Review

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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

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	$^2H_2$–$^{13}C_2$–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)	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: $^2H_2$-$^{13}C_2$-risperidone and $^2H_2$-$^{13}C_2$-9OH-risperidone	SPE (10cc/130mg Bond Elut Certify)	3–μm C18 BDS–Hypersil column (100 × 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

Table 2. *(Continued)*

Author (Year)	Volume [ml]	Drugs	IS	Extraction	Column	Mobile Phase	Detection mode	Validation data
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

Table 3. *(Continued)*

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

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

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

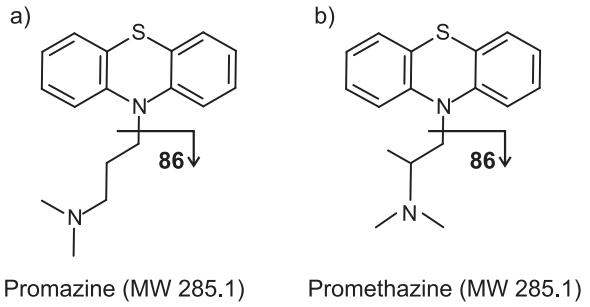

Figure 1. Structures of promazine (a) and promethazine (b), their molecular weights and the side-chain fragmen-tation resulting in the most abundant fragment for both compounds (m/z=86).

(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,

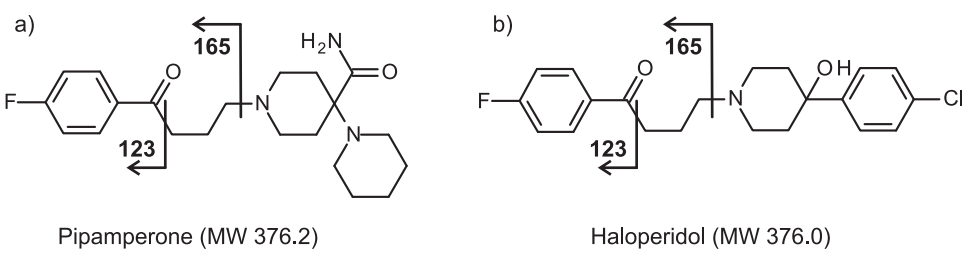

Figure 2. Structures of pipamperone (a) and haloperidol (b), their molecular weights and the fragmentations resulting in the two most abundant fragment for both compounds (m/z=123 and m/z=165).

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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