously published method [22] and was found to be ''drug-free''. All blood samples were stored at − 20 °C prior to analysis.

3. Methods of detection

3.1. Apparatus

The LC–MS/MS system consisted of an Applied Biosystems 3200 Q-TRAP® linear ion-trap quadrupole mass spectrometer (Applied Biosystems, Melbourne, VIC, Australia) equipped with a Turbo V ion source, operated in the electron spray ionization (ESI) mode, and an Agilent Technologies (AT) 1200 Series HPLC system (Agilent, Melbourne, VIC, Australia) which consisted of a degasser, a binary pump and an autosampler.

3.2. Preparation of stock solutions and extraction

Preparation of stock solutions, calibration standards and stability samples and extraction procedures were performed as published previously [22]. The extraction consisted of a liquid–liquid extraction of the APs from whole blood using butylchloride.

4. Stability experiments

4.1. Preliminary experiment

Preliminary samples (designated ''P'') were prepared at two concentration levels: ''LOW'' and ''HIGH'' (at a low therapeutic concentration and a supratherapeutic concentration of the respective drug [22]).

The P LOW samples were prepared using 20 mL of blank blood pooled in a volumetric flask and an independently prepared mixture of the 30 APs in methanol at a concentration 100 times higher than the concentrations of the corresponding P LOW samples. After inversion for 30 min on a rotary wheel, 100 μL aliquots were transferred to 2 mL Sarstedt tubes (Sarstedt, Mawson Lakes, SA,). P LOW samples ($n = 160$) were divided into four groups ($n = 40$ samples) of different storage temperatures (20 °C, 4 °C, −20 °C, and −60 °C).

P HIGH samples were prepared according to the same protocol, but using a ''high'' therapeutic concentration of the respective drugs instead.

Sample extraction was performed twice weekly in duplicate over a period of 10 weeks and the samples were analysed according to the procedure described previously [22]. The concentrations of the analytes in the P samples were calculated using the daily calibration curves included in each assay.

4.2. Final experiment

From the P experiment, haloperidol and risperidone were shown to be stable under all conditions and were used as control references, as they are also commonly detected in cases sent to the authors' laboratory for toxicological testing. Eight drugs (chlorpromazine, chlorprothixene, droperidol, fluspirilene, olanzapine, thioridazine, triflupromazine and ziprasidone) which did not meet the acceptance criteria for stability under at least one storage condition, were selected for further investigation and designated as ''F'' samples. F samples were prepared at the respective HIGH concentration of each drug using 100 mL of blank pooled blood aliquoted in 10× 10 mL volumetric flasks. Each blood aliquot was spiked with a methanolic solution of one of the drugs of interest at a concentration 100 times higher than the concentrations of the corresponding F sample. After inversion for 30 min on a rotary wheel 100 μL aliquots of each flask were transferred to 2 mL Sarstedt tubes ($n = 800$) and labelled accordingly. F samples of each drug ($n = 80$) were divided into four groups ($n = 20$ samples) at different storage temperatures (20 °C, 4 °C, −20 °C, and −60 °C).

Sample extraction was performed once weekly ($n = 1$ per drug and storage temperature) over a period of 20 weeks and the samples were analysed according to the procedure described above. The concentrations of the analytes in the F samples were calculated via the daily calibration curves included in each assay.

4.3. Stability evaluation/acceptance criteria

As the degradation of a drug over time was of interest, fewer replicates were analysed with more frequency in contrast with papers that seek to establish a statistically significant difference between "control" samples at the start of the experiment and "stability" samples at the end of the experiment. In order to determine all possible instabilities of the target drugs, the approach introduced by Wieling et al. in 1996 for the determination of autosampler stability was used in the experiments [23]. The calculated drug concentration was plotted versus time and a curve of best fit was visually evaluated. Outliers have been determined via a residual plot using a 95% CI and removed from the data prior to interpretation of the results.

As all drugs included in this study (with the exception of bromperidol, buspirone and perphenazine) had proven to fulfill the acceptance criteria for accuracy of being ± 15% of target value in the previously published method validation [22], a loss of >15% of the initial drug concentration in the P experiment was considered a possible instability and the drug was incorporated in the F experiment for further investigation.

Olanzapine was of great interest in this study as it is known to be an exceptionally unstable drug. Since olanzapine was excluded from a previously published method validation [22] due to difficulties mainly regarding processed sample stability, the authors attempted to overcome this problem by reducing the time of olanzapine samples on the autosampler to a minimum (<1 h). However, the reader must be aware that the measured concentrations can only be seen as approximate values with a higher variation than the other drugs included in this study.

5. Results

Table 1 shows the standardised losses of the P experiment: A loss <15% is referred to as "1" (acceptable), a loss ≤30% – ≥15% is referred to as "2" (acceptable if present only at 20 °C), a loss ≥30% is referred to as "3" (unacceptable). Therefore, for drugs that only showed losses at 20 °C, a loss of >30% ("3") (twice the minimum accuracy requirement) was considered unacceptable.

In the P experiment the majority of the 30 drugs ($n = 24$) appeared to be stable at 4 °C and lower temperatures. An additional six drugs showed some losses (<30%) when stored at 20 °C. Table 1 shows the standardised results of the P experiment. Haloperidol and risperidone were chosen as stability controls as they did not show significant losses under any storage condition after 10 weeks. Since instability was either found to be independent of concentration or else only the "HIGH" concentration was affected by degradation in the P experiment, the F experiment was performed using only a "HIGH" concentration of the respective drug. Towards the end of the P experiment, an error in storage conditions of the sample set P LOW −60 °C was discovered. Therefore, these samples were excluded from this study as their integrity was compromised.

Table 2 lists all drugs that were included in the F experiment and their respective concentrations over 20 weeks of storage while Table 3 shows their respective best fits. An instrument failure during the F experiment rendered the data collected in week 15 and 17 unusable; therefore these weeks were excluded prior to data analysis.

Haloperidol showed losses of ~25% after 20 weeks of storage at 20 °C and >15% degradation after 20 weeks of storage at 4 °C. None of the other storage temperatures seemed to have an effect on the

Table 1
Results of the P experiment, showing standardised* losses after 10 weeks of storage at 20 °C, 4 °C, −20 °C and −60 °C.

	20 °C		4 °C		−20 °C		−60 °C
ID	LOW	HIGH	LOW	HIGH	LOW	HIGH	HIGH
9-OH Risperidone	**2**	**2**	1	1	1	1	1
Amisulpride	1	1	1	1	1	1	1
Aripiprazole	1	1	1	1	1	1	1
Bromperidol	1	1	1	1	1	1	1
Buspirone	1	1	1	1	1	1	1
Chlorpromazine	**2**	**3**	**2**	**2**	1	**2**	1
Chlorprothixene	**2**	**3**	**3**	**2**	1	1	1
Clozapine	1	1	1	1	1	1	1
Droperidol	**2**	**3**	1	1	1	1	1
Fluphenazine	1	1	1	1	1	1	1
Fluspirilene	**3**	**3**	**3**	**3**	1	1	1
Haloperidol	1	1	1	1	1	1	1
Levomepromazine	**2**	**2**	1	1	1	1	1
Melperone	1	1	1	1	1	1	1
Olanzapine	**3**	**3**	**3**	**3**	1	1	**3**
Perazine	**2**	**2**	1	1	1	1	1
Perphenazine	**2**	**2**	1	1	1	1	1
Pimozide	1	1	1	1	1	1	1
Pipamperone	1	1	1	1	1	1	1
Promazine	**2**	**2**	1	1	1	1	1
Promethazine	**2**	**2**	1	1	1	1	1
Quetiapine	1	1	1	1	1	1	1
Risperidone	1	1	1	1	1	1	1
Sulpiride	1	1	1	1	1	1	1
Thioridazine	1	**2**	1	**2**	1	1	1
Trifluoperazine	1	1	1	1	1	1	1
Triflupromazine	**2**	**3**	1	**2**	1	1	1
Ziprasidone	**3**	**3**	1	1	1	1	1
Zotepine	1	1	1	1	1	1	1
Zuclopenthixol	1	1	1	1	1	1	1

Drugs in bold indicate that at least one acceptance criteria** for stability was not met and drug was therefore included in the F experiment.
* Losses: 1 = stable (<15%); **2** = some losses (≥ 15% – ≤ 30%); **3** = unstable (≥ 30%).
** 1 = acceptable; **2** = unacceptable, unless only 20 °C is affected; **3** = unacceptable.

Table 2
Relative concentrations of drugs in F experiment stored at 20 °C, 4 °C, −20 °C and −60 °C over 20 weeks.

Drug	Time (weeks)	Storage temperature			
		20 °C	4 °C	−20 °C	−60 °C
Risperidone	0	100 (95)	100 (104)	100 (100)	100 (90)
	1	99	114	109	102
	2	86	89	84	77
	3	83	79	–	82
	4	82	–	91	77
	5	90	107	102	75
	6	–	103	92	89
	7	81	94	89	84
	8	101	107	96	91
	9	87	91	77	67
	10	**77 (82)**	**77 (88)**	**75 (84)**	**68 (79)**
	11	89	90	93	93
	12	85	94	–	79
	13	66	77	86	68
	15	68	67	74	59
	17	78	72	61	64
	18	75	90	74	86
	19	65	64	62	69
	20	**69 (69)**	**76 (72)**	**63 (68)**	**72 (68)**
Haloperidol	0	100 (90)	100 (100)	100 (99)	100
	1	101	107	93	100
	2	85	93	90	87
	3	85	88	92	96
	4	83	–	90	100
	5	87	105	113	102
	6	95	111	110	102
	7	87	95	95	80
	8	78	86	89	99
	9	81	99	107	106
	10	**77 (83)**	**86 (92)**	**95 (95)**	**99 (92)**
	11	72	83	94	84
	12	73	100	104	99
	13	74	76	87	83
	15	80	88	97	80
	17	74	86	84	82
	18	83	92	80	78
	19	82	86	96	89
	20	**87 (75)**	**89 (84)**	**93 (91)**	**99 (86)**
Chlorpromazine	0	100 (98)	100 (99)	100 (93)	100 (91)
	1	95	100	100	102
	2	–	–	67	75
	3	–	68	72	95
	4	71	77	74	88
	5	69	86	88	102
	6	72	80	94	93
	7	57	63	68	–
	8	–	62	67	78
	9	56	50	59	–
	10	**50 (60)**	**61 (60)**	**71 (65)**	**81 (87)**
	11	50	50	50	70
	12	50	69	82	85
	13	50	54	58	67
	15	64	72	–	98
	17	50	55	71	80
	18	51	68	30	78
	19	48	57	26	93
	20	**47 (52)**	**60 (65)**	**30 (36)**	**97 (83)**
Chlorprothixene	0	100 (91)	100 (84)	100 (108)	100 (110)
	1	90	90	115	–
	2	93	70	80	110
	3	90	77	99	119
	4	68	87	104	98
	5	81	75	118	99
	6	81	93	105	–
	7	67	61	94	101
	8	71	60	96	119
	9	59	45	74	89
	10	**60 (68)**	**53 (67)**	**85 (83)**	**90 (101)**
	11	61	59	63	99
	12	62	70	74	111
	13	54	57	49	95
	15	66	61	81	103
	17	58	60	–	90
	18	49	69	102	99
	19	50	53	72	80
	20	**46 (45)**	**52 (50)**	**62 (57)**	**101 (93)**
Droperidol	0	100 (86)	100 (92)	100 (110)	100 (92)
	1	85	107	122	85
	2	72	91	100	93
	3	72	69	98	84
	4	67	72	89	86
	5	81	76	100	87
	6	67	77	106	80
	7	75	95	126	72
	8	78	83	120	85
	9	70	99	121	99
	10	**62 (66)**	**68 (79)**	**94 (100)**	**78 (80)**
	11	63	78	93	67
	12	69	88	97	91
	13	57	59	89	72
	15	72	78	94	–
	17	55	73	88	72
	18	60	86	91	77
	19	50	55	–	66
	20	**59 (57)**	**64 (67)**	**92 (90)**	**65 (68)**
Fluspirilene	0	100 (102)	100 (90)	100 (94)	100 (89)
	1	65	88	100	–
	2	48	59	71	76
	3	–	–	–	–
	4	49	–	76	69
	5	43	52	73	88
	6	49	47	90	80
	7	43	30	86	73
	8	42	26	73	66
	9	51	31	100	77
	10	**42 (35)**	**27 (27)**	**88 (79)**	**70 (72)**
	11	38	27	85	85
	12	49	29	92	–
	13	36	25	–	69
	15	37	23	83	65
	17	28	19	81	64
	18	21	24	65	64
	19	16	9	–	51
	20	**16 (26)**	**14 (20)**	**70 (65)**	**46 (55)**
Olanzapine	0	100 (83)	100 (70)	100 (160)	100 (62)
	1	23	30	34	–
	2	12	16	19	22
	3	10	13	23	22
	4	–	–	25	26
	5	11	17	32	25
	6	12	16	31	35
	7	8	12	29	20
	8	7	11	19	34
	9	9	13	23	34
	10	**5 (4)**	**12 (8)**	**2 (8)**	**28 (23)**
	11	6	12	20	30
	12	6	3	34	33
	13	5	9	25	24
	15	–	–	–	–
	17	2	6	1	17
	18	3	6	2	15
	19	1	4	1	13
	20	**0 (2)**	**3 (5)**	**1 (4)**	**17 (18)**
Thioridazine	0	100 (107)	100 (101)	100 (94)	100 (98)
	1	105	103	86	100
	2	–	88	–	–
	3	92	81	84	87
	4	103	104	92	89
	5	108	112	91	80
	6	98	–	94	99
	7	100	74	78	77
	8	73	88	74	88
	9	99	83	77	86
	10	**81 (88)**	**93 (84)**	**81 (84)**	**91 (83)**
	11	82	80	98	

Table 2 (*Continued*)

Drug	Time (weeks)	Storage temperature			
		20 °C	4 °C	−20 °C	−60 °C
	12	89	85	102	93
	13	96	–	96	–
	15	85	83	90	79
	17	78	75	47	69
	18	–	–	–	–
	19	63	56	–	57
	20	**58 (68)**	**60 (67)**	**72 (74)**	**69 (67)**
Triflupromazine	0	100 (101)	100 (88)	100 (110)	100 (116)
	1	112	91	99	100
	2	89	74	101	104
	3	89	87	125	136
	4	–	–	117	123
	5	100	83	122	124
	6	83	94	113	135
	7	77	71	99	113
	8	60	60	91	100
	9	65	53	73	76
	10	**54 (69)**	**56 (68)**	**70 (91)**	**76 (99)**
	11	66	53	88	75
	12	73	69	81	79
	13	56	54	58	66
	15	55	65	90	100
	17	48	58	88	100
	18	52	68	93	104
	19	41	44	75	69
	20	**41 (38)**	**54 (48)**	**78 (73)**	**92 (79)**
Ziprasidone	0	100 (88)	100 (112)	100 (94)	– (96)
	1	80	122	98	100
	2	61	91	77	89
	3	52	103	80	91
	4	–	–	78	81
	5	35	111	89	87
	6	33	115	–	99
	7	25	104	87	88
	8	18	97	98	82
	9	16	94	85	99
	10	**11 (15)**	**91 (90)**	**66 (81)**	**77 (84)**
	11	11	90	77	96
	12	10	87	83	87
	13	8	77	70	75
	15	5	79	73	75
	17	4	74	88	64
	18	5	–	87	78
	19	3	63	57	80
	20	**2 (2)**	**57 (68)**	**58 (69)**	**72 (73)**

Figures in brackets represent the expected relative concentration defined by the best curve, '–' indicates value is outlier and therefore excluded prior to data analysis. Bold values indicate >15% loss at 10 and 20 weeks (highlighted in grey).

haloperidol concentration, even after 20 weeks of storage. Risperidone revealed losses of 15–20% under all storage temperatures after 10 weeks of storage, with losses increasing up to ~35% after 20 weeks of storage under all storage conditions.

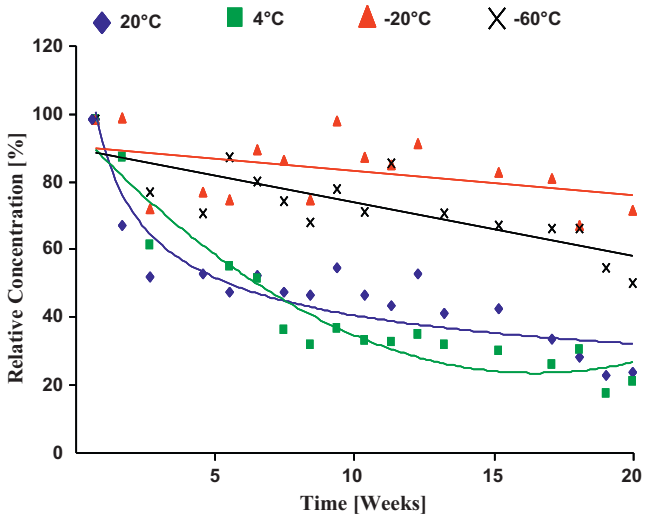

Fig. 1. Relative concentration [%] of fluspirilene samples in the F experiment stored for 20 weeks at 20 °C, 4 °C, −20 °C and −60 °C.

Similar to the P experiment, chlorpromazine showed losses of ~40% after 10 weeks of storage at 20 °C, with the concentration decreasing by another 10% over the following 10 weeks. Storage at 4 °C confirmed the observation from the P experiment with losses of ~35–40% after 20 weeks of storage. Interestingly, the most extensive losses of chlorpromazine (~65–70%) could be seen after 20 weeks of storage at −20 °C with the main degradation occurring after 17 weeks.

Chlorprothixene and droperidol showed comparable losses to chlorpromazine when stored at 20 °C and 4 °C. Interestingly, droperidol appeared to be stable when stored at −20 °C but showed losses similar to storage at 4 °C and 20 °C (~35%) when stored at −60 °C.

Degradation of fluspirilene in samples was significant after 20 weeks under all storage conditions. Extensive degradation was seen with ~80% drug loss when stored at 20 °C and 4 °C, but samples were also significantly affected by degradation of up to 50% when stored at −20 °C and −60 °C, respectively (Fig. 1).

Olanzapine showed more extensive losses in the F experiment compared with the P experiment. All storage temperatures were affected by severe degradation of up to almost 100% after 20 weeks.

The observation for storage of ziprasidone samples at 20 °C for 10 weeks was consistent with the P experiment and ~85% of degradation and a clear pattern of break-down. The concentration decreased further to almost 100% loss of the drug after 20 weeks of storage. Storage at 4 °C, −20 °C and −60 °C seemed favorable with ~30–40% losses at the end of the experiment (Fig. 2).

Table 3
Best fit equations for all drugs in the F experiment after 20 weeks of storage at 20 °C, 4 °C, −20 °C, and −60 °C.

Drug	Storage temperature			
	20 °C	4 °C	−20 °C	−60 °C
Risperidone	$y = -1.3153x + 96.295$	$y = -1.5722x + 105.46$	$y = -1.9006x + 104.27$	$y = -1.1067x + 90.791$
Haloperidol	$y = -0.7531x + 91.201$	$y = -0.7647x + 100.34$	$y = -0.4103x + 99.447$	$y = -0.6584x + 99.723$
Chlorpromazine	$y = 0.21x^2 - 6.908x + 104.82$	$y = 0.214x^2 - 6.433x + 105.27$	$y = -2.8443x + 95.887$	$y = -0.4328x + 91.718$
Chlorprothixene	$y = -2.3091x + 93.077$	$y = -1.698x + 85.712$	$y = -1.8638x + 106.14$	$y = -0.7149x + 108.06$
Droperidol	$y = 0.0525x^2 - 2.623x + 88.981$	$y = -1.2579x + 93.006$	$y = -0.9654x + 110.64$	$y = -1.1841x + 93.007$
Fluspirilene	$y = 102.34x^{-0.4499}$	$y = 0.2749x^2 - 9.549x + 99.169$	$y = -0.7773x + 91.083$	$y = -1.6946x + 90.483$
Olanzapine	$y = 82.925x^{-1.2202}$	$y = 69.783x^{-0.8876}$	$y = 160x^{-1.2546}$	$y = 62.485x^{-0.4135}$
Thioridazine	$y = -1.9333x + 109.02$	$y = -1.6814x + 102.5$	$y = -1.038x + 95.44$	$y = -1.5651x + 100.11$
Triflupromazine	$y = -3.1384x + 103.75$	$y = -2.0125x + 90.267$	$y = -1.8487x + 111.68$	$y = -1.6893x + 116.27$
Ziprasidone	$y = 106.44e - 0.1819x$	$y = -2.5004x + 117.04$	$y = -1.1653x + 93.15$	$y = -1.1592x + 97.221$

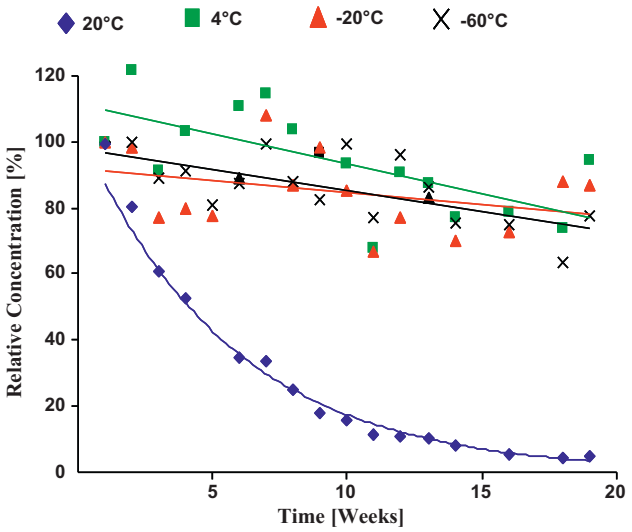

Fig. 2. Relative concentration [%] of ziprasidone samples in the F experiment stored for 20 weeks at 20 °C, 4 °C, −20 °C and −60 °C.

6. Discussion

Following the unexpected death of a person, it can take days or even weeks until the individual is discovered. During this time, any drugs that the individual may have been exposed to prior to their death remain in whole blood. The blood is therefore susceptible to the temperature conditions to which the deceased is exposed, potentially compromising the quality of the sample. PMR also becomes a concern for bodies that remain undiscovered for longer periods of time [24].

Once the body has been discovered and admitted to the mortuary, the deceased is stored at 4 °C until autopsy is performed; a period that typically averages two to three days at our Institute [25] but could vary in other organizations. The blood samples taken at autopsy for toxicological testing are stored at 4 °C, −20 °C, or −60 °C, depending on the individual laboratory policies. The testing may not occur for several weeks, during which time some drugs could degrade. Depending on the state of decomposition, it is often not possible to separate the blood cells from the blood sample in order to obtain plasma; therefore toxicological tests are routinely performed using whole blood [26].

In consideration of these factors, the choice of whole blood as a matrix for stability studies appears logical in order to obtain data that correlates more closely to the data obtained during analysis in many real cases. The storage temperatures of 20 °C, 4 °C, −20 °C, and −60 °C were selected as drugs in post-mortem specimens are likely to be exposed to these temperatures prior to toxicological testing.

In the P experiment, no stability issues were observed for haloperidol samples after 10 weeks under any storage condition. To the authors' knowledge, the only published study investigating the long-term stability of haloperidol in plasma (3 months at −20 °C) was in 1987 by Haring et al. [27] and no stability problems were discussed. The discrepancy between the P and the F experiment regarding the storage at 20 °C for 10 weeks could possibly be explained by the inaccuracy of the method; with an expected 83% of the initial concentration still present after 10 weeks, the concentration is just outside the acceptance criteria of a 15% loss.

The same reasoning could also explain the 10–20% losses of risperidone under all storage conditions after 10 weeks of storage in the F experiment, while the drug appeared to be stable in the P experiment.

Chlorpromazine showed higher losses when stored at −20 °C than at 20 °C and 4 °C. This phenomenon has been seen for different drugs where lower storage temperatures seemed to be less favorable than higher temperatures [28,29]. To the authors' knowledge, all studies to date have predominately investigated the stability of chlorpromazine in serum [30] and plasma [12], where no stability issues were discovered. A study carried out in 1984 by McKay et al. [31] found chlorpromazine in whole blood samples to be stable over 84 days when stored at −20 °C; a longer storage interval has not been published. It seems surprising that the main degradation at −20° took place after four months of storage, further research needs to be undertaken to investigate this phenomenon.

For droperidol, the results for the stability of samples stored at −60 °C were unexpected. That the instability at −60 °C was consistent throughout the entire storage period (and similar to results at 20 °C and 4 °C) suggests that an unknown mechanism could be responsible. There have not been extensive stability studies conducted at −60 °C (as the refrigeration units required to reach these temperatures are costly and thus uncommon), and the authors have not seen this phenomenon described elsewhere in the literature.

The contradictory results of the olanzapine samples were not entirely unexpected, as several authors have reported conflicting results of stability studies involving olanzapine [7,9,32,33]. A possible explanation for the differing results in P and F experiment could be the different batches of blank blood being used. Similar discrepancies between different blood samples have been reported by Nilsson et al. [21] in the determination of the stability of zopiclone. As it is unknown at this stage what is causing the instability of olanzapine in blood samples, different matrix influences might contribute to conflicting results. This needs always to be taken into consideration when interpreting olanzapine concentrations in blood samples in order to avoid inaccurate conclusions.

7. Conclusion

Even though the majority of the tested drugs appeared to be stable over 20 weeks of storage in whole blood, instability appears to be a serious issue for several drugs when stored at certain temperatures.

Overall, −20 °C and −60 °C seem to be preferable for all drugs investigated in this study. All laboratories involved in the handling, processing and analysis of specimens need to identify these potential risks and incorporate processes to adequately accommodate for them. As the storage conditions and turnaround times of analysis differ between laboratories, short-term stability experiments tailored to the laboratory's individual needs should be performed.

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Appendix 1.4

Identification of 2-Hydroxymethyl-olanzapine as a Novel Degradation Product of Olanzapine

Saar, E., Gerostamoulos, D., Drummer, O.H., Beyer, J
Forensic Sci Int, 2012. **220** (1-3): p. 74-79

Forensic Science International 220 (2012) 74–79

Contents lists available at SciVerse ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Identification of 2-hydroxymethyl-olanzapine as a novel degradation product of olanzapine

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ARTICLE INFO

Article history:
Received 21 September 2011
Received in revised form 12 January 2012
Accepted 28 January 2012
Available online 23 February 2012

Keywords:
Olanzapine
Stability
Degradation
2-Hydroxymethyl-olanzapine

ABSTRACT

Olanzapine (OLZ) is amongst the most commonly prescribed antipsychotic drugs and is associated with substantial instability. The aim of this study was to investigate the instability of OLZ and to identify the degradants formed from its breakdown. Three experiments were conducted to monitor the degradation of OLZ and the formation of degradants in blood (1), water (2), and post-extraction at 4 °C (3). All three sample sets were analysed in duplicate and repeated in the absence (A) and presence (B) of 0.25% ascorbic acid. One degradant was identified in sample sets 2A and 3A with *m/z* 329 and confirmed as 2-hydroxymethyl-OLZ (2-OH-OLZ) using LC–MS techniques. The addition of 0.25% ascorbic acid slowed the degradation of OLZ down in all three experiments and inhibited the formation of 2-OH-OLZ in sample sets 2A and 3A.

To investigate the influence of oxygen on the degradation of OLZ and the formation of 2-OH-OLZ in water, an additional experiment (4) was conducted. Sample sets were prepared containing different vortexing or sonication steps in order to alter the oxygen content in the samples. Statistical analysis confirmed that degradation increased significantly following vortexing for 1 min while sonication did not affect the rate of degradation of OLZ further suggesting the involvement of oxygen in the degradative processes. 2-OH-OLZ was only identified as a degradant of OLZ in aqueous solutions. It also degrades over time but its product is currently unknown and is under investigation.

1. Introduction

The atypical antipsychotic drug olanzapine (OLZ, Fig. 1a) is amongst the most commonly prescribed antipsychotic drugs, not only for adults [1–5] but also for youths [6,7]. Treatment with OLZ is associated with several health risks, including cardiovascular complications, an increased risk of sudden cardiac death (SCD) [8], diabetic complications ranging from "mild glucose intolerance to diabetic ketoacidosis" [9], a lowered seizure threshold level in epilepsy [10,11] and fatal status epilepticus [12]. It is therefore not surprising that OLZ is commonly present in post-mortem cases [13].

A problem regularly associated with OLZ is its instability in blood. The stability in plasma and serum samples has been discussed in scientific publications for over a decade. Olesen and Linnet [14] performed several stability experiments and found that OLZ was unstable in human serum and ascorbic acid could reduce loss in stored samples [15]. In contrast, Lakso [16] found OLZ to be unstable in calf serum but stable in human serum. The stability in spiked and authentic human plasma samples without addition of an antioxidant was confirmed by Dusci et al. [17]. There have been several methods published, both with [18,19] and without [20–22] antioxidants added to plasma samples prior to analysis. With contradictory results regarding the stability of OLZ in serum and plasma, it remains unclear whether anti-oxidants are necessary.

However, all studies of the stability of OLZ in whole blood have shown it to be unstable [23,24], unless an antioxidant has been added to the blood. OLZ had to be excluded from a recently published method as the stability in post-mortem blood and processed sample stability could not be assured during validation studies [25]. Post-mortem drug testing is most often performed in whole blood as plasma or serum is hard or impossible to obtain. While therapeutic drug monitoring (TDM) methods frequently describe the addition of an antioxidant such as ascorbic acid to samples suspected to contain OLZ, this is not common practice in post-mortem cases, where a wide range of drugs may be present. In death investigations, delays between the actual time of death, sampling and analysis, further increase the risk of significant OLZ losses.

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doi:10.1016/j.forsciint.2012.01.029

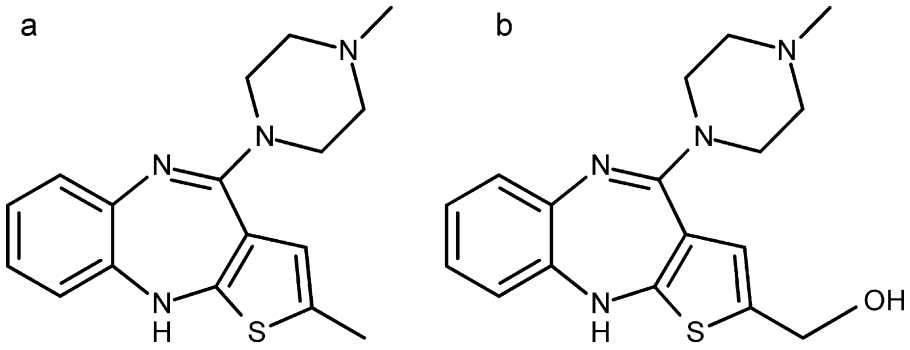

Fig. 1. Chemical structure of OLZ (a) and 2-OH-OLZ (b).

Two degradation product studies using solid oral formulations of OLZ have been published to date and confirmed six degradation products in OLZ tablets: OLZ-lactam, OLZ-ketolactam, OLZ-ketothiolactam, OLZ-N-oxide, OLZ-keto-oxim and a dimeric compound [26,27]. It is not known if any of these are formed in blood or plasma or whether other products are formed.

The aim of this study was to study the instability of OLZ in blood, to identify the degradants formed and if possible, study their formation and ultimate loss.

2. Materials and methods

2.1. Chemicals and reagents

OLZ was obtained from the Division of Analytical Laboratories, (Lidcombe, NSW, Australia). 2-Hydroxymethyl OLZ (2-OH-OLZ) and OLZ N-oxide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The isotope-labeled internal standard haloperidol-d_4 was purchased from Cerilliant (Round Rock, TX, USA). Acetonitrile (ACN), ammonium formate, 1-chlorobutane, methanol, and formic acid were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from BDH Chemicals (Kilsyth, VIC, Australia). Ascorbic acid was obtained from Crown Scientific (Minto, NSW, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia).

2.2. Specimens

Whole blood for degradation product experiments and preparation of calibration curves was obtained from the local blood bank in lithium heparin-coated plastic bags (500 mL). The blood was aliquoted into 10 mL polypropylene tubes containing 200 mg sodium fluoride and 30 mg potassium oxalate. Blood was analyzed using a previously published method and was found to be "drug-free" [25]. All blood samples were stored at −20 °C prior to analysis.

2.3. Equipment

The LC–MS/MS system used for the determination of the degradation products of OLZ consisted of an AB SCIEX Q-TRAP® 5500 linear ion-trap quadrupole mass spectrometer (AB SCIEX, Melbourne, VIC, Australia), equipped with a Turbo V ion source, operated in the electron spray ionization (ESI) mode coupled with an Shimadzu Prominence high performance liquid chromatography (HPLC) system (Shimadzu, Melbourne, VIC, Australia) which consisted of a degasser, two eluent pumps, a column oven and an autosampler.

The LC–MS/MS system used for the high resolution confirmation of the identified degradation product of OLZ consisted of an AB SCIEX TripleTOF™ 5600 system (AB SCIEX, Shanghai, China) coupled with a HPLC system as described above.

Gradient elution was performed on an Agilent Zorbax Eclipse XDB-C_{18} (4.6 mm × 150 mm, 5 μm particle size; Biolab, Scoresby, VIC, Australia). The gradient elution using 50 mmol/L aqueous ammonium formate adjusted to pH 3.5 with formic acid (eluent A) and ACN containing 0.1% formic acid (eluent B) has been described in a previous publication [25].

Experiments using the AB SCIEX Q-TRAP® 5500 system and the AB SCIEX TripleTOF™ 5600 system for mass spectrometric identification and confirmation of degradants used the following inlet conditions for Q1 scan and Product Ion Scan 329: gas 1 nitrogen (90 psi; 620.5 kPa); gas 2 nitrogen (90 psi; 620.5 kPa); ion-spray voltage (IS) 5500 V; ion-source temperature 750 °C; curtain gas, nitrogen (10 psi; 68.9 kPa). For the Product Ion Scan 329, the following additional settings were chosen: collision energy (CAD) was set at medium; declustering potential (DP) 140.0.

2.4. Preparation of standards and extracts

Stock solutions were prepared at a concentration of 1 mg/ml by separate weighings dissolved in ACN.

Working solutions of each analyte were prepared using ACN by independent dilution from each stock solution at the following concentrations: 0.1 mg/ml, 0.01 mg/ml, and 0.001 mg/ml. All solutions were stored at −60 °C.

Five calibration standards were prepared at concentrations of 0.01 mg/L, 0.05 mg/l, 0.1 mg/L, 0.25 mg/L and 0.5 mg/L using 50 μL of Eluent B and 450 μL of Eluent A and spiking solutions prepared from the working solutions as mixtures of OLZ and 2-OH-OLZ at concentrations 10 times higher than the corresponding calibration standards.

The liquid–liquid extraction using 1-chlorobutane and trizma buffer was described in a previous publication [25].

2.5. Stability experiments and identification criteria

Three experiments (set 1–3) were set-up to examine the degradation of OLZ in blood and water in the absence (A) and presence (B) of 0.25% ascorbic acid. All sample sets were prepared in duplicate at 0.1 mg/L and stored at 4 °C for 21 days in order to monitor the degradation of OLZ and the formation of potential degradation products (Table 1).

Set 1 contained blood spiked with OLZ and the extraction and analysis was performed daily over three weeks.

Set 2 contained distilled water spiked with OLZ left on the autosampler.

Set 3 contained blood spiked with OLZ, extracted immediately, reconstituted into 50 mmol/l aqueous ammonium formate adjusted to pH 3.5 with formic acid and ACN containing 0.1% formic acid and left on the autosampler.

For sample sets 2 and 3, injection was performed daily over three weeks.

The identification of degradation products involved direct comparison with reference standards, rather than a library match. Therefore, match factors were not defined for positive identification. Accurate mass deviation limits of 5 ppm were applied and considered acceptable.

2.6. Influence of oxygen on the degradation of OLZ

To investigate the influence of oxygen on the degradation of OLZ in aqueous solutions, to replicate a real life situation post-extraction, a series of experiments were conducted in which OLZ was present in distilled water and the solution was variously agitated to alter the exposure to air.

Table 1
Overview over sample sets 1–4 and the concentration of OLZ.

SET	A	B	C
1	Blood 0.1 mg/L	Blood 0.1 mg/L + ascorbic acid	–
2	Water 0.1 mg/L	Water 0.1 mg/L + ascorbic acid	–
3	Reconstitution post-extraction 0.1 mg/L	Reconstitution post-extraction 0.1 mg/L + ascorbic acid	–
4	Water 0.2 mg/L 'normal'	Water 0.2 mg/L vortexed	Water 0.2 mg/L sonicated

Set 4 consisted of three subsets A, B and C which were stored at 4 °C for 21 days. All of these sets were prepared in triplicate and contained OLZ in distilled water at 0.2 mg/L. Set 4A had no further treatment, set 4B was vortexed for 1 min and set 4C was sonicated for 5 min before loading onto the autosampler. Injection and analysis was performed daily over three weeks.

2.7. Statistical analysis

Statistics were performed using IBM SPSS Statistics 19. A repeated measures analysis of variance (ANOVA) test was performed to determine the influence of different oxygen levels on the degradation of OLZ and the formation of 2-OH-OLZ. Sphericity was tested for by Mauchly's test and the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. $p < 0.05$ was considered statistically significant. The F ratio, degrees of freedom, outcome and significance values are reported.

3. Results and discussion

3.1. Loss of OLZ and identification of 2-OH-OLZ as degradant

In order to investigate the loss of OLZ and formation of a degradant, a Q1 scan and product ion scan for all six m/z of

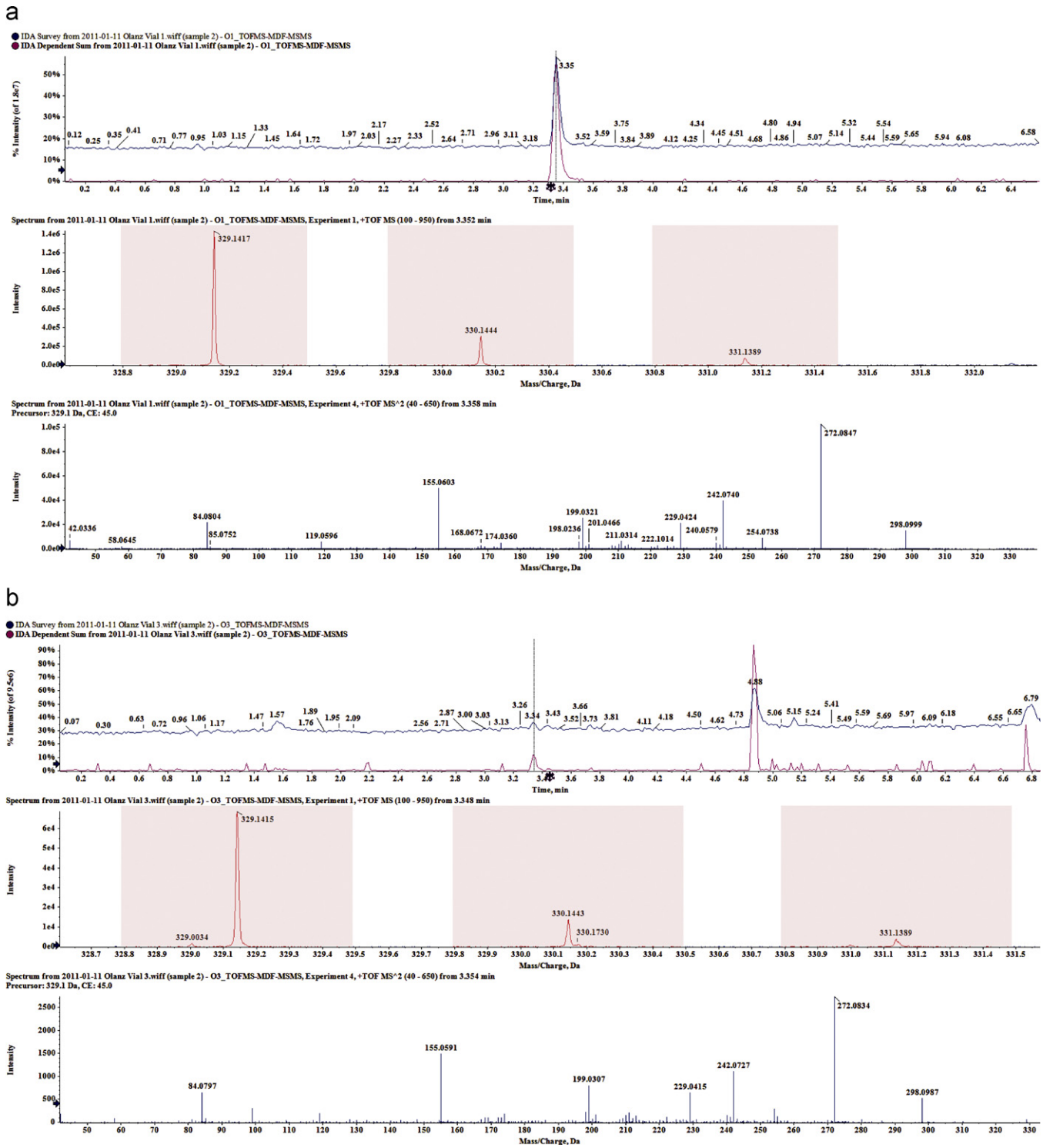

Fig. 2. Chromatogram of information dependant acquisition (IDA) scan (top row), TOF scan at 3.34 min (middle row), TOF-MS/MS spectra of m/z 329 at 3.34 min (bottom row) of the synthetic standard of 2-OH-OLZ (a) and the degradation product identified in sample set 2A and 3A (b).

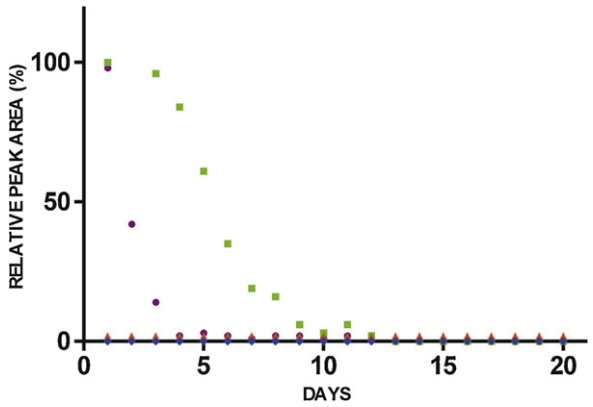

Fig. 3. Degradation of OLZ in set 1A (blood) without ascorbic acid (●) and set 1B in blood samples with 0.25% ascorbic acid (■) over three weeks at 4 °C and the corresponding formation of a peak at *m/z* 329 without (▲) and with ascorbic acid (◆).

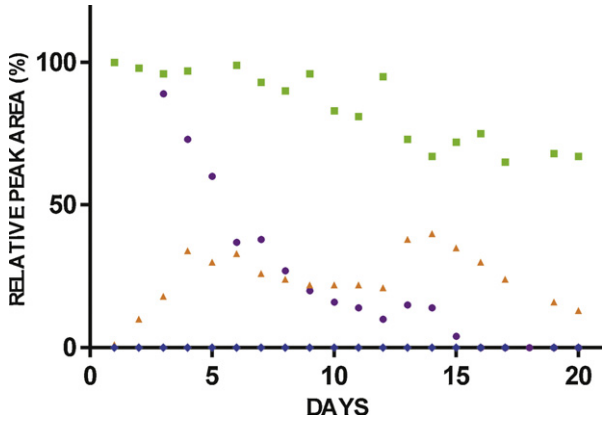

Fig. 4. Degradation of OLZ in set 2A (water) without ascorbic acid (●) and set 2B in water samples with 0.25% ascorbic acid (■) over three weeks at 4 °C and the corresponding formation of a peak at *m/z* 329 without (▲) and with ascorbic acid (◆).

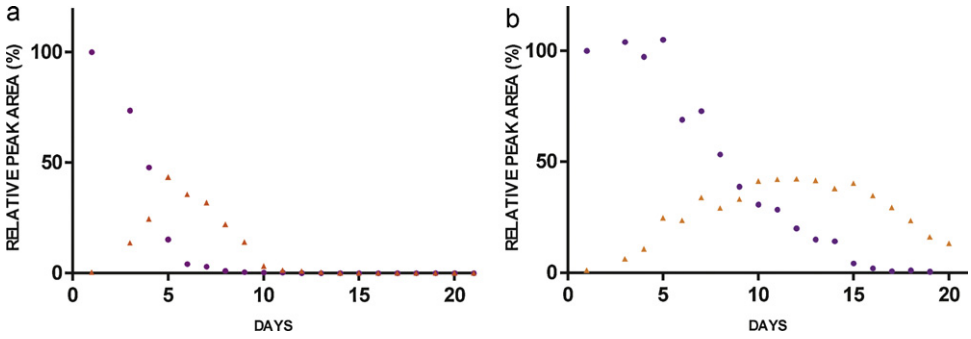

Fig. 5. (a) Degradation of OLZ in sample 1 of set 2A (water) without ascorbic acid (●) and the corresponding formation of a peak at *m/z* 329 without ascorbic acid (▲) over three weeks at 4 °C. (b) Degradation of OLZ in sample 2 of set 2A without ascorbic acid (●) and the corresponding formation of a peak at *m/z* 329 without ascorbic acid (▲) over three weeks at 4 °C.

previously described degradation products of OLZ in solid oral formulations [26,27] was carried out daily on sample sets 1–3 (and sub-sets A and B).

The only significant peak in comparison with a blank sample was found for *m/z* 329 in sample sets 2A and 3A, possibly corresponding to OLZ N-oxide as described by Baertschi et al. [26]. The respective peak area was plotted over time in the following experiments. The R.S.D. between sample duplicates was always <15% unless otherwise indicated.

The degradant with *m/z* 329 was compared with the commercially purchased standards of OLZ N-oxide and 2-OH-OLZ (Fig. 1b), a minor in vivo metabolite of OLZ [28], both giving an ion at *m/z* 329 using the AB SCIEX TripleTOF™ 5600 system. Fig. 2 shows the comparison between accurate mass and fragmentation patterns of a commercial standard of 2-OH-OLZ (a) and the degradant with *m/z* 329 (b). The detected mass and the isotopic pattern of the commercial standard and the degradation product match a theoretical mass of 2-OH-OLZ within acceptance criteria.

The OLZ concentration in sample set 1A containing OLZ spiked in blood stored at 4 °C decreased rapidly with a 100% loss of drug after four days of storage (Fig. 3). The addition of ascorbic acid slowed the loss but all OLZ was lost after ten days of storage at 4 °C (set 1B). Despite the rapid and substantial loss of OLZ, no 2-OH-OLZ was found in the stored blood samples (set 1). This indicated that the loss of OLZ in stored blood samples resulted in a different degradation product than 2-OH-OLZ. Comparison with a reference standard of OLZ N-oxide also did not provide a match.

However, the degradation of OLZ in water at 4 °C (set 2A) was associated with the formation of 2-OH-OLZ. When averaging the duplicate of sample set 2A (Fig. 4), the R.S.D. for degradation of OLZ and the formation of 2-OH-OLZ was >15%. This indicates that there were significant differences between these two single samples that formed the duplicate. The peak areas of the duplicate samples of set 2A are therefore plotted separately (Fig. 5a and b). In both samples OLZ degraded over time and 2-OH-OLZ was formed. Additionally, 2-OH-OLZ appeared to be unstable, as its degradation was seen in sample one of the duplicate (Fig. 5a) after five days and in sample two (Fig. 5b) after 15 days. The degradation of OLZ was

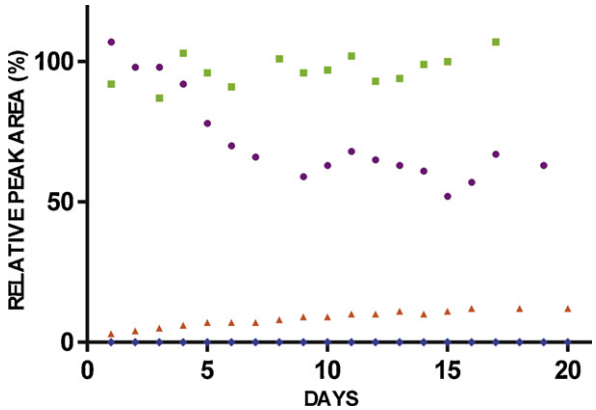

Fig. 6. Degradation of OLZ in set 3A (post-extraction) without ascorbic acid (●) and set 3B post-extraction with 0.25% ascorbic acid (■) and the corresponding formation of a peak at *m/z* 329 without (▲) and with ascorbic acid (◆) over three weeks at 4 °C.

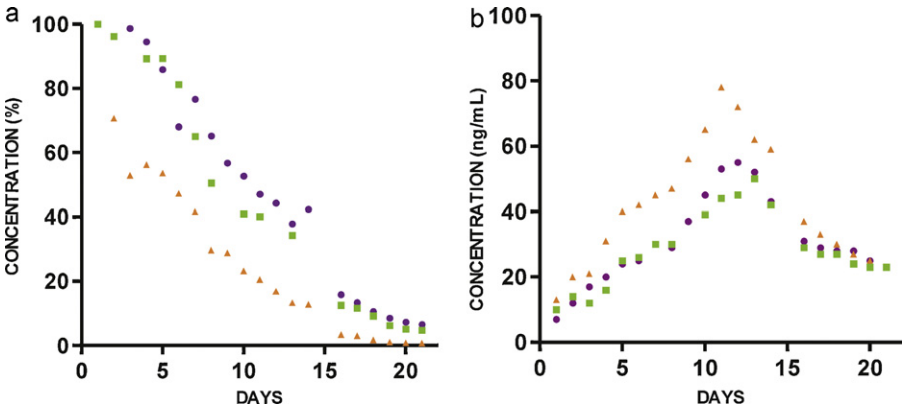

Fig. 7. (a) Degradation of OLZ and (b) formation of 2-OH-OLZ in water kept over three weeks at 4 °C without further preparation (set 4A ●), after 1 min of vortexing (set 4B ▲), and after 5 min of ultrasonication (set 4C ■).

slower in sample two (Fig. 5b), with a total loss of OLZ after 15 days of storage at 4 °C. OLZ degradation in sample one (Fig. 5a) was seen after five days at 4 °C.

Interestingly, the time and rate of degradation of 2-OH-OLZ appears to correspond to the respective OLZ concentration. The total conversion rate from OLZ to 2-OH-OLZ was approximately 50% in both samples.

Set 2B, distinguishable to set 2A in the addition of ascorbic acid to the samples prior to storage (Fig. 4) showed a much slower decrease in OLZ concentration, with a ~30% loss after three weeks at 4 °C. 2-OH-OLZ was not formed in these samples. It appears that the addition of ascorbic acid to aqueous OLZ samples does not only slow the degradation of OLZ down but also completely inhibits the formation of 2-OH-OLZ.

Set 3A was used to investigate the processed sample stability of OLZ over three weeks. This set showed ~35% degradation of OLZ over three weeks and a small formation of 2-OH-OLZ (Fig. 6).

Sample set 3B was used to investigate the processed sample stability and formation of a degradant after addition of ascorbic acid. This set showed very little instability of OLZ and no formation of 2-OH-OLZ (Fig. 6). Again, the addition of ascorbic acid slowed the rate of degradation of OLZ, similar to the previous sample sets.

The significant loss of OLZ in sample set 3A was not unexpected. Major degradation in extracted OLZ samples post-extraction was observed in a previous publication when extraction was performed using the same method [25].

3.2. Influence of oxygen on the degradation of OLZ and the formation of 2-OH-OLZ

Sample sets 4A–C were prepared in order to study the effect of different oxygen concentrations on the degradation of OLZ and the formation of 2-OH-OLZ. While sample set 4A was prepared by simple addition of an OLZ spike solution to water, sets 4B and 4C were either vortexed (4B) or sonicated (4C) prior to sample analysis, in order to alter the oxygen content in the sample. The R.S.D. was <15% from the mean for all analyzed sample triplicates and therefore in an acceptable range.

Fig. 7a shows the decrease in OLZ concentration over 21 days in sample sets 4A–4C.

Statistical analysis revealed that the degradation of OLZ was significantly different between the three sample sets, $F(1.416, 21.243) = 33.25$, $p < .05$. Post hoc tests confirmed that the visual observation of the degradation of OLZ in sample set 4B (containing an additional vortexing step) was significantly different from sample sets 4A and 4C, while sample set 4A and 4C were not different from each other.

Sample set 4B also showed the highest formation of 2-OH-OLZ and the most rapid increase (Fig. 7b). Statistical analysis revealed that the formation of 2-OH-OLZ was significantly different between the analyzed sample sets, $F(1.175, 21.41) = 29.8$, $p < .05$. Post hoc tests revealed that sample set 4B was significantly different from sample set 4A and 4C, whereas 4A and 4C were not different from each other. It appears that even 1 min of vortexing resulted in a statistically significant loss of OLZ compared with the normal sample preparation that only included a few seconds of vortexing. 5 min of ultrasonication did not create a significant difference between sample set 4C and any other sample set. The conversion rate of OLZ to 2-OH-OLZ appeared to be between 25% and 40%.

4. Conclusions

Ascorbic acid slows down the degradation of OLZ in stored blood samples (~50%) and completely inhibits instability in processed samples over three weeks.

2-OH-OLZ was identified as a degradation product of OLZ that is formed in aqueous solutions and accounted for approximately 25–50% of the loss of OLZ. 2-OH-OLZ also appears to be unstable and subsequently degrades to a product that remains unknown.

The formation of 2-OH-OLZ is affected by different oxygen concentrations which might be part of the reason for the discrepancies in different reports regarding the stability of OLZ. It is recommended to keep vortexing steps consistent during sample preparation in order to preserve sample integrity. However, 2-OH-OLZ was not formed in whole blood and its formation is inhibited by the addition of ascorbic acid in aqueous solutions. As the instability of OLZ in blood samples remains a major problem in analytical toxicology, further investigations for the identification of degradation products in blood are required.

Acknowledgments

The authors would like to thank AB SCIEX and especially Dr. Brad Patterson for the opportunity to use the AB SCIEX TripleTOF™ 5600 system in Shanghai (China) for this study and his helpful advice.

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Appendix 1.5

The Time-dependent Post-mortem Redistribution of Antipsychotic Drugs

Saar, E., Beyer, J., Gerostamoulos, D., & Drummer, O.H.
Forensic Sci Int. 2012 *(ePub)*

Forensic Science International xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

The time-dependant post-mortem redistribution of antipsychotic drugs

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ARTICLE INFO

Article history:
Received 11 May 2012
Received in revised form 29 May 2012
Accepted 30 May 2012
Available online xxx

Keywords:
Antipsychotic drugs
Post-mortem redistribution
LC–MS/MS

ABSTRACT

The post mortem redistribution of ten commonly prescribed antipsychotic drugs (APs) was investigated. Femoral blood was collected from 273 cases at admission to mortuary (AD) and at post-mortem (PM). The PM samples were collected at various times up to nine days after admission and the sample pairs analysed using LC–MS/MS. The drugs included in this study were 9OH-risperidone (paliperidone), amisulpride, chlorpromazine, clozapine, haloperidol, olanzapine, promethazine, quetiapine, risperidone, and zuclopenthixol. Haloperidol, quetiapine and risperidone showed minimal changes between AD and PM specimens, whereas the majority of drugs showed significant changes between the sample pairs collected at different time points post mortem ($p < 0.01$) in addition to an average concentration change greater than the uncertainty of measurement of the applied method. Average increases in blood concentrations after admission to the mortuary ranged up to 112% (chlorpromazine and olanzapine) but also decreases up to −43% (9OH-risperidone) were seen. There were large standard deviations between sample pairs and substantial day-to-day unpredictable changes that highlight the difficulty in the interpretation of drug concentrations post-mortem. Based on the presented data, we recommend that specimens for toxicological analysis should to be taken as soon as possible after admission of a deceased person to the mortuary in order to minimise the effects of the PM interval on the drug concentration in blood.

1. Introduction

Post mortem redistribution (PMR) is a well-recognised but under-explored phenomenon that complicates the interpretation of drug concentrations in medico-legal death investigations. It is believed to occur by diffusion of drug from tissue-bound stores at higher concentrations adjacent to blood vessels into blood after death, therefore increasing blood concentrations post-mortem [1]. The two main factors that appear to influence the PMR of a drug are sampling site and time of sampling relative to the time of death. Peripheral blood is regarded as more suitable for post-mortem drug testing because of its distance from central organs and the gastrointestinal tract [2,3].

PMR has been most associated with a large volume of distribution (V_d) >3 L/kg and a high degree of lipophilicity [2,4–7]. Basic drugs are considered to be more susceptible to PMR as their ionised fraction increases with the mainly aqueous content of cells as they become more acidic post-mortem. During post-mortem lysis of cells basic drugs diffuse more easily into hydrophilic body fluids, which can potentially cause increases in drug concentrations in blood [8]. Since antipsychotic drugs (APs) are basic and generally lipophilic with a large V_d (Table 1) they are likely to be susceptible to PMR, however, this has not been studied in detail.

Currently published data on the PMR for APs has been obtained from animal studies, targeting one or a few analytes [9–11], or from human tissue distribution studies in post-mortem cases [12–19]. These studies focused predominantly on the impact of sampling site on a post-mortem drug concentration, rather than the influence of the post-mortem time interval (PMI). This is probably due to the difficulty in obtaining relevant specimens for testing and ethical restrictions on human experimentation on deceased persons.

Since an autopsy is unlikely to be carried out immediately following admission of a body to a mortuary, a PMI of a few to several days is common increasing the likelihood of substantial post-mortem changes in concentrations.

The Victorian Institute of Forensic Medicine (VIFM) is able to obtain a peripheral blood specimen on admission to the mortuary as part of its ability to conduct preliminary examinations prior to a coroners order on whether an autopsy should be conducted. The order to conduct an autopsy can take several days. This allows an opportunity to compare the blood concentrations on admission and the subsequent concentrations

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0379-0738/$ – see front matter © 2012 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.forsciint.2012.05.028

Table 1
Volumes of distribution (V_d), protein binding (F_b), and lipophilicity (log P) values for APs of interest.

Drug	V_d	F_b	log P
Amisulpride	13–16	0.17	1.5
Chlorpromazine	10–35	0.98	5.18
Clozapine	2–7	0.95	3.67
Haloperidol	18–30	0.9	3.7
Olanzapine	10–20	0.93	2.65
Promethazine	13	0.93	4.52
Quetiapine	8–12	0.83	2.93
Risperidone	0.7–2.1	0.9	3.27
9OH-risperidone	U/K	U/K	2.3
Zuclopenthixol	15–20	0.98	4.46

V_d and F_b are obtained from Baselt [29], log P values are calculated using ALGOPS 2.1. U/K: unknown.

from blood taken at autopsy, in order to study the effect of time on the PMR.

2. Methods

2.1. Case selection

Cases were selected in which both an admission to mortuary blood specimen (AD) and a post-mortem peripheral blood specimen (PM) taken at autopsy had been collected and showed the presence of at least one AP drug during routine toxicological testing. Only cases in which the investigation by the coroner was completed were included in this study.

Several exclusion criteria were applied. Cases that contained insufficient sample volume following routine toxicological analysis and subsequent long-term storage of 2 mL of specimen were excluded as were suspicious death cases. Additionally, all cases where the time interval between death and sampling of the AD sample was greater than 24 h were excluded from the study. Cases were also excluded where the circumstances of the death indicated significant trauma prior to death. In these instances, the integrity of the blood vessels was likely to have been compromised. Samples in this study that showed signs of decomposition (visually evaluated) were also excluded.

A total of 273 cases (546 paired specimens) were selected that showed the presence of at least one AP and matched the criteria described above. A total of ten APs were detected in these cases including 9OH-risperidone (paliperidone), amisulpride, chlorpromazine, clozapine, haloperidol, olanzapine, promethazine, quetiapine, risperidone, and zuclopenthixol.

2.2. Ethical review process

Ethics approval was granted by the Ethical Review Committee of the VIFM (Reference number: EC 5/2011).

2.3. Analysis of specimens

All specimens were analysed using a previously published validated tandem LC–MS method using three transitions per drug [20]. A matrix-matched freshly spiked seven-point calibration curve was extracted with every assay and used to calculate the respective concentrations of the drugs. Quality control (QC) samples were run after every ten samples. The assay was only accepted if all QCs were within 20% of the target concentration. All 273 sample pairs were re-analysed despite some of them having had the AD specimen or the PM specimen tested during routine toxicological analysis. This was done in order to minimise differences in drug concentration potentially caused by different analysis times due to instability of compounds. The following formula was used to evaluate the change in concentration [%] between AD and PM sample:

$$\frac{\text{Conc (PM)} - \text{Conc (AD)}}{\text{Conc (AD)}} \times 100 = \Delta\text{Conc [\%]}$$

where Conc = concentration.

If ΔConc [%] > 0 an increase in concentration was observed between AD and PM sample, if ΔConc [%] < 0 a decrease in concentration was observed between AD and PM sample.

2.4. Statistical evaluation

All AD specimens were compared with their respective PM sample using a two-tailed Wilcoxon Matched-Pairs Rank-Sum Test, with samples grouped according to the AP. This non-parametric test was chosen to evaluate the results, as normal distribution cannot be assumed for the sample set. The two-tailed approach was chosen, as concentration changes in any direction needed to be considered. Significance values were only evaluated for individual PMI where six or more sample pairs were available, as the Wilcoxon Matched-Pairs Rank-Sum Test requires at last six matched pairs to be significantly different before assuming significant differences within a group of pairs. Subsequently, individually paired cases and their concentration change at defined time points post-mortem were combined in a group and used to evaluate a trend over time. The p-value was reported for all cases containing one drug. If there were six or more sample results for any given PMI, the significance value was provided for the individual PMI, in addition to the group value (Supplement 1). Additionally, n-values, the mean and standard deviation for each PMI are reported.

3. Results and discussion

In order to evaluate the post-mortem drug concentration changes of each drug, several factors have been taken into consideration. In addition to the statistical evaluation, the average concentration change over the investigated PMI has been determined in order to make the data comparable with the outcomes of previous studies (Table 2). As inaccuracies (RSD) caused by the analytical method used in this study have proven to be under 20% for all drugs with the exception of olanzapine (OLZ) (which was excluded from method validation due to its instability [20]), concentration changes greater than 40% (2RSDs) were considered likely to be caused by reasons other than method inaccuracy. Additionally, the drug concentration change on every day of the PMI has been determined along with the standard deviation, giving more detailed information on the change over time (Supplement 1).

The majority of drugs showed significant changes between AD and PM specimens ($p < 0.01$) in addition to an average concentration change greater than 40%. Average increases in blood concentrations after admission to the mortuary ranged up to 112% (chlorpromazine and olanzapine) but also decreases up to −43% were observed (9OH-risperidone).

Table 2
Number of sample pairs per drug (n), average concentration change including range [%], the investigated PMI (time) [days] and p-value (p=) for the studied antipsychotic drugs.

Drug	n	Mean ΔConc [%] [min, max]	Time [days]	Significance (p=)
Amisulpride	11	**57 [43, 84]**	2–8	**<0.01**
Chlorpromazine	17	**112 [25, 216]**	1–9	**<0.01**
Clozapine	15	**41 [16, 74]**	2–6	**<0.01**
Haloperidol	18	2 [−30, 49]	1–9	0.83
Olanzapine	95	**112 [17, 234]**	1–9	**<0.01**
Promethazine	22	**63 [13, 174]**	1–7	**<0.01**
Quetiapine	57	25 [16, 38]	1–7	<0.01
Risperidone	33	−15 [−36, 12]	2–7	<0.01
9OH-risperidone	35	**−43 [−68, −26]**	2–8	**<0.01**
Zuclopenthixol	15	**62 [28, 146]**	1–7	**<0.01**

Bold: mean ΔConc > 2RSD (>40%) and $p < 0.01$.

Please cite this article in press as: E. Saar, et al., The time-dependant post-mortem redistribution of antipsychotic drugs, Forensic Sci. Int. (2012), http://dx.doi.org/10.1016/j.forsciint.2012.05.028

9OH-risperidone was the only analyte which showed a reduction in drug concentration over all time points (35 sample pairs from cases with PMI ranging from 1–8 days, $p < 0.01$), with an average loss of 43%. 9OH-risperidone is the main-metabolite of the atypical AP risperidone and is also available in some countries as paliperidone. It is formed by cytochrome (CYP) P450 enzymes, specifically CYP2D6, and is likely to contribute to the in vivo effects of risperidone [21]. In a clinical setting risperidone is rapidly metabolised and concentrations have been shown to be generally lower than 9OH-risperidone [22]. Hence, 9OH-risperidone is commonly measured in addition to risperidone, providing an indication, wherever possible, of prior risperidone ingestion in cases where risperidone can no longer be detected. With protein binding estimated at ~77% in human plasma and a partition coefficient (log *P*) of 2.3, 9OH-risperidone is less lipophilic than its parent compound, risperidone (protein binding = 90%, log *P* = 3.0) [23]. Consequently, 9OH-risperidone should be less likely than risperidone to distribute into organs and fatty tissue after death. However, results of this study showed losses of more than 65% after a PMI of eight days, with a loss in concentration of approximately 40% after four days. The significant losses of 9OH-risperidone over the time frame examined are interesting, considering a previous study investigating the stability of 9OH-risperidone in spiked whole blood samples did not reveal any significant losses over ten weeks of storage at 4°, −20°, and −60° [20]. However, whole blood samples in the stability study were preserved with 200 mg sodium fluoride and 30 mg potassium oxalate, which is likely to improve the stability of 9OH-risperidone.

Risperidone was the only analyte in which the concentrations decreased although a slight increase occurred at day seven (Fig. 1). Risperidone showed an average loss of −15% over the investigated PMI ($p < 0.01$). Interestingly, Rodda et al. reported that the heart to femoral ratio of 9OH-risperidone reflected that of risperidone [19]. This observation combined with the results of this study for the post-mortem drug concentration changes of risperidone and 9OH-risperidone, emphasise that despite sharing a similar heart to femoral ratio, drugs may undergo different patterns of post-mortem changes over time. Furthermore, risperidone shows the smallest V_d of all investigated drugs (0.7–2.1 L/kg), suggesting that it is not likely to be susceptible to significant PMR in the first few days; a response that is supported by our data in the early PMI period. As the average concentration change was less than 40%, the PMR of risperidone was considered not significant as it is unlikely to materially affect the interpretation of its likely effects.

Only two drugs (chlorpromazine and olanzapine) showed consistent increases in concentration over the nine days PMI ($p < 0.01$). These increases were generally greater than the uncertainty of measurement. Chlorpromazine showed an increase in concentration over time, with an average increase of ~112% over a PMI of nine days. This is consistent with reported heart/femoral blood ratios ranging from 1.57 (1.0–2.7; unknown sample size) up to 2.0 (0.8–7.2, $n = 6$) and even 4.0 (1.0–8.0, $n = 5$) [24] that have been reported in the literature, suggesting that chlorpromazine is subject to substantial PMR. This raises doubt over what can be said of blood concentrations that could be caused by redistribution since these changes could be mistaken for drug misuse and toxicity [25]. Olanzapine concentrations increased on average ~112% over the investigated PMI of nine days (>100% increase after four days) suggesting that this drug is highly susceptible to PMR. While the SDs of olanzapine were large (seven out of nine were greater than 30%), this was not entirely unexpected due to the large case to case variation. The drug is also known to be inherently unstable [20] and it is likely that larger increases occurred but some drug was lost to degradation. While the analysis of Horak and Jenkins [13] found the PMR of olanzapine to be "minimal" with a heart to femoral ratio of 1.24, this is supported by previous case studies, where the heart to femoral blood ratio of olanzapine has been reported to range from 1.1 to 1.4 [17,26]. However, the observed variability in the detection of olanzapine highlights the limited value of single case studies as large variations in detection are likely to give misleading results. With a total of 95 sample pairs analysed in this study, olanzapine is highly likely to undergo PMR over time, however the true extent of PMR cannot be determined due to its instability.

Clozapine and promethazine showed the most significant increases in the first three days of the PMI. The largest increase in concentration occurred at four days for clozapine (>70%) and three days for promethazine (>170%). As the drug concentration decreased from this point onwards, drug results obtained after a longer PMI (four days onwards) intriguingly appear to be more likely to represent drug concentrations at the time of admission of a deceased person. Both drugs appear to undergo a pattern of increase in drug concentration followed by decrease, causing large inter-day concentration differences (Fig. 1). Flanagan et al. investigated the PMR of clozapine in the domestic pig [9]. Two pigs were administered with a single dose of 10 mg/kg of clozapine. After death, blood was taken from a peripheral vein at different time points over a 24 h period. Interestingly, both pigs showed an increase in blood concentration initially, followed by a decrease. Clozapine was no longer detectable in one of the pigs after 24 h. Consequently, the observed pattern of post-mortem behaviour of clozapine is supported by the results of our study.

Amisulpride and zuclopenthixol showed slower increases in concentration with the largest increase reached after four (amisulpride, >80%), and five days (zuclopenthixol, >145%), averaging 57% and 62%, respectively.

The two remaining drugs (in addition to risperidone) appeared to have undergone only minor post-mortem changes.

Quetiapine showed an average concentration change of 25% over the investigated PMI (seven days). Following a tissue distribution study in 2000, Anderson et al. concluded that quetiapine was likely to undergo PMR [18]; this finding was also supported by Parker & McIntyre in 2005, who reported a heart to femoral ratio of 1.4, suggesting some propensity for PMR [27]. However, our results highlight that different conclusions may be reached depending on the time since death. With an average increase of less than 40% over seven days of PMI, the concentration change is within the inaccuracy of the method and also would not materially affect any interpretations made.

Haloperidol was the only drug included in this study where post-mortem concentration changes were statistically not significant over the whole time frame average concentration change being only 2% (Table 2). The only published study investigating the PMR of haloperidol is a tissue distribution study in the rat which showed an increase six hours after death [10]. No additional blood samples were collected after this time, making conclusions regarding a longer PMI difficult.

There were limitations to this study. The PM sample was taken during the autopsy process, therefore the possibility of contamination through collection of non-femoral blood, urine, faeces, serous fluid that has leaked from the chest cavity or stomach contents cannot be fully excluded. Furthermore, despite having excluded putrefied samples, a previous study has shown that even non-decomposed samples can result in altered extraction efficiencies and variable matrix effects compared with ante-mortem blood samples [28]. These outcomes suggest that variations are likely to be even higher if the sample group is not controlled. Another drawback is the unpredictability of the change in drug concentration that may have occurred in the time frame between death and taking of the AD sample.

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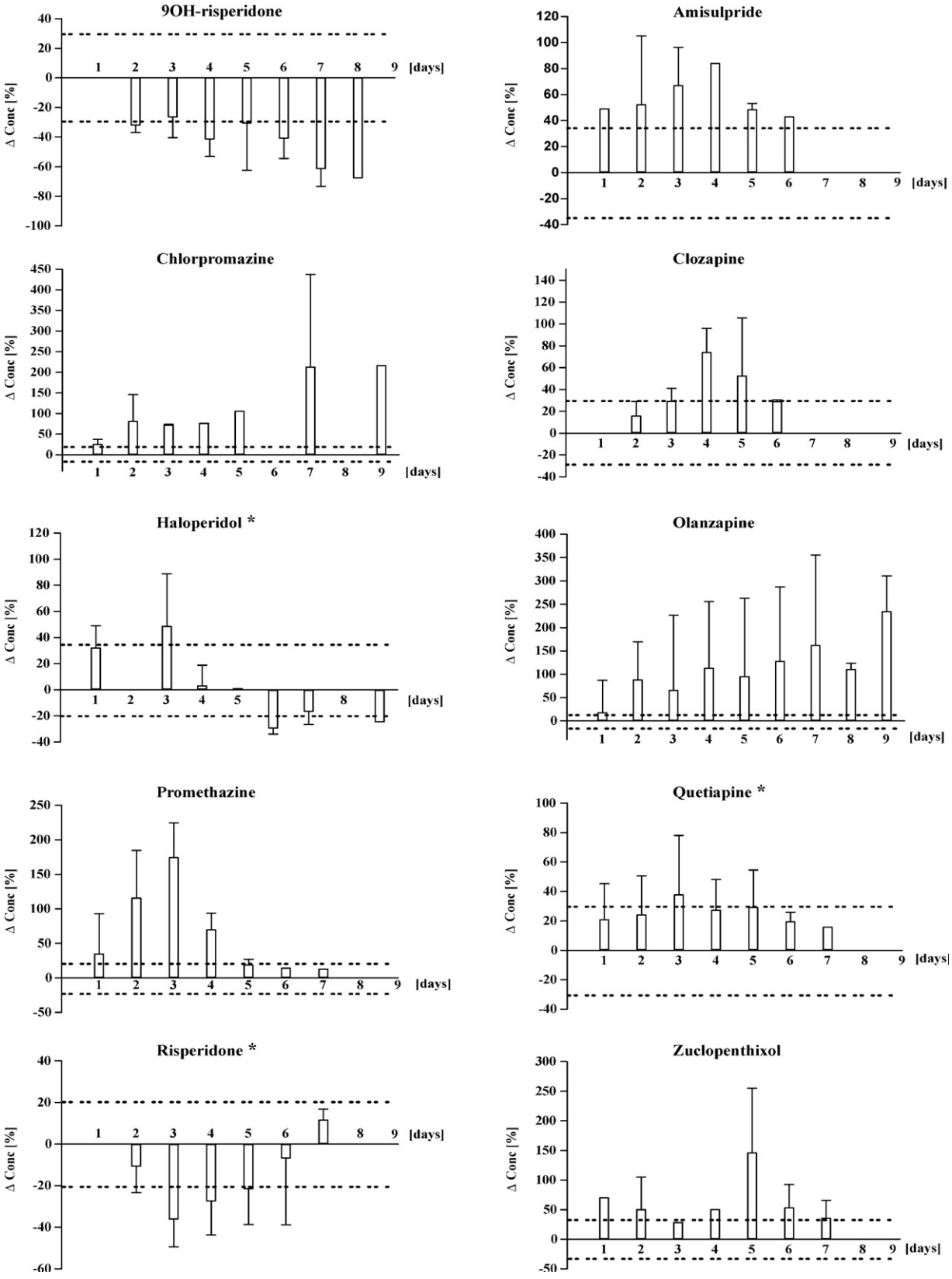

Fig. 1. Concentration change between AD and PM specimen over the PMI, displaying twice the RSD (—); ΔConc, change in concentration; *, mean ΔConc < 2RSD. Please note that the scales are set to different ranges.

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4. Conclusions

In conclusion, the majority of drugs showed significant changes between AD and PM specimens ($p < 0.01$) in addition to an average concentration change greater than the uncertainty of measurement of the applied method. Haloperidol, quetiapine and risperidone did not show concentration changes greater than the extent of the uncertainty of measurement, therefore their risk to undergo significant post mortem redistribution was considered low. The outcomes of this study highlight the limitations of reporting post-mortem concentration changes. While average values as reported in this study can give an indication of whether or not a drug is subject to PMR, the analysis of samples collected over various days of the PMI has shown that individual variations between different time points of the PMI are can be significant. In addition to large standard deviations, this complicates the interpretation of post-mortem drug results, especially when a long or unknown time frame has passed between death and sampling of a specimen for toxicological analysis. Specimens for toxicological analysis need to be taken as soon as possible after admission of a deceased person to the mortuary. However, the large variations in reported results highlight that speculation concerning the magnitude of a post-mortem drug concentration change are impractical. It is more important to be aware of the variability of the change that is likely to occur.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2012.05.028.

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Please cite this article in press as: E. Saar, et al., The time-dependant post-mortem redistribution of antipsychotic drugs, Forensic Sci. Int. (2012), http://dx.doi.org/10.1016/j.forsciint.2012.05.028

Appendix 2

Other Publications relating to Ph.D candidature

Appendix 2.1

Comparison of Extraction Efficiencies and LC-MS-MS Matrix Effects Using LLE and SPE Methods for 19 Antipsychotics in Human Blood

Saar, E., Gerostamoulos, D., Drummer, O.H., Beyer, J
Anal Bioanal Chem, 2009. **393**(2): p. 727-34

Anal Bioanal Chem (2009) 393:727–734
DOI 10.1007/s00216-008-2498-6

ORIGINAL PAPER

Comparison of extraction efficiencies and LC–MS–MS matrix effects using LLE and SPE methods for 19 antipsychotics in human blood

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Received: 25 August 2008 / Revised: 20 October 2008 / Accepted: 24 October 2008 / Published online: 11 November 2008

Abstract Antipsychotic drugs are frequently associated with sudden death investigations. Detection of these drugs is necessary to establish their use and possible contribution to the death. LC–MS(MS) methods are common; however accurate and precise quantification is assured by using validated methods. This study compared extraction efficiency and matrix effects using common liquid–liquid and solid-phase extraction procedures in both ante-mortem and post-mortem specimen using LC–MS–MS. Extraction efficiencies and matrix effects were determined in five different blank blood specimens of each blood type. The samples were extracted using a number of different liquid–liquid extraction methods and compared with a standard mixed-mode solid-phase extraction method. Matrix effects were determined using a post-extraction addition approach—the blank blood specimens were extracted as described above and the extracts were reconstituted in mobile phase containing a known amount of analytes. The extraction comparison of ante-mortem and post-mortem blood showed considerable differences, in particular the extraction efficiency was quite different between ante-mortem and post-mortem blood. Quantitative methods used for determination of antipsychotic drugs in post-mortem blood should establish that there are no differences in extraction efficiency and matrix effects, particularly if using ante-mortem blood as calibrator.

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Keywords Antipsychotics · Matrix effects · Extraction efficiency · Blood · LC–MS–MS

Introduction

Antipsychotic drugs are frequently associated with sudden death investigations. Patients receiving antipsychotic drugs have been reported to be 1.4 times more likely to experience sudden unexpected death than individuals who are free from antipsychotic drugs [1–7]. Detection of these drugs is necessary to establish their use and possible contribution to the death. Reliable qualitative and quantitative detection forms the basis of a competent interpretation of the possible role of the drug in death. Increasingly, LC–MS(MS) methods are being commonly utilized for the detection of antipsychotic drugs in a wide range of tissues including blood [2–11]. LC–MS(MS) methods provide the required sensitivity and selectivity and, in contrast with gas chromatography, do not require thermal stability of the drugs in question [12]. However, accurate and precise quantification needs to be assured by the use of validated methods [13, 14]. Matrix effects can limit the usability of LC–MS(MS) methods, especially using electrospray ionization [15] Therefore, an assessment of matrix effects should be included in method validation when using LC–MS(MS) [13, 16, 17]. The most efficient approach to reduce matrix effects is elimination of the sample constituents which are responsible for matrix effects [18–20]. This can be achieved by improvement of sample pre-treatment. The most common pretreatment of specimens (i.e. blood, urine) either involves liquid–liquid extraction (LLE) or solid-phase extraction (SPE). Irrespective of whether sample pre-treatment results in 100% extraction efficiency for all analytes, improvement of the method by

avoiding matrix effects is essential. Generally, SPE methods are likely to produce cleaner extracts, particularly with ante-mortem blood, plasma, or serum specimens [21–23]. However, the extraction of non-decomposed post-mortem (N-DEC) or heavily decomposed post-mortem (DEC) samples can be difficult. Another major drawback in post-mortem analysis is that validation studies have usually been performed on ante-mortem blood [24–30]. Even if matrix effects are not present and extraction recoveries high in ante-mortem blood samples, the converse can be true for post-mortem specimens (i.e. considerable matrix effects and poor extraction recoveries). Therefore, the aim of this study was to compare extraction efficiency and matrix effects using common LLE and SPE methods using different ante-mortem and post-mortem blood samples.

Materials and methods

Chemicals and reagents

Chlorpromazine, haloperidol, thioridazine, trifluperazine, and Trizma base were purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Clozapine was provided by Sandoz (Pyrmont, NSW, Australia); 9-OH-Risperidone and pimozide were obtained from Janssen–Cilag (North Ryde, NSW, Australia). Amisulpride, aripiprazole, buspirone, olanzapine, promethazine, quetiapine, risperidone, and zuclopenthixol were obtained from the Division of Analytical Laboratories, (Lidcombe, NSW, Australia). Droperidol, perphenazine, promazine, and sulpiride were provided by Australian Government Analytical Laboratories (Pymble, NSW, Australia). 1-Chlorobutane, ethyl acetate, isopropanol, methanol, and formic acid were purchased from Merck (Darmstadt, Germany) Sodium sulfate, sodium bicarbonate, and ammonium formate were provided by Ajax Finechem (Taren Point, NSW, Australia). Acetic acid was purchased from BDH Chemicals (Kilsyth, VIC, Australia). All chemicals were of analytical grade or better and water was purified using a Milli-Q Ultrapure Water System (Waters, Rydalmere, NSW, Australia).

Phosphate buffer (pH 6) was prepared by dissolving 13.72 g NaH_2PO_4 and 4.28 g of Na_2HPO_4 in 1 L water. Trizma buffer (pH 9.2) was prepared by dissolving 242 g Trizma base in 1 L water. The HPLC eluents were as follows: eluent A contained 50 mmol L^{-1} ammonium formate in water, adjusted to pH 3.5 with formic acid; eluent B contained 0.1% formic acid in acetonitrile.

Specimens

Drug-free ante-mortem (AM) blood samples were obtained from drug-free volunteers. Samples were collected into spray coated K_2EDTA preserved plastic tubes. Post-mortem blood samples were submitted to the authors' laboratory for routine toxicological analysis. The post-mortem blood samples were regarded as drug free if none of the existing tests showed the presence of the studied drugs in any specimen (including blood, liver, and urine). Classification of the post-mortem blood samples as N-DEC and DEC was based upon pathology description of the body from which the specimen was taken. All post-mortem blood samples were collected into plastic tubes containing 1% fluoride–oxalate. This is the standard collection tube at autopsy used across Australia and New Zealand. All blood samples were stored at −20°C prior to analysis.

Sample preparation

Liquid–liquid extraction

In a 10-mL glass tube, 0.5 mL blood was mixed with 50 μL eluent A. For comparison, either 1 mL Trizma buffer, 1 mL saturated sodium sulfate solution, or 100 mg solid $NaHCO_3$ were added. The blood–buffer mixtures were extracted with 8 mL of three different solvents or solvent mixtures, resulting in nine different extraction procedures. The extraction solvents were: ethyl acetate, mixture of diethyl ether and ethyl acetate (50:50) or 1-chlorobutane. The samples were extracted for 30 min on a rotating wheel. After a brief centrifugation to separate layers, the solvent layer was transferred to an 8-mL disposable borosilicate tube and evaporated to dryness using a TurboVap LV Evaporation System (Millennium Science, Melbourne, VIC, Australia) at 40°C for 27 min. The residue was reconstituted in 100 μL of a mixture of eluent A and eluent B (90:10) and transferred to an autosampler vial. The final extract (10 μL) was injected into the LC–MS–MS system.

Solid-phase extraction

In a 10-mL glass tube, 0.5 mL blood was mixed with 50 μL eluent A, and 1 mL phosphate buffer. The mixture was ultrasonicated for 10 min prior to centrifugation (10 min at 1800 *g*). The supernatant (1 mL) was added to 4 mL phosphate buffer and loaded on to SPE cartridges previously conditioned with 3 mL purified water, 3 mL methanol, and 3 mL phosphate buffer. The loaded SPE cartridges (XtrackT, XRDAH203; UCT, Bristol. PA, USA) were treated sequentially with 6 mL purified water, 1 mL acetic acid, and 3 mL methanol. The analytes were eluted with 3 mL 84% ethyl acetate, 12% isopropanol, and 4% ammonia. The eluates were evaporated to dryness using an LV Evaporation System (Millennium Science) at 40°C for 27 min. The residue was reconstituted in 100 μL of a mixture of eluent A and eluent B (90:10) and transferred to an autosampler vial. The final

extract (10 μL) was injected into the LC–MS–MS system. Solid phase extractions were automated using a Rapidtrace Solid-Phase Extraction unit 50000 (Millennium Science).

Apparatus

The LC–MS–MS system consisted of an Applied Biosystems 3200 Q-TRAP linear ion-trap quadrupole mass spectrometer (Applied Biosystems, Melbourne, VIC, Australia) with Analyst software (Version 1.4.2) and equipped with a Turbo V ion source, operated in the electron spray ionization (ESI) mode, and an Agilent Technologies (AT) 1200 Series HPLC system (Agilent, Melbourne, VIC, Australia) which consisted of a degasser, a binary pump, and an autosampler.

HPLC conditions

Gradient elution was performed on an Agilent Zorbax Eclipse XDB-C18 (4.6 mm ×150 mm, 5 μm particle size; Biolab, Clayton, VIC, Australia). The mobile phase consisted of 50 mmol L^{-1} aqueous ammonium formate adjusted to pH 3.5 with formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). During use, the mobile phase was degassed by the integrated Agilent 1200 degasser. Before starting the analysis, the HPLC system was equilibrated for 10 min with a mixture of 90% eluent A and 10% eluent B. The HPLC system was additionally equilibrated for 4 min prior to each run. The flow rate and gradient were programmed as follows: equilibration time (−4.00 min–0.00 min) 10% eluent B, flow rate 1.4 mL min^{-1}; 0.00–1.00 min: 10% eluent B, flow rate 1.4 mL min^{-1}; 1.01–18.00 min: gradient increase to 100% eluent B, flow rate increase to 2.2 mL min^{-1}; 18.01–20.00 min: 100% eluent B, flow rate 2.2 mL min^{-1}. The column oven was set at 60°C. The autosampler was operated at room temperature; the autosampler needle was rinsed using a wash vial filled with a mixture of eluent A and eluent B (90:10).

MS–MS conditions

For detection and quantification, the following ESI inlet conditions were applied: gas 1, nitrogen (90 psi; 620.5 kPa); gas 2, nitrogen (90 psi; 620.5 kPa); ion-spray voltage, 5,500 V; ion-source temperature, 750°C; curtain gas, nitrogen (10 psi; 68.9 kPa).

The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode with the following settings: collision gas was set at medium, the collision cell exit potential was 4.00 V, the dwell time was set at 15 ms. All other settings were analyte-specific and were determined using Analyst software in the quantitative optimization mode. The settings are summarized in Table 1. Q1 and Q3 were operated in unit resolution.

Extraction efficiency, matrix effects, and process efficiency

Extraction efficiencies, matrix effects, and process efficiencies were estimated in a post-extraction addition approach, as previously described [31, 32]. Three sets of samples were prepared. Samples set 1 consisted of neat standard containing the analytes at concentrations of 10 μg mL^{-1} in eluent B–eluent A (10:90). For preparation of samples set 2 (matrix effects), blank blood samples from 15 different sources were first extracted, as described previously, using 15 different blood samples (5× AM, 5× N-DEC, and 5× DEC). Then, the dry residues were reconstituted in 50 μL eluent A containing the analytes at a concentration of 10 μg mL^{-1}. For preparation of samples set 3 (extraction efficiency), blank blood samples from the same sources as those in set 2 were spiked with 50 μL eluent A containing the analytes at a concentration of 10 μg mL^{-1}. Thereafter, they were extracted as described previously, and the dry residues were reconstituted in 50 μL eluent A. Extraction efficiencies were estimated by comparison of the peak areas from the samples from set 3 with those from the corresponding samples of set 2 and reported as percentages. Matrix effects were estimated by comparison of the peak areas from the samples of set 2 with those from the corresponding samples of set 1 and reported as percentages. Hence, values below 100% indicate ion suppression whereas values above 100% indicate ion enhancement. Finally, process efficiencies (combination of extraction efficiencies and matrix effects) were estimated by comparison of the peak areas of the samples from set 3 with those from the corresponding samples of set 1.

Results and discussion

Determination of extraction efficiencies and matrix effects is a major part of LC–MS method validation according to international guidelines [13, 22, 23]. For detection of drugs in blood or plasma, sample cleanup is used to reduce the possibility of matrix effects. This study describes the comparison of commonly used LLE and SPE methods in terms of extraction efficiencies and matrix effects. Prior to mass spectrometric detection, the extracts were separated using HPLC. A representative chromatogram of the separation and detection is given in Fig. 1.

For some analytes, the concentrations selected to compare extraction efficiencies and matrix effects were not based on therapeutic concentrations. It is recognized that for postmortem blood some of these drugs will exhibit a high degree of redistribution resulting in concentrations exceeding those deemed to be therapeutic in ante mortem specimens.

Table 1 Analytes, multiple reaction monitoring (MRM) transitions, and the settings declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), and collision cell energy (CE) used in LC–ESI-MS–MS

Name	Q1 Mass	Q3 Mass	DP	EP	CEP	CE
9-OH Risperidone	427.0	207.2	61	4.5	18	39
		110.2	61	4.5	18	59
		69.1	61	4.5	18	75
Amisulpride	370.1	242.2	61	8.0	32	41
		195.9	61	8.0	32	55
		112.1	61	8.0	32	39
Aripiprazole	448.0	285.2	71	9.5	20	33
		176.1	71	9.5	20	43
		98.2	71	9.5	20	51
Buspirone	386.1	122.2	71	10.0	32	43
		79.0	71	10.0	32	105
		95.2	71	10.0	32	75
Chlorpromazine	319.1	86.1	46	5.0	14	31
		58.2	46	5.0	14	55
		246.1	46	5.0	14	33
Clozapine	327.1	270.2	51	4.5	30	29
		192.2	51	4.5	30	59
		164.1	51	4.5	30	95
Droperidol	380.1	123.1	41	5.5	16	63
		194.2	41	5.5	16	21
		165.1	41	5.5	16	39
Haloperidol	376.0	123.1	56	4.5	26	57
		165.2	56	4.5	26	35
		95.0	56	4.5	26	93
Olanzapine	313.1	256.2	56	4.5	14	31
		198.1	56	4.5	14	53
		84.2	56	4.5	14	33
Perphenazine	404.0	171.1	56	10.5	18	31
		143.2	56	10.5	18	39
		100.2	56	10.5	18	57
Pimozide	462.1	109.1	396	10.5	56	71
		328.3	396	10.5	56	33
		147.1	396	10.5	56	55
Promazine	285.1	86.2	46	4.5	34	27
		58.1	46	4.5	34	53
		180.1	46	4.5	34	51
Promethazine	285.1	86.1	36	4.5	32	27
		198.1	36	4.5	32	35
		71.2	36	4.5	32	57
Quetiapine	384.1	253.2	61	5.0	18	29
		221.3	61	5.0	18	53
		279.2	61	5.0	18	33
Risperidone	411.1	191.2	56	9.0	18	41
		110.2	56	9.0	18	69
		82.2	56	9.0	18	81
Sulpiride	342.0	112.2	66	4.5	40	37
		214.1	66	4.5	40	45
		84.1	66	4.5	40	57
Thioridazine	371.1	126.2	51	8.5	16	33
		98.3	51	8.5	16	47
		70.0	51	8.5	16	87
Trifluperazine	408.1	70.0	61	5.0	34	67
		113.2	61	5.0	34	39
		141.3	61	5.0	34	31

Table 1 (continued)

Name	Q1 Mass	Q3 Mass	DP	EP	CEP	CE
Zuclopenthixol	401.0	231.2	66	4.5	38	55
		221.1	66	4.5	38	69
		271.0	66	4.5	38	37

Comparison of different LLE procedures

Different conditions for LLE are described in the literature. Prior to extraction of drugs with an organic solvent, blood samples are usually adjusted to a certain pH value. Additionally, use of buffer solutions with a high salt content improves the extraction efficiency as the high salt content decreases the solubility of the drugs of interest in blood. This forces the partly lipophilic drugs to be transferred into the organic phase and therefore increases the extraction efficiency. For the LLE of antipsychotics, the blood is usually buffered to a slightly basic pH, as these drugs generally have basic properties and a basic pH decreases the solubility in the aqueous blood even further. The most common buffers used for the extractions of drugs and drugs of abuse are Trizma buffer [33], saturated sodium sulfate [34, 35], or solid sodium hydrogen carbonate. Typically, the most common organic solvents for the LLE of drugs and drugs of abuse include ethyl acetate, 1-chlorobutane [36–38], or the mixture diethyl ether–ethyl acetate (50:50) [34, 35]. We therefore compared the extraction efficiencies of 19 antipsychotics and LC–MS matrix effects using these solvents and buffers.

In the first experiment, the 19 antipsychotics were extracted from spiked whole blood samples at a concentration of 10 μg mL^{-1} each, using nine different combinations of buffers and organic solvents. Of all the buffers used in these experiments, the highest extraction efficiencies from ante-mortem blood samples were obtained using Trizma buffer, irrespective of the solvent used. The extraction efficiencies obtained when using neutral Na_2SO_4 were generally lower than those obtained with basic buffer, which suggests that antipsychotics are better extracted from blood samples using basic buffer.

For the majority of drugs, 1-chlorobutane gave the highest extraction efficiencies of the three different solvents

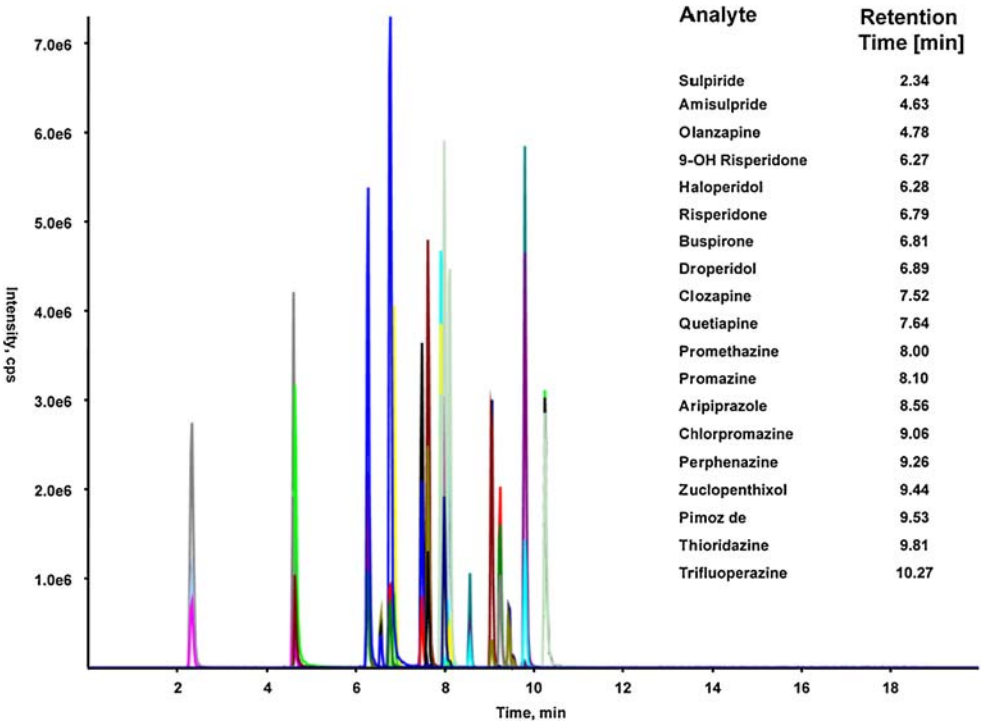

Fig. 1 Representative chromatogram of the separation and detection of 19 antipsychotics in blood using HPLC–MS–MS

Table 2 Comparison of extraction recoveries from ante-mortem blood (AM), non-decomposed post-mortem blood (N-DEC), and decomposed post-mortem blood (DEC) using liquid–liquid extraction and solid-phase extraction (n=5 for each combination)

Analyte	Liquid–liquid extraction (LLE)			Solid-phase extraction (SPE)		
	AM Mean [range]	N-DEC Mean [range]	DEC Mean [range]	AM Mean [range]	N-DEC Mean [range]	DEC Mean [range]
9-OH-Risperidone	74 [70–79]	74 [70–80]	74 [56–76]	45 [37–59]	41 [37–44]	56 [38–65]
Amisulpride	63 [55–67]	63 [62–64]	***62 [27–74]***	45 [39–60]	38 [34–43]	***61 [36–64]***
Aripiprazole	54 [51–61]	54 [43–60]	25 [16–43]	11 [8–19]	21 [17–29]	24 [9–30]
Buspirone	75 [65–79]	74 [68–76]	65 [63–80]	51 [44–56]	50 [48–55]	58 [48–66]
Chlorpromazine	60 [47–65]	61 [50–63]	***37 [33–78]***	18 [14–26]	28 [24–30]	23 [7–32]
Clozapine	74 [69–77]	72 [66–86]	67 [55–81]	40 [36–42]	44 [43–46]	43 [29–50]
Droperidol	65 [59–71]	66 [60–66]	47 [44–58]	32 [27–35]	31 [24–32]	31 [11–39]
Haloperidol	70 [70–73]	71 [64–76]	62 [56–64]	46 [29–48]	43 [40–48]	***44 [20–51]***
Olanzapine	68 [51–71]	64 [55–68]	***40 [0–87]***	34 [19–43]	39 [34–41]	39 [34–46]
Perphenazine	64 [60–67]	53 [52–74]	***36 [34–77]***	19 [8–25]	22 [20–26]	20 [4–31]
Pimozide	58 [51–71]	66 [51–83]	23 [10–36]	14 [7–26]	25 [22–31]	25 [11–33]
Promazine	63 [55–64]	61 [56–67]	***61 [44–84]***	29 [27–36]	36 [31–39]	36 [16–40]
Promethazine	64 [61–68]	62 [60–71]	55 [44–69]	39 [28–41]	38 [37–40]	***42 [19–45]***
Quetiapine	73 [71–76]	70 [65–77]	68 [58–78]	46 [41–50]	49 [45–51]	56 [44–58]
Risperidone	77 [76–79]	79 [76–87]	***78 [55–85]***	52 [46–56]	54 [50–57]	57 [53–63]
Sulpiride	6 [5–7]	7 [6–9]	7 [2–8]	42 [33–55]	33 [30–37]	49 [32–56]
Thioridazine	66 [56–68]	57 [52–68]	***30 [23–150]***	15 [11–28]	31 [26–34]	29 [8–40]
Trifluperazine	60 [52–63]	52 [46–60]	***25 [15–74]***	9 [4–18]	20 [18–24]	15 [1–25]
Zuclopenthixol	60 [58–68]	60 [42–69]	***30 [0–43]***	19 [5–25]	23 [22–30]	19 [4–32]

Table 3 Comparison of matrix effects from ante-mortem blood (AM), non-decomposed post-mortem blood (N-DEC), and decomposed post-mortem blood (DEC) using liquid–liquid extraction and solid-phase extraction (n=5 for each combination)

Analyte	Liquid-liquid extraction (LLE)			Solid phase extraction (SPE)		
	AM Median [range]	N-DEC Median [range]	DEC Median [range]	AM Median [range]	N-DEC Median [range]	DEC Median [range]
9-OH-Risperidone	82 [75–83]	83 [80–84]	81 [66–98]	78 [75–85]	83 [80–87]	81 [66–98]
Amisulpride	74 [70–78]	79 [74–82]	***83 [75–107]***	75 [70–76]	78 [76–79]	***91 [76–113]***
Aripiprazole	117 [107–121]	114 [99–123]	***80 [58–108]***	138 [123–143]	***153 [129–158]***	***125 [91–144]***
Buspirone	95 [94–103]	100 [95–103]	***108 [80–116]***	105 [101–112]	107 [106–109]	***109 [100–131]***
Chlorpromazine	116 [111–121]	107 [102–121]	***101 [25–155]***	138 [119–147]	145 [134–150]	***144 [120–167]***
Clozapine	81 [76–84]	86 [81–91]	***88 [27–131]***	88 [81–93]	91 [89–95]	***98 [90–126]***
Droperidol	122 [115–132]	126 [119–135]	116 [98–134]	134 [120–151]	148 [146–157]	***134 [111–145]***
Haloperidol	92 [91–97]	99 [88–100]	***85 [76–119]***	92 [84–99]	98 [96–101]	***102 [95–133]***
Olanzapine	86 [86–93]	97 [82–100]	***89 [0–102]***	87 [85–96]	96 [92–98]	91 [86–101]
Perphenazine	146 [141–155]	145 [128–155]	***125 [36–155]***	162 [156–172]	177 [167–186]	***138 [113–180]***
Pimozide	***112 [106–139]***	80 [77–100]	***82 [46–99]***	***125 [123–169]***	***145 [129–168]***	***127 [70–137]***
Promazine	100 [94–111]	98 [96–102]	***98 [24–143]***	105 [100–113]	111 [108–117]	***112 [105–137]***
Promethazine	82 [76–83]	81 [78–83]	***77 [24–124]***	81 [78–84]	87 [83–89]	***93 [80–143]***
Quetiapine	74 [71–74]	75 [74–78]	***77 [54–117]***	73 [71–76]	78 [75–79]	***95 [75–125]***
Risperidone	70 [69–72]	71 [69–73]	***73 [63–123]***	70 [70–70]	71 [69–72]	***87 [66–121]***
Sulpiride	95 [91–97]	96 [93–100]	***99 [90–136]***	100 [95–107]	104 [97–109]	***106 [98–130]***
Thioridazine	94 [93–96]	90 [88–95]	***77 [8–137]***	98 [91–104]	105 [100–107]	104 [92–104]
Trifluperazine	159 [148–167]	***145 [124–154]***	***101 [30–139]***	***180 [145–193]***	190 [176–197]	***157 [104–192]***
Zuclopenthixol	98 [96–106]	93 [87–98]	***74 [36–100]***	102 [86–110]	105 [100–110]	98 [85–103]

used. Sulpiride was the only antipsychotic which showed considerable lower extraction efficiency using 1-chlorobutane in comparison to the other solvents.

Considerable matrix effects in extracted blood samples were observed for olanzapine when using ethyl acetate as solvent. Otherwise, matrix effects were similar when using different buffers and solvents. However, it was observed that the use of 1-chlorobutane resulted in fewer matrix effects than other solvents.

Overall, LLE using Trizma buffer and 1-chlorobutane showed the best results in terms of extraction efficiencies and matrix effects. Therefore, this extraction method was chosen for further comparison of LLE and SPE.

Comparison of LLE and SPE

LLE extracts and SPE extracts of spiked AM, N-DEC, and DEC blood samples were compared. Five different blood samples from each group were used for this comparison.

Table 2 shows median values, standard deviations, and the range of extraction efficiencies using the LLE and SPE techniques. Data sets where the range is more than ±20% difference of the median value (not acceptable) are marked bold and underlined. With the exception of sulpiride, extraction efficiencies were comparable between LLE and SPE. For sulpiride extraction efficiencies were much lower (6% vs. 42% in AM samples) using LLE.

Comparison of AM, N-DEC, and DEC specimens shows that the median extraction efficiencies are comparable for most drugs, but the range of values determined in different DEC specimens varies. In comparison with SPE, this variation is more observable using LLE. The generally lower extraction recoveries for SPE for most analytes can be explained by the extraction procedure used. Blood from decomposed bodies can be viscous and with an oily consistency and often these samples block SPE cartridges. Despite use of extraction cartridges with a larger pore size, blockages or slow elution rates occurred using diluted blood. Therefore, all blood samples were diluted, ultrasonicated, and centrifuged prior to loading on to the SPE cartridge. One millilitre of a total volume of 1.5 mL of diluted blood samples was used to avoid blockages. As this reduced volume used for analysis was not corrected in calculations, results for SPE can theoretically not exceed 66.6% extraction efficiency.

Table 3 shows median values and the range of matrix effects using LLE or SPE. Data sets where the range is more than ±20% difference of the median value (not acceptable) are marked bold and underlined. With some exceptions, the variations of matrix effects over five different blood samples in the same group were acceptable for AM and N-DEC samples. The application of this experiment to DEC samples showed considerable variations over five different samples, even though the median value was still comparable with the value determined in AM and N-DEC samples. The variation in matrix effects between the DEC blood samples was slightly better using SPE compared with LLE, however, these variations were still more than 20% for most analytes. Because of these considerable differences between different blood samples, methods for detection of antipsychotic drugs in post-mortem material should also consider validation experiments in blank post-mortem blood. Experiments conducted in this research did not compare preservative agents or anticoagulants pertaining to collection tubes. Further studies may be necessary to define what contribution different collection tubes make to matrix effects and extraction efficiency. To our knowledge, this is the first publication describing the differences in matrix effects of different qualities of blood samples for antipsychotics.

Conclusion

The study presented here compares extraction efficiencies and LC–MS matrix effects for 19 antipsychotic drugs in AM, N-DEC, and DEC blood. The study shows that LLE and SPE methods in ante-mortem blood are generally comparable; both extraction methods show good and reliable extraction efficiencies and low matrix effects in these samples. However, the study also shows considerable differences between clinical and post-mortem blood from decomposed bodies in terms of extraction efficiency and LC–MS matrix effects. Therefore, methods for detection of antipsychotic drugs in post-mortem material should also consider validation experiments in drug-free post-mortem blood. We also suggest that validation experiments for post-mortem analysis methods should always include extraction efficiency and matrix effect studies in N-DEC and DEC samples.

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Appendix 3

Conference Oral Presentations

Orals presented at domestic and international conferences during candidature and deriving from the research included the following:

1. Saar, E., Beyer, J., Gerostamoulos, D. & Drummer, O. H. Application of LC-MS/MS Analysis for the Detection of Antipsychotics in Blood. Australasian Forensic Toxicology Meeting (AFTA): 12 - 14 October 2009. Melbourne, Australia.

2. Saar, E., Beyer, J., Gerostamoulos, D. & Drummer, O. H. Stability of Antipsychotic Drugs in Stored Blood Samples Paper 62, TIAFT: 29 August - 3 September 2010. Bonn, Germany.

3. Saar, E., Gerostamoulos, D., Drummer, O. H. & Beyer, J. The identification of a new olanzapine degradation product using high-resolution mass spectrometry. Paper 7, FACTA: 31 July - 3 August 2011, Melbourne, Australia*

4. Saar, E., Gerostamoulos, D., Drummer, O. H. & Beyer, J. A new degradation product of olanzapine. TIAFT, Paper 18, 28 - 30 September 2011, San Francisco, USA**

5. Saar, E., Beyer, J., Gerostamoulos, D. & Drummer, O. H. The time-dependent post-mortem redistribution of antipsychotic drugs. TIAFT, Paper 84, 3 - 8 June 2012, Hamamatsu, Japan

   * Awarded "Best Oral Presentation" by the FACTA board.
   ** Awarded with the "Young Scientist Award" by TIAFT as "Best Oral Presentation"