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Evaluation of electrospray ionisation liquid chromatography–tandem mass spectrometry for rational determination of a number of neuroleptics and their major metabolites in human body fluids and tissues

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Abstract

A study of liquid chromatography–triple quadrupole mass spectrometry (LC–MS–MS) with positive electrospray ionisation (ESI) for the determination of selected drugs in human tissues and body fluids such as blood, urine and hair is described. The possibility to screen for and quantify the 19 most commonly prescribed neuroleptics on the Swedish market and determine the presence of their major metabolites within a single LC–MS–MS analysis was evaluated on a PE Sciex API2000 instrument. Chromatographic conditions were optimised and the best separation, with individual retention times for most of the analytes, was obtained on a Zorbax SB-CN column within a 9-min gradient run. The MS–MS fragmentation conditions were optimised for each compound in order to obtain both specific fragments and high signal intensity. Since neuroleptics are a heterogeneous group of compounds, a markedly difference in collision energy needed to achieve fragments of the selected parent ions was seen and the number of fragments achieved varied as well. For sensitive quantification the transition of the most intense fragment of the protonated molecular ion $(M+1)^+$ was selected for multiple reaction monitoring analysis. More than 70 transitions were finally included in the assay. Detection levels down to the lower ng/ml level were achieved for all analytes, but between analytes more than a 10-fold difference in signal response was seen. By evaluation of extracted ion chromatograms from the analysis of authentic human blood, urine and hair sample the proposed concept for rational drug analysis was found to be both selective and sensitive for the neuroleptics included. A great number of metabolites could be determined in blood, urine and hair as well. A full method validation was not performed since the objective was to evaluate the method design rather than to validate a final method set-up.
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Keywords: Electrospray ionisation; Neuroleptics

1. Introduction

In forensic science the neuroleptics are of considerable interest because of their subject to abuse and their involvement in suicides and intoxication. Based on chemistry, pharmacokinetics and pharmacodynamics they form a heterogeneous group of compounds and could be divided in subgroups based on their chemical structures [1]. In this study 19 neuroleptics registered on the Swedish market were studied represented by phenthiazine derivatives such as chlorpromazine and fluphenazine; thioxantene derivatives such as, chlorprothixene and zuclopenthixol; butyrophenon derivatives such as melperone and haloperidol; dibensodiazepines such as clozapine and others [2], structures are shown in Fig. 1.

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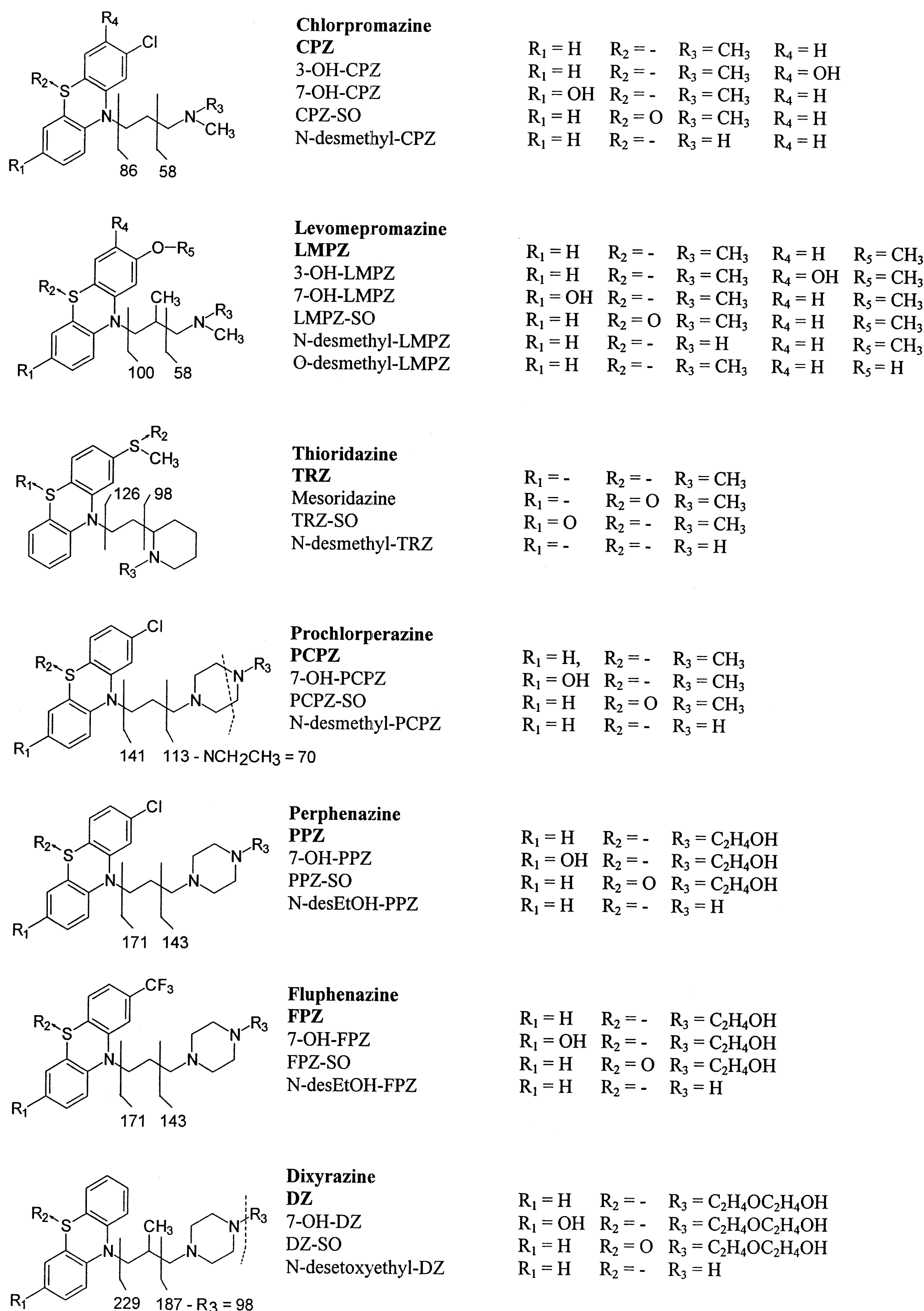


Fig. 1. Structures of 19 neuroleptics and their major metabolites arranged after their major structure elements. [a] Lines, proposed mechanism for the MS–MS CAD fragmentation.

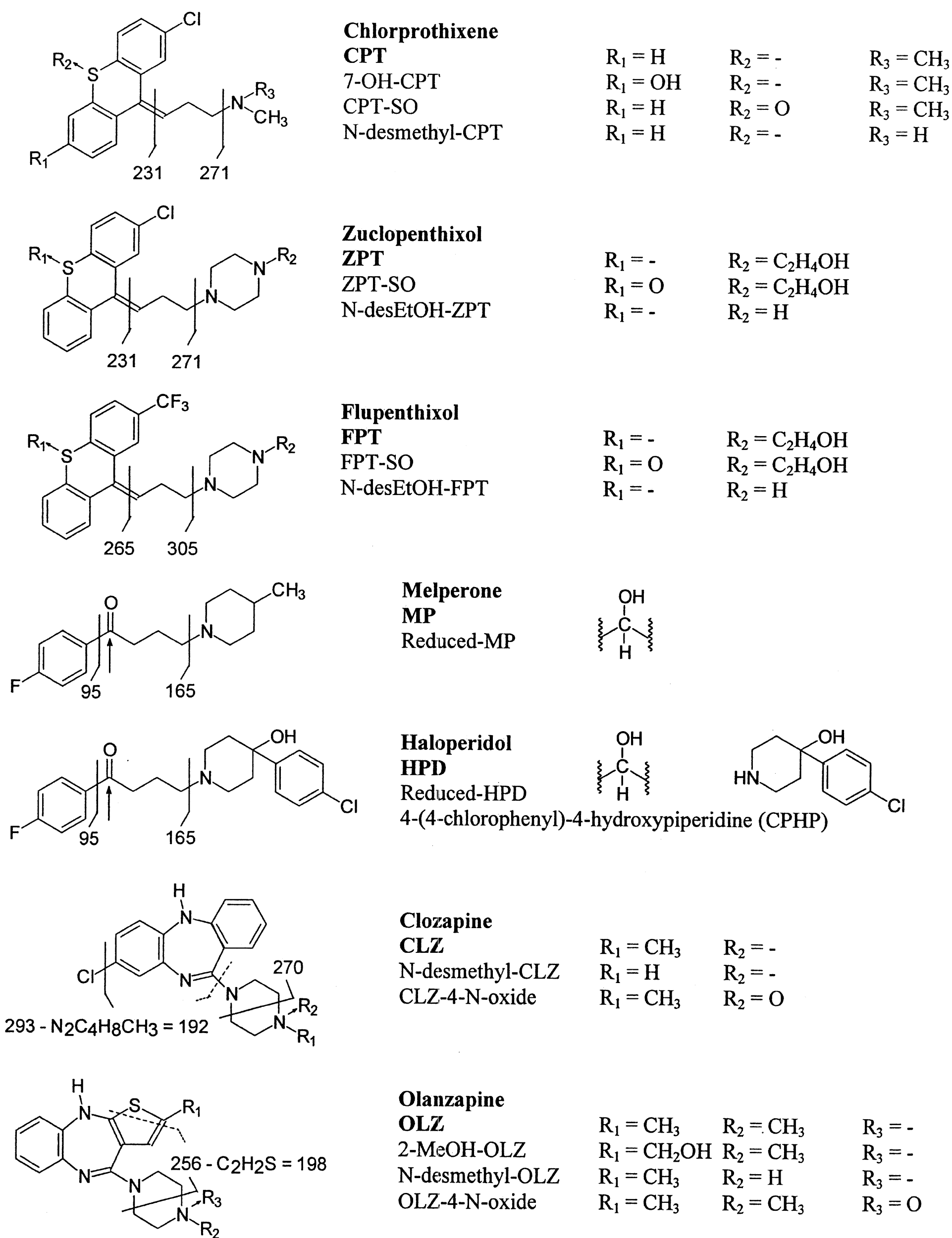


Fig. 1. (*continued*)

Gas chromatography–mass spectrometry (GC–MS) has for a long time been the golden standard in drug analysis within the forensic field. GC–MS methodology offers high sensitivity and high selectivity and generates mass spectra that can be used as fingerprints for the compounds. In recent years several new low-dosage neuroleptics have entered the market and have put further demands on already complex screening assays and thus alternative methods for analysis need to be investigated. Liquid chromatography–mass spectrometry (LC–MS) using electrospray (ESI) and atmospheric pressure chemi-

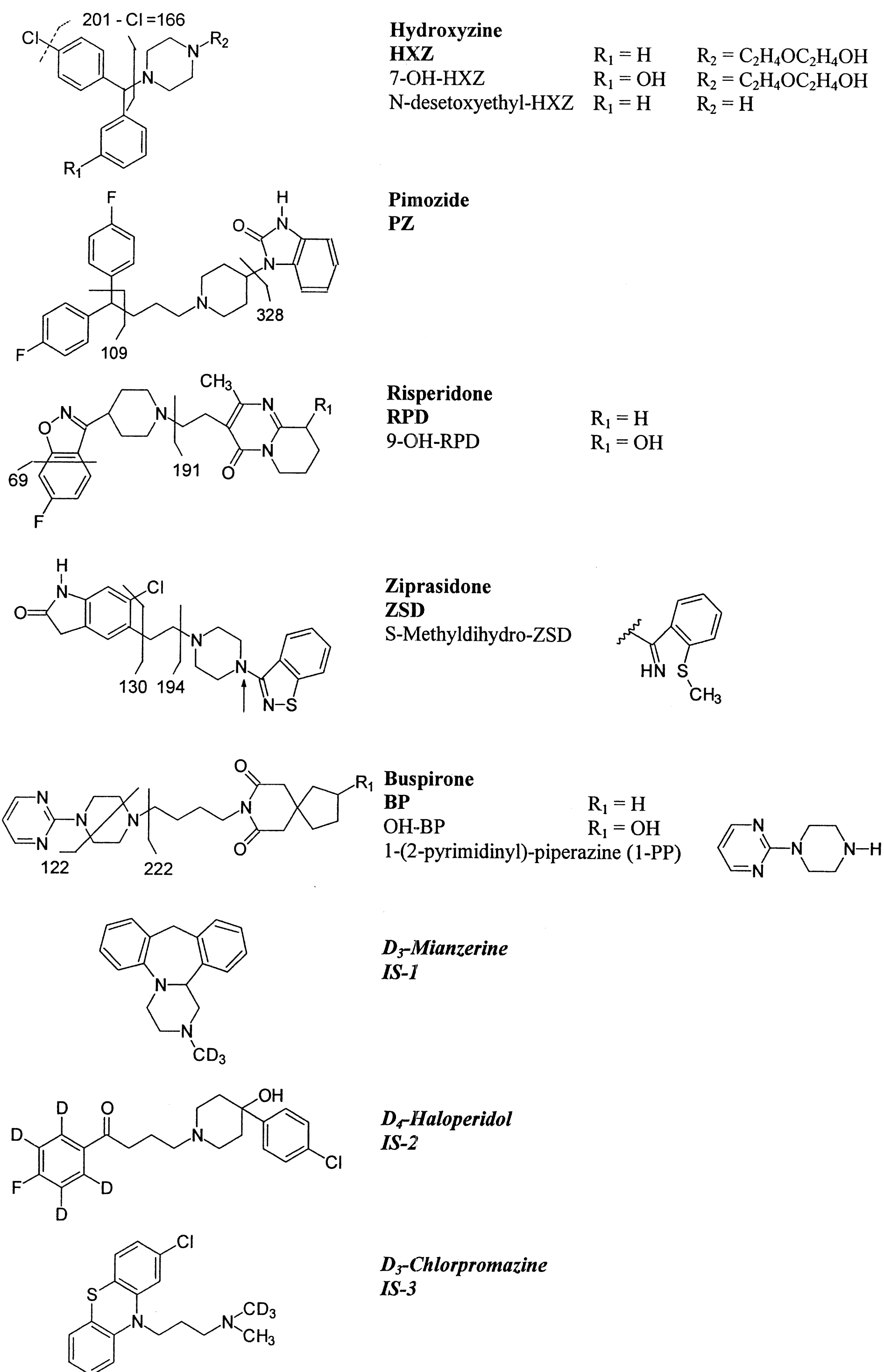


Fig. 1. (*continued*)

cal ionisation (APCI) are soft ionisation techniques, and enable MS analysis without prior derivatisation of the analytes, making LC–MS a powerful alternative to GC–MS [3]. Moreover, since LC–MS allows analysis of compounds with high molecular mass and/or very high polarity it has become useful for metabolite identification [4–6].

The application of LC–MS in the forensic field has recently been reviewed [3,4,7,8] but more extensive assays for determination of neuroleptics are rare [9–11]. However, LC–MS applications for the analysis of a single or a limited number of neuroleptics for clinical purposes have lately been described for several compounds [8–22]. In some studies the metabolism of the drugs in human has been more thoroughly evaluated with LC–MS and/or LC–MS–MS. The presence of different metabolites, their structures as well as their MS fragmentation has been described for buspirone [12,13], chlorpromazine [9], clozapine [14], flupenthixol [9], haloperidol [15], olanzapine [17], risperidone [9,20], ziprasidone [21]. These studies show that most of the neuroleptics are extensively metabolised to their glucuronides, sulfoxides, hydroxyls and dealkylated analogues. These evaluations give valuable information about the ionization and fragmentation of related compounds as well.

The aim of this study was to evaluate the possibility to develop a fast and sensitive LC–MS–MS assay for the selective screening and quantitation of a number of neuroleptics and determination of their major metabolites in various matrices such as human blood, urine and hair samples. The work was focused on method design of chromatography and mass detection while sample preparation and quantitation was more briefly evaluated.

2. Experimental

2.1. Materials

2.1.1. Chemicals and reagents

Acetonitrile and methanol, gradient grade, acetonitrile, methanol, butyl acetate, isopropanol and dichloromethane, all analytical-reagent grade, were purchased from Merck (Darmstadt, Germany). Formic acid (98%), acetic acid (100%), ammonia (21%) and sodium acetate, all analytical-reagent grade, were purchased from Merck as well. Ammonium formiate, analytical-reagent grade, was purchased from Fluka (Buchs, Switzerland) and *Escherichia coli* β-glucuronidase (68.6 U/mg 37 °C) was from Roche (Mannheim, Germany). Bond Elute Certify columns (130 mg/10 ml) were purchased from Varian (Middelburg, The Netherlands).

2.1.2. Reference compounds

Reference compounds were: buspirone (Bristol Mayers, Syracuse, NY, USA), dixyrazine and hydroxyzine (UCB, Brüssels, Belgium), fluphenazine and perphenazine (Schering Plough, Rathdrum, Ireland), flupenthixol, chlorprothixene, melperone and zuclopenthixol (Lundbeck, Copenhagen, Denmark), haloperidol, reduced haloperidol, pimozide and risperidone (Jansen, Beerse, Belgium), chlorpromazine, levomepromazine and prochlorperazine (Rhone Poulenc Rorer, Vitry-Alforville, France), clozapine, dm-clozapine and thioridazine (RBI, Natick, MA, USA), olanzapine (Lilly, Indianapolis, IN, USA), ziprasidone (Pfeizer, Ringaskiddy, Ireland).

Internal standards were: d_3-chlorpromazine, d_4-haloperidol and d_3-mianzerine (Cerilliant, Austin, TX, USA).

2.1.3. Solutions

Stock solutions of the reference compounds were prepared at 1.0 mg/ml in methanol. A stock solution of the 19 neuroleptics was prepared at 0.01 mg/ml in methanol. Working solutions for preparations of calibration standards were prepared at 1.0, 0.1 and 0.01 μg/ml in methanol by consecutive dilution of the mixed stock solution. Internal standard solutions of the three deuterated compounds were prepared at 4.0 μg/ml in methanol.

2.1.4. Samples

Authentic femoral blood and corresponding urine samples were obtained from post mortem cases. Samples where neuroleptics previously had been found in blood were selected. Corresponding urine and hair samples were obtained from psychiatric patients under steady state medication. In these cases the dosages of the drug were known.

2.2. Sample preparation

Urine samples (0.5 ml) were treated with 20 μl β-glucuronidase for 30 min at ambient temperature in order to hydrolyse the conjugated drugs and metabolites. In this study no pH adjustment was performed before hydrolysis. A mixture of urine–mobile phase buffer (1:1, v/v) was transferred to high-performance liquid chromatography (HPLC) vials for analysis on the LC–MS–MS system, without any further extraction or processing.

Blood samples were extracted on Bond Elute Certify solid-phase extraction (SPE) columns. The columns were conditioned before use with 2 ml of acetonitrile and 2 ml of 50 m*M* sodium acetate buffer, pH 6. Blood (1 g) and 25 μl internal standard solution were dissolved in 3 ml of buffer and treated with ultrasonic agitation for 15 min. After centrifugation at 5000 rpm for 10 min the supernatant was transferred to the SPE column. The solution was applied to the column with a gentle under pressure (<5 mmHg; 1 mmHg=133.322 Pa). The columns were then washed with 1 ml of a 0.1 *M* formic acid solution followed by 2 ml of a solution of 15% acetonitrile and 15% acetone in 0.1 *M* formic acid, and were then dried for 2 min. The samples were eluted with 2 ml of a solution of 2% ammonia in dichloromethane–isopropanol (80:20, v/v) into 10-ml glass tubes. The solvents were evaporated to dryness with N_2 at 40 °C in a TurboVap evaporation station. Samples were reconstituted in 100 μl of mobile phase A (see Section 2.5) and transferred to HPLC vials for analysis.

Unwashed hair samples (10–20 mg) were incubated for 15 min in 0.5 ml 1 *M* NaOH (40 °C) in a water bath with orbital shaking. After cooling the pH was adjusted to 9.5 with 25 m*M* Tris buffer. Extraction was performed with 2 ml of butyl acetate for 10 min and the organic phase was transferred to a new tube. A back-extraction into 200 μl of 1% formic acid was performed and the aqueous layer was then transferred to HPLC vials for analysis.

2.3. Calibration

For quantitation in urine, calibration standards were prepared in drug free urine by addition of 5, 10, 20, 50, 100, 200, 500 and 1000 ng/ml of the reference compound mixture. A mixture of urine–mobile phase buffer (1:1, v/v) were transferred to HPLC vials for analysis by the LC–MS–MS system without any further extraction or processing.

For quantitation in blood, calibration standards were prepared in drug-free blood by addition of 0.5, 1, 5, 10, 20, 50, 100, 200 and 500 ng/ml of the reference compound mixture. After addition of internal standard solution the samples were extracted on SPE columns as described above.

2.4. Instrumentation

The LC–MS–MS system used consisted of a Perkin-Elmer 200 chromatographic system equipped with two micro pumps, a solvent degasser and an autosampler with a 10-μl loop (Norwalk, CT, USA), and a Keystone Scientific Hotpocket column oven (Bellafonte, PA, USA). Mass detection was performed on a Sciex API 2000 triple quadrupole instrument equipped with a turbo ion-spray interface (PE Sciex, Ontario, Canada) operating in the positive ion mode. The interface probe was set at 350 °C and the ion-spray needle was operated at +5000 V. Nitrogen was used as nebulizer, auxiliary, curtain and collision activated dissociation (CAD) gas and was set at 25, 50, 30 p.s.i. and a value of 5, respectively (1 p.s.i.=6894.76 Pa). Infusion experiments were performed with the built-in Hamilton syringe pump at a flow-rate of 10 μl/min.

Instrument control, integration and calculation were performed with the personal computer-based PE Sciex software, Analyst 1.1.

2.5. Chromatography

HPLC was carried out on a Zorbax Stable Bond Cyano column 50×2.1 mm I.D., 3.5 μm particles (Rockland Technologies, USA) equipped with an Opti-Solv 2 μm column inlet filter (Optimize, Portland, OR, USA). The mobile phases consisted of methanol–acetonitrile–20 m*M* ammonium formiate buffer, pH 4, of 2:8:90 (v/v/v) for phase A and 24:36:40 (v/v/v) for phase B. A linear chromatographic gradient from 50 to 80% B-phase over 10 min was run including the time for reconditioning of

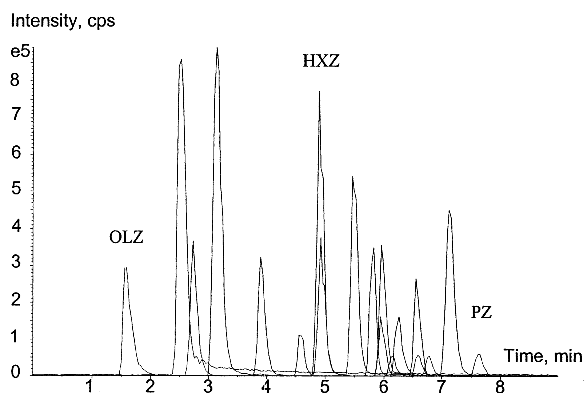


Fig. 2. Chromatographic separation of 19 neuroleptics on a Zorbax SB-CN column (50×2.1 mm I.D., 3.5 μm particles) with a linear gradient. An amount of 50 ng of each compound injected, MRM analysis of the most intense transition with a dwell time of 100 ms for each.

the HPLC column. A flow-rate of 0.25 ml/min at 20 °C was used. A reference chromatogram is shown in Fig. 2 and the retention times are shown in Table 1.

Table 1
Retention time for 19 neuroleptics and three deuterated internal standards

Compound	t_R (min)
Olanzapin (OLZ)	1.6
Melperone (MP)	2.5
Buspirone (BP)	2.7
Risperidone (RPD)	3.1
d_3-Mianzerine (I.S.-1)	3.7
Clozapine (CLZ)	3.9
Ziprasidone (ZSD)	4.6
d_4-Haloperidol (I.S.-2)	4.8
Haloperiodol (HPD)	4.9
Hydroxyzin (HXZ)	4.9
Levomepromazine (LMPZ)	5.5
Dixyrazine (DZ)	5.8
Perphenazine (PPZ)	5.9
d_3-Chlorpromazine (I.S.-3)	5.9
Chlorpromazine (CPZ)	6.0
Chlorprothixene (CPT)	6.2
Zuclopenthixol (ZPT)	6.2
Fluphenazine (FPZ)	6.6
Prochlorperazine (PCPZ)	6.6
Flupenthixol (FPT)	6.8
Thioridazine (TRZ)	7.2
Pimozide (PZ)	7.7

Chromatogram in Fig. 2.

2.6. Mass spectrometry

The detection and quantitation of the selected compounds were performed in the multiple reaction monitoring (MRM) mode. MS–MS product ion spectra were produced by CAD of the protonated molecule ion $(M+1)^+$. The most favourable transitions were selected and the instrument parameter settings were optimised individually for each reference compound by constant infusion at 10 μl/min of a 500 ng/ml solution.

The declustering potential (DP) for the molecular ions varied from 20 V for the lowest masses to 80 V for the highest. The collision energy (CE) needed for fragmentation ranged from 25 to 95 V. The optimum CE settings found for the selected transition are presented in Table 2. An MRM method was prepared including the two most intense transitions for each drug and if applicable two transitions for different metabolites. However, as seen in Fig. 1 and Table 2 one transition was often in common for a substance's hydroxyl and sulfoxide metabolites. The final MRM method included 75 transitions with a dwell time of 20 ms for each transition resulting in a total scan time close to 2 s. The signal intensity varied by a factor greater than 10 when the most intense transitions for each compound were compared. Transitions and MRM conditions for the metabolites included in this study were selected by combining information found from previous performed studies (cited in Table 2) and/or by comparison of related structures and their fragmentation and instrumental settings (Fig. 1, Table 2). These parameters were verified for several metabolites by analysis of authentic urine samples (Tables 4 and 5). An MRM method with 19 transitions (i.e., one for each drug) and a dwell time of 100 ms was used as well (Fig. 2). Reference MS–MS spectra were collected individually using flow injection analysis of 10 μl of the infusion solution at a flow of 250 μl/min (Fig. 3)

3. Results and discussion

3.1. Methodological aspects

In the forensic field there is a certain need for high accuracy in the identification of drugs in human body

Table 2
Selected transitions (Q1/Q3) for 19 neuroleptics and their major metabolites

Compound	Q1 (u)	Q3 (u)	CE (V)	RSI† (%)	Metabolite(s)	Q1 (u)	Q3 (u)	CE (V)	Refs.
Buspirone (BP)	386	222	38	5	OH-BP	402	122	37	[12,13]
	*386	122	37	45	1-PP	165	122	37	
Chlorpromazine (CPZ)	319	86	30	40	OH-CPZ, CPZ-SO	335	58	60	[9,23]
	*319	58	60	45	*N*-Desmethyl-CPZ	305	44	60	
Chlorprothixene (CPT)	316	271	25	20	OH-CPT, CPT-SO	*332*	*287*	*25*	[24]
	*316	231	40	90	*N*-Desmethyl-CPT	*302*	*271*	*25*	
Clozapine (CLZ)	*327	270	30	45	*N*-Desmethyl-CLZ	313	192	*50*	[11,14]
	327	192	60	20	CLZ-4-*N*-oxide	343	256	30	
Dixyrazine (DZ)	*428	229	32	45	OH-DZ, DZ-SO	*444*	*229*	32	-
	428	98	65	25	*N*-Desetoxyethyl-DZ	*340*	*141*	*32*	
Flupenthixol (FPT)	435	305	40	5	FPT-SO	451	281	40	[9,11,25]
	*435	265	50	10	*N*-desEtOH-FPT	391	265	50	
Fluphenazine (FPZ)	*438	171	35	30	OH-FPZ, FPZ-SO	*454*	*171*	*35*	[25]
	438	143	40	20	*N*-desEtOH-FPZ	*394*	*127*	*35*	
Haloperidol (HPD)	*376	165	30	50	Reduced-HPD	378	360	23	[10,15,16]
	376	95	95	15	CPHP	212	194	30	
Hydroxyzine (HXZ)	*375	201	25	100	OH-HXZ	*391*	*217*	*25*	–
	375	166	55	30	*N*-Desetoxyethyl-HXZ	*287*	*201*	*25*	
Levomepromazine	329	100	25	50	OH-LMPZ, LMPZ-SO	*345*	*58*	*60*	[27]
(LMPZ)	*329	58	60	70	*N*-Desmethyl-LMPZ	*315*	*44*	*60*	
					O-Desmethyl-LMPZ	*315*	*58*	*60*	
Melperone (MP)	*264	165	25	120	Reduced-MP	*266*	*248*	*20*	–
	264	95	70	40		*266*	*167*	*25*	
Olanzapine (OLZ)	*313	256	30	40	2-MeOH-OLZ	329	272	30	[17–19]
	313	198	60	–	*N*-Desmethyl-OLZ	299	198	50	
					OLZ-4-*N*-oxide	329	213	30	
Perphenazine (PPZ)	*404	171	32	20	OH-PPZ, PPZ-SO	*420*	*171*	*32*	[28]
	404	143	40	15	*N*-desEtOH-PPZ	*360*	*127*	*31*	
Pimozide (PZ)	462	328	40	5	Not included				[10]
	*462	109	70	10					
Prochlorperazine	*374	141	30	10	OH-PCPZ, PCPZ-SO	*390*	*141*	*30*	–
(PCPZ)	374	70	65	5	*N*-Desmethyl-PCPZ	*360*	*127*	*30*	
Risperidone (RPD)	*411	191	40	120	OH-RPD	427	207	40	[20]
	411	69	80	–					

Table 2. Continued

Compound	Q1 (u)	Q3 (u)	CE (V)	RSI† (%)	Metabolite(s)	Q1 (u)	Q3 (u)	CE (V)	Refs.
Thioridazine (TRZ)	*371	126	30	55	Mesoridazine, TRZ-SO	*387*	*126*	*30*	[11,29]
	371	98	50	50	*N*-Desmethyl-TRZ	*357*	*112*	*30*	
Ziprasidone (ZSD)	*413	194	40	15	*S*-Methyldihydro-ZSD	429	194	40	[21]
	413	130	80	–					
Zuclopenthixol (ZPT)	*401	271	35	10	ZPT-SO	417	287	35	[11,22]
	401	231	50	5	*N*-desEtOH-ZPT	357	271	35	

The selected optimum collision energy (CE) and the relative intensity (RSI) for the MRM signals.
Italics denote theoretically determined transitions for expected metabolites.
*, Transition with the most intense MRM signal.
†, Signal intensity compared with the signal for 375→201 (i.e., hydroxyzine).

fluids and tissues. However, the quality of the available samples varies and the number of xenobiotics present and their concentration within the samples are often unknown. Thus, rational methods with high selectivity and high specificity for verification of a drug intake are of paramount importance. Furthermore, an advantage would be if the same methodology could be used for samples from several matrices with minor variation in sample preparation.

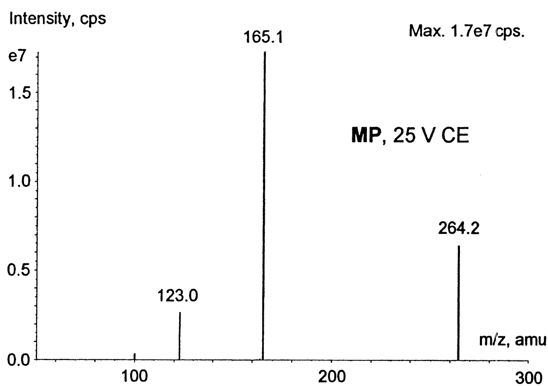
With the LC–MS technique this is theoretically possible to achieve. However, when analysing low concentrations of structurally very similar compounds in complex matrices there are some limitations with single quadrupole LC–MS analysis. Identification based only on the determination of the molecular ions is unreliable and co-determination of one or more specific fragment ions is recommended. However, since fragmentation often is limited in LC–MS and structure analogues often have fragments in common identification with high specificity can be difficult. In this study LMPZ and the OLZ metabolites 2-MeOH-OLZ and OLZ-4-*N*-oxide have equivalent molecular ions at 329 u (Table 2). Moreover, several other analytes have one or more fragments in common. One example is CPT, *N*-desmethyl-CPT, ZPT and *N*-desEtOH-ZPT that all have fragments at 271 u. In addition, CPT and ZPT are poorly separated in the present chromatographic system (Table 1).

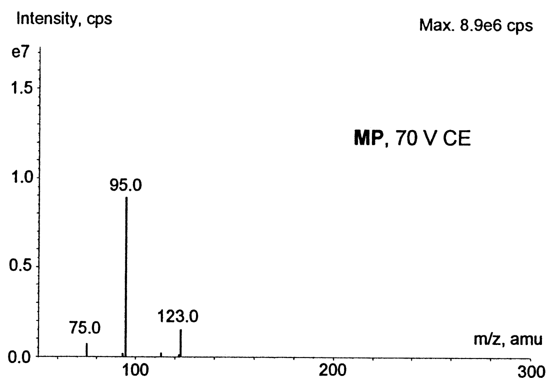
With LC–MS–MS though, the selection of a specific molecular ion and the subsequent isolated fragmentation to specific product ions gives much higher specificity compared with LC–MS and by MRM [tandem selected ion monitoring (SIM/SIM)] analysis it is possible to achieve high sensitivity allowing accurate quantitations at low concentrations. A risk for ion-suppression by artefacts such as other xenobiotics co-eluting with the analyte to be determined is however still true for LC–MS–MS [3], thus selective sample preparation and chromatography are still important. For direct injections of urine samples it is of certain importance to have a good separation of the analytes from the void in order to avoid ion-suppression from early eluting polar compounds and salts (Fig. 2). For blood samples, however there is a greater risk for ion-suppression of more non-polar compounds.

3.2. Chromatography

The chromatography of the pure drug substances was investigated in order to increase the selectivity of the method and reduce the risk for ion-suppression. Since access to pure standards for the metabolites was limited they were not included in this evaluation. The three Zorbax stable bond column materials octadecyl-, phenyl- and cyano-silica were studied. On the octadecyl material extensive peak tailing was seen even for early eluting analytes. The cyano-silica gave the best peak shape and the best selectivity for the 19 neuroleptics. However, owing to the limited resolution in liquid chromatography, baseline separation of all analytes was not possible to achieve within a reasonable elution time. The aim though, was to get individual retention times for a maximum number of peaks, which was achieved by

(A)





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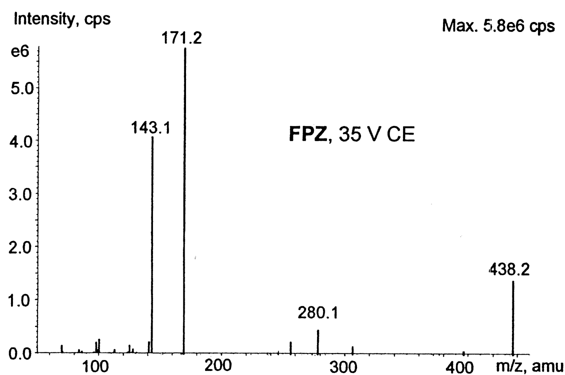
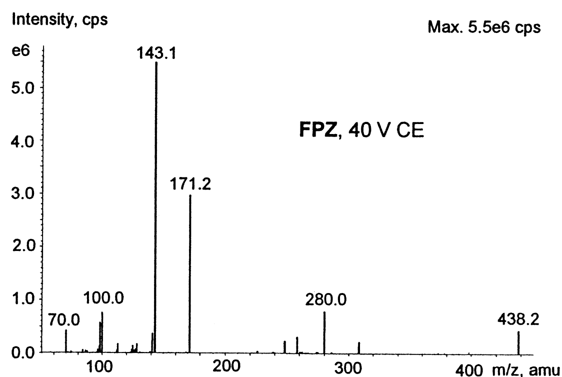
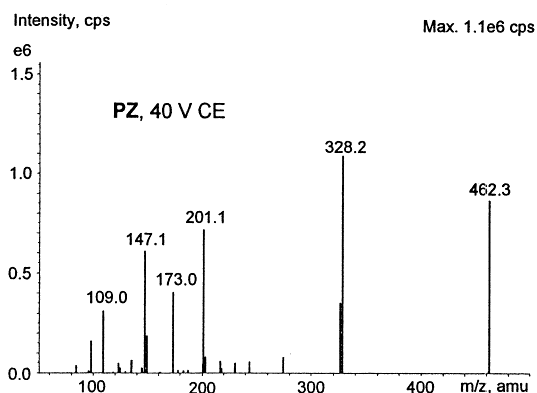




(C)



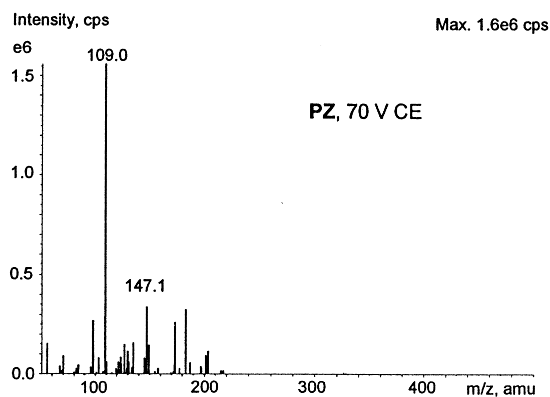


Fig. 3. Example of different MS–MS CAD fragmentation patterns for three neuroleptics; melperone (A), fluphenazine (B) and pimozide (C), at high and low collision energies (CEs). Product ion mass spectra obtained by collision induced dissociation of the protonated molecular ions $(M+1)^+$. Spectra obtain after flow injection analysis, 2 μg reference compound injected.

a gradient elution (Table 1, Fig. 2). Different chromatographic resolutions were found with methanol or acetonitrile as the organic modifier in the mobile phase and an optimum chromatography was achieved with a gradient run using a combination of the two solvents and by changing their ratio over time. Furthermore, by changing from a standard sized narrow bore column (150×2.0 mm I.D., 5.0 μm) to a shorter column with smaller silica particles (50×2.0 mm I.D., 3.5 μm) the time for elution was reduced from nearly 20 to less than 10 min without any loss of resolution. This clearly reduced the sample turnover making the assay more suitable for rational routine analysis. When analysing authentic samples the chromatography was found to work well for the determination of the metabolites as well. Some of the metabolites eluted early in the chromatogram and most were well separated from their corresponding parent compounds (Fig. 4). This was also true for metabolites with the same masses e.g., OH-LMPZ and LMPZ-SO.

3.3. Mass spectrometry

The analytes studied could be ionised with the electrospray interface and formed stable protonated molecular ions $(M+1)^+$. No adducts were observed but some relatively unstable $(M+1)^+$ (e.g., ziprasidone) were to some extent fragmented up-front, in the interface, thus giving lower MS–MS signals compared with the other analytes.

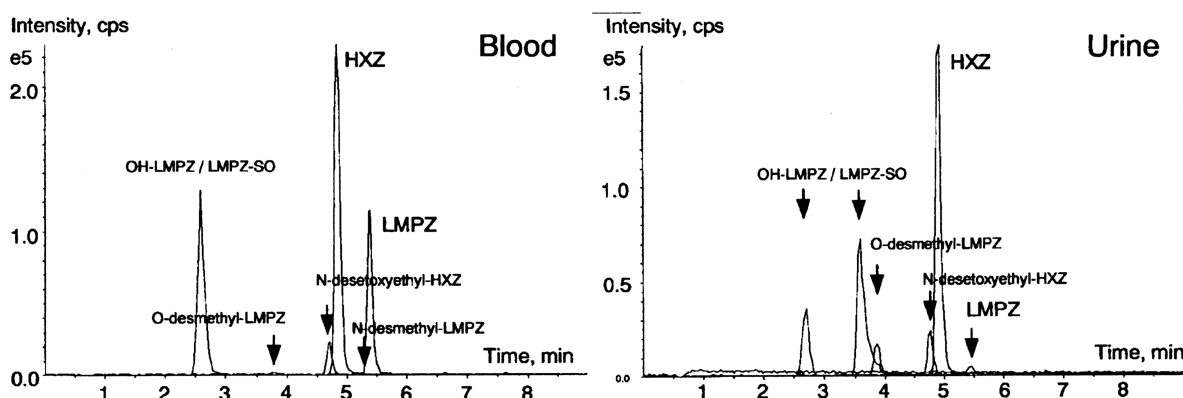
With respect to specificity and confirmation of analyte identity the formation of more than one specific fragment is favourable. However, our results show the difficulties in achieving more extensive fragmentation and also that it often results in lower signal and lower sensitivity for the analyte. The pattern of in-source CAD fragmentation, in the collision cell, for the neuroleptics in this study could be divided into three major groups: (1) analytes that were easily fragmented (20–40 V CE) and gave one product ion with a relatively high mass, e.g., HXZ, MP and RPD (Fig. 3A). (2) Analytes that needed a higher electron voltage for fragmentation (30–50 V CE) causing two or more fragments where the most intense product ion often was of a low mass, e.g., FPZ and PPZ (Fig. 3B). (3) Analytes that needed a comparable high voltage for fragmentation (CE>50 V) giving a number of fragments with relatively low masses and low intensity, e.g., FPT and PZ, (Fig. 3C). These factors resulted in a 10-fold difference in analyte sensitivity when transitions with the highest intensities were selected (Table 2). Moreover, a requirement of two transitions for confirmation of identity would considerably deteriorate the limit of detection for several analytes, e.g., CPT, HXZ and MP and would be hard to achieve for others, e.g., RPD and ZSD. A considerable increase in dwell time (e.g., from 20 to 100 ms) for a selected transition in order to increase sensitivity showed no or little effect on the signal intensity but resulted in less baseline noise. Since the effect on the sensitivity was low, the same dwell time was used for all transitions. An alternative to the use of additional transitions for confirmation of analyte identity could be the codetermination of one or more metabolites since they often are present at high concentrations, and have different elution time and other ion masses than their parent compounds.

3.4. Quantitation

A 10-fold difference in dosage between individuals is usual for many of the neuroleptics and between compounds a 100-fold difference in dosage is seen (Table 3). A great variation in blood, hair and urine concentration would thus be expected in authentic samples. Consequently a more sensitive assay would be required for some of the low dosage compounds (e.g., FPT, RPD and HPD) while the sensitivity most likely would be less critical for others (e.g., CPT, MP and CLZ). Moreover, the expected concentrations would be markedly lower in the blood samples compared with the urine samples. Thus the blood samples in this study were concentrated during sample preparation (Section 2.2).

In order to evaluate the limitations of the suggested assay the quantification of the 19 neuroleptics was briefly studied. A full method validation was not performed since the objective was to evaluate the method design rather than to validate a final method set-up.

After dilution in buffer urine samples were injected directly on the LC–MS–MS system, thus no internal standards were needed to compensate for losses during processing. The limit of quantitation



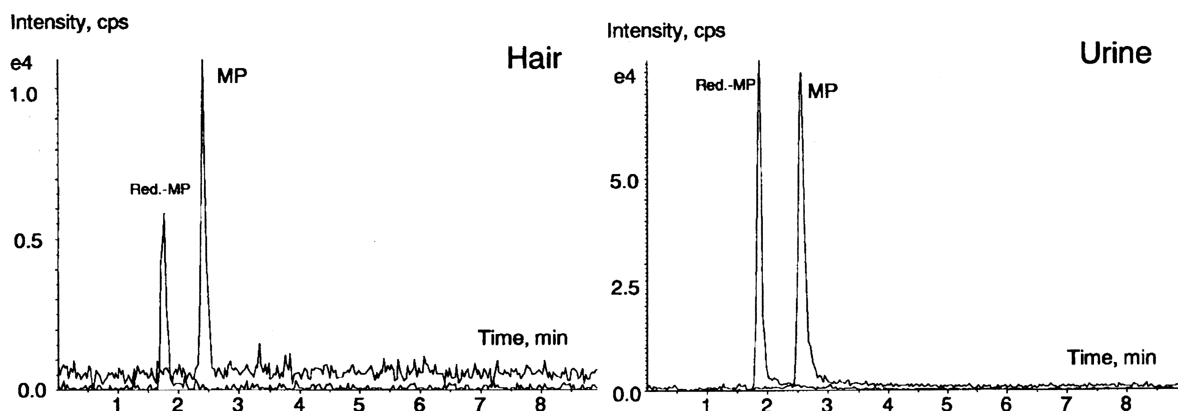


Fig. 4. Overlay of some extracted ion chromatograms (XIC) from analysis of authentic urine, blood and hair samples analysed with MRM. [a] Urine and blood extracts from post mortem samples, case C01 in Table 4. [b] Urine and hair extracts from patient samples, sample S05 in Table 5.

(LOQ) and working range for quantitation were briefly studied by analysis of urine standards spiked with the 19 neuroleptics in the concentration range 1–1000 ng/ml and when using quadratic curve fit a good correlation between signal intensity and concentration was achieved with $r \geq 0.998$ for all analytes. The lowest standard used for calibration varied between analytes depending on their LOQ (Table 3). The limit of quantification was estimated by evaluations of the extracted ion chromatograms (XIC). Five replicates of the lowest standards in the calibrations were analysed. With a definition of LOQ as a signal-to-noise ratio (S/N) greater than 10 and a relative standard deviation better than 20% the LOQ ranged from 2 ng/ml for RPD to 50 ng/ml for PZ (Table 3). As expected the method was most sensitive for the analytes with one major intense fragment (e.g., RPD) and least sensitive for the analytes fragmented to several less intense fragments (e.g., PZ).

For blood samples more extensive sample preparation (SPE) with a pre-concentration of the extracts was needed. Since several steps of volume transfers

Table 3
Usual dosage range (Rng) for 19 neuroleptics and their relative MRM signal intensity (RSI), limit of quantification (LOQ) and calibration range in human blood and urine samples

Compound	Dose Rng		CE (V)	RSI (%)	LOQ		Calibration Rng	
	Min (mg)	Max (mg)			Blood (ng/g)	Urine (ng/ml)	Blood (ng/g)	Urine (ng/ml)
Risperidone (RPD)	1	8	40	**120**	<0.5	2	0.5–500	1–500
Flupenthixol (FPT)	1	15	50	*10*	10	30	5–500	10–1000
Haloperidol (HPD)	1	15	30	50	2	10	1–500	5–500
Pimozide (PZ)	2	12	70	*10*	10	50	5–500	20–500
Fluphenazine (FPZ)	2	20	35	30	5	15	5–500	10–1000
Olanzapine (OLZ)	5	20	30	40	5	10	5–500	5–1000
Perphenazine (PPZ)	8	32	32	*20*	5	5	5–500	5–500
Buspirone (BP)	10	30	37	45	1	10	0.5–500	5–1000
Zuclopenthixol (ZPT)	10	50	35	*10*	10	10	5–500	10–500
Dixyrazine (DZ)	20	50	32	45	2	5	1–500	5–500
Hydroxyzin (HXZ)	20	150	25	**100**	2	10	1–500	5–1000
Ziprasidone (ZSD)	20	160	40	*15*	1	10	0.5–500	10–500
Prochlorperazine (PCPZ)	30	150	30	*10*	10	20	10–500	10–1000
Levomepromazine (LMPZ)	25	400	60	70	2	10	1–500	10–1000
Melperone (MP)	25	400	25	**120**	1	5	0.5–200	5–1000
Chlorprothixene (CPT)	50	400	25	*20*	5	20	5–500	10–1000
Clozapine (CLZ)	150	450	30	45	2	10	1–500	10–1000
Thioridazine (TRZ)	150	600	30	55	5	10	5–500	5–1000
Chlorpromazine (CPZ)	200	800	60	45	2	10	1–500	10–1000

Dose Rng, usual p.o. dosage interval. Based on data from; Baldessarini [1] and Hedstrand [2].
RSI, Relative signal intensity compared with the MRM signal for hydroxyzine. Bold for high and italic for low signal intensity.
LOQ, roughly estimated from calibration samples. For urine samples based on replicated direct injections and for blood samples on single injections of extracted samples.

were done an internal standard mixture was added before sample preparation. The mixture consisted of d_3-mianzerine, d_4-haloperidol and d_3-chlorpromazine. For most of the compounds a linear fit was applicable and a good correlation between signal intensity and concentration was achieved with $r \geq 0.995$. The LOQ and working range for quantitation was briefly studied by analysis of blood standards spiked with the 19 neuroleptics in the concentration range 0.5–500 ng/ml.

For blood samples the LOQs were roughly estimated from the lowest standards in the calibration curve with a signal to noise greater than 10. No estimation of extraction recovery was made. The LOQs ranged from <0.5 ng/ml for RPD to 10 ng/ml, e.g., PZ (Table 3).

No calibration was made for hair, thus the hair samples were only qualitatively evaluated in this study.

This study showed that the LOQ for RPD, that has a high MRM signal for the major fragment, was sufficient for blood and urine quantification (C11, Table 4 and S01, Table 5). However, the determination of trace concentrations of FPT and PZ in blood, might be more problematic since the signal intensities were more than 10-times less than for RPD (Table 3). For other compounds with a low MRM signal, e.g., ZSD, CPT and PCPZ this might not be true since their dosage is markedly higher and subsequently the expected blood concentrations would be higher (C07, Table 4). A more extensive evaluation of authentic sample with neuroleptics at therapeutic and sub-therapeutic concentrations is needed for a complete validation of the power of the assay.

3.5. Metabolite determination

Glucuronidated metabolites of the neuroleptics are often found in urine. In order to achieve the free fraction of the compounds the authentic urine samples in this study were hydrolysed with β-

Table 4
Neuroleptics found in samples from human post mortem autopsy

Case	Compound	Concentration		Metabolites found in blood	Metabolites found in urine
		Blood (μg/g)	Urine (μg/ml)		
C01	Hydroxyzine	0.4	>1.0	*N*-Destoxyethyl-HXZ	*N*-Destoxyethyl-HXZ
	Levomepromazine	>1	0.05	*OH-LMPZ/LMPZ-SO *N*-Desmethyl-LMPZ *O*-Desmethyl-LMPZ	*OH-LMPZ/LMPZ-SO *O*-Desmethyl-LMPZ
C02	Thioridazine	0.7	0.9	*Mesoridazine/TRZ-SO *N*-Desmethyl-TRZ	*Mesoridazaine/TRZ-SO *N*-Desmethyl-TRZ
	Perphenazine	0.02	NF	*N*-desEtOH-PPZ	NF
C03	Buspirone	0.2	0.07	NF	NF
	Melperone	0.6	0.6	Red.-MP	NF
	Levomepromazine	0.06	0.03	*OH-LMPZ/LMPZ-SO *N*-Desmethyl-LMPZ	†*OH-LMPZ/LMPZ-SO *N*-Desmethyl-LMPZ *O*-Desmethyl-LMPZ
C04	Hydroxyzine	0.7	0.3	*N*-Destoxyethyl-HXZ	NF
	Haloperidol	0.01	NF	Red.-HP	NF
C05	Dixyrazine	NA	0.5	NA	*OH-DZ/DZ-SO *N*-Destoxyethyl-DZ
C06	Clozapine	0.2	0.4	*N*-Desmethyl-CLZ	*N*-Desmethyl-CLZ CLZ-4-*N*-oxide
C07	Perphenazine	0.2	0.004	**N*-desEtOH-PPZ OH-PPZ/PPZ-SO	OH-PPZ/PPZ-SO
	Chorprothixene	0.03	NF	*N*-Desmethyl-CPT	*N*-Desmethyl-CPT
	Chlorpromazine	0.01	NF	NF	NF
C08	Flupenthixol	0.02	NA	NF	NA
C09	Chlorprothixen	1.0	0.3	*N*-Desmethyl-CPT	*N*-Desmethyl-CPT
	Chlorprothixen	0.01	NF	CPZ-SO	NF
C10	Zuclopenthixol	0.03	NA	*N*-desEtOH-ZPZ	
C11	Risperidone	0.002	0.004	OH-RPD	OH-RPD
C12	Olanzapine	0.4	NF	**N*-Desmethyl-OLZ 2-MeOH-OLZ	*N*-Desmethyl-OLZ
C13	Chlorpromazine	0.1	0.6	*CPZ-SO *N*-Desmethyl-CPZ	*OH-CPZ/CPZ-SO *N*-Desmethyl-CPZ

NA, Not available.
NF, Not found.
*, The most intense MRM response.
†, More than one peak determined (several metabolites present).

Table 5
Neuroleptics found in samples from patients during steady-state medication

Sample	Compound (oral dosage)	Found hair	Concentration urine (μg/ml)	Metabolites found in hair	Metabolites found in urine
S01	Clozapine (375 mg)	Yes	0.2	*N*-Desmethyl-CLZ, trace	*N*-desmethyl-CLZ CLZ-4-*N*-oxide
	Risperidone (8 mg)	Yes	NF	OH-RPD	NF
S02	Zuclopenthixol (NA)	Yes	0.05	*N*-desEtOH-ZPT, trace	NF
	Levomepromazine (100 mg)	NF	0.04	OH-LMPZ/LMPZ-SO	†OH-LMPZ/LMPZ-SO *N*-desmethyl-LMPZ **O*-Desmethyl-LMPZ
S03	Haloperidol (7 mg)	NA	0.07	NA	Red.-HP
S04	Olanzapine (30 mg)	NA	0.4	NA	**N*-desmethyl-OLZ OLZ-4-N-oxide
	Levomepromazine (50 mg)	NA	NF	NA	NF
S05	Melperone (150 mg)	Yes	0.1	Red.-MP	Red.-MP
	Flupenthixol (7 mg)	NF	NF	NF	NF

NA, Not available.
NF, Not found.
*, The most intense MRM response.
†, More than one peak determined (several metabolites present).

glucuronidase for at least 30 min before analysis. However, the time for hydrolysis was not optimised for each drug individually. Repeated injections after more than 12 h indicate that the time for hydrolysis was sufficient but for single analytes a longer time than 30 min for hydrolysis might be recommended (e.g., C03, Table 4) [13,21].

The transition for at least one major metabolite for each drug was included in the MRM method. The selection was based on previous findings [9–29]. For some neuroleptics however more than two metabolites could be determined since several of their metabolites have transitions in common, e.g., 3-OH-PPZ, 7-OH-PPZ and PPZ-SO (Table 2, Fig. 1). However, due to limited access of pure references of the metabolites it was not possible to verify these transitions, optimise the instrument parameter settings and estimate their relative signal intensity. Thus the interpretation of the metabolite pattern must be based on experience of the analysis of a number of authentic samples and the comparison with previous findings. Moreover, comparison of the relative concentration of different metabolites within a single sample cannot be based on the peak area since the signal intensity for the actual transitions can vary 10-fold as shown in Table 2.

All the neuroleptics included in method except BP, PZ, PCPZ and ZSD were identified in authentic blood samples and several of their metabolites were seen in blood as well as in corresponding urine samples (Table 4). Some of the neuroleptics and their metabolites were also identified in hair (Table 5). The findings in case 01 (Fig. 4) show the power of co-determination of metabolites. Several metabolites were found that strengthens the confirmation of the drug intake and could give additional information on the time for, route and amount of the intake. In blood LMPZ and the three metabolites OH-LMPZ or LMPZ-SO (not verified), *N*-desmethyl-LMPZ and *O*-desmethyl-LMPZ were found, while the three metabolites OH-LMPZ, LMPZ-SO and *O*-desmethyl-LMPZ were found in urine.

3.6. Method design and application

The proposed concept with a rapid and selective chromatography in combination with an extensive and selective MRM method gave the flexibility needed for different applications. The methodology could easily be adapted to different demands depending on the sample matrices to be analysed. However, for blood and hair analysis where the presence of metabolites are limited or have less interest the number of transitions can be reduced. Instead, the

measurement of an additional transition for accurate identification can be more valuable. Furthermore, fewer transitions in total admit longer dwell time settings that may increase signal-to-noise ratio and enhance the limit of quantification. The more extensive sample preparation needed for blood and hair analysis requires the addition of an internal standard to obtain accurate quantitations. Ideally the corresponding deuterated analogue is used, but when a number of analytes are included in the assay this will not be cost effective. Moreover, few deuterated analogues of the neuroleptics are commercially available. If all 19 neuroleptics are included in the method it is recommended to use a limited number of internal standards with different polarity. In this study deuterated analogues to analytes early, in the middle and in the end of the chromatogram were found suitable (Table 1 and Fig. 2). For drug screening in urine on the other hand, the possibility to analyse several different metabolites is of greater interest while the need for measurement of several transitions for each analyte is questionable. Since the urine samples can be injected directly without extensive sample preparation there is no need for internal standards.

Finally, the proposed concept can be used for initial screening followed by quantitation. Then a set of individually designed MRM methods can be used for the quantitation. The same instrumentation and chromatography can be used for this second injection of the sample allowing rational analysis.

4. Conclusions

This study proves that LC–MS–MS with gradient chromatography is a powerful tool for the rapid, sensitive, and selective screening for a great number of drugs and their metabolites. By using MRM analysis all relevant information could be achieved within a single run. The same methodology might be used both for screening and quantitation. The possibility to co-determine one or several metabolites, strengthen drug identification notably and increases the amount of information for interpretation of forensic cases.

Furthermore, the development and evaluation of selective screening methods for a number of structurally related analogues gives valuable information on the selectivity and the specificity of the method as well. However, a rational selection of analytes included is recommended in order to keep the complexity of the method manageable.

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