Application note

Drug Testing and Analysis

Received: 21 March 2011 Revised: 5 May 2011 Accepted: 6 May 2011 Published online in Wiley Online Library: 13 September 2011

(www.drugtestinganalysis.com) DOI 10.1002/dta.303

Toxicological screening of basic drugs in whole blood using UPLC-TOF-MS

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Ultra performance liquid chromatography (UPLC) coupled with time-of-flight (TOF) mass spectrometry (MS) was established for toxicological screening of basic drugs in whole blood and tested on authentic samples. Whole blood samples (0.2 ml) were extracted using a Gilson apparatus equipped with Bond Elut Certify columns. Screening was performed for 175 compounds (psychotropic, cardiovascular, designer, and abused drugs). The drugs were separated in 15 min using a UPLC system (Waters ACQUITY BEH C18, 1.7 μm, 2.1 mm × 100 mm column) coupled to an LCT Premier XE (Waters) instrument. Data were processed by ChromaLynx XS using identification criteria of ±0.2 min retention time, and ±5 mDa mass tolerance. Whole blood was spiked with the 175 compounds in concentrations from 5–100 μg/kg to assess approximately the lowest concentrations that could be identified. This method was further applied to 119 samples from forensic investigations, leading to 302 hits, of which 291 (96%) were subsequently verified, in concentrations exceeding the lower limit of quantification (LLOQ), by a liquid chromatography (LC)-MS/MS confirmation method. In conclusion, this UPLC-TOF-MS method is a useful and effective screening method for basic drugs in whole blood.

Keywords: UPLC-TOF-MS; ChromaLynx XS; whole blood; SPE; toxicological screening.

Introduction

Various approaches may be used for screening of drugs in the area of forensic toxicology. Often a combination of procedures is used, for example, a combined approach based on gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) with UV-diode-array detection, and immunoassays.[1] Although such combined procedures may provide reasonable results, there are several drawbacks. The sensitivity of GC-MS is moderate, unless derivatization is carried out, which makes the procedure more cumbersome. Also, the sensitivity and specificity of LC-UV procedures are limited. Immunoassays can only be used for common drugs, where commercial antibodies are available. In order to carry out screening in a more efficient and rational way, there has been increasing interest in using methods based on liquid chromatography-mass spectrometry (LC-MS).[2,3] These procedures may encompass drugs with a wide range of polarity, and so in a single analytical step it is possible to accomplish a very broad screening for compounds. LC-ion trap MS/MS^n methods have been presented for screening of several hundred drugs.[4–6] More recently there has been increased interest in LC-time-of-flight (LC-TOF) procedures, which have the potential for screening of a very large number of drugs based on estimation of the accurate mass of the molecules.[7–10] In forensic toxicology, LC-TOF methods have mainly been used to screen urine, whereas screening of blood has been less studied. In many contexts (such as traffic cases), however, the primary interest lies in detecting compounds in blood; drug effects are related to their concentrations in blood, suggesting that blood screening may be preferable in many cases. Here we present an ultra performance liquid chromatography (UPLC-TOF-MS) screening method for basic drugs in whole blood. This represents an extension and improvement of previously presented methods for measurement of compounds in urine and hair.[11,12]

Materials and methods

Chemicals and reagents

Some of the reference compounds, and the internal standard, diazepam D5, were obtained from Lipomed GmbH (Bad Säckingen, Germany); others were gifts from pharmaceutical companies. All chemicals were analytical reagent grade. LCMS-grade acetonitrile and methanol were obtained from Fisher Scientific UK (Loughborough, UK), and formic acid 98–100% GR for analysis was obtained from Merck (Darmstadt, Germany). Purified water was generated from a Millipore Synergy UV water purification system (Millipore A/S, Copenhagen, Denmark). Acidic water (0.1% formic acid in water) was prepared and stored at 4 °C. Blank human whole blood was obtained from the Blood Bank at Copenhagen University Hospital (Copenhagen, Denmark) and preserved by adding 1% sodium fluoride. The blank human blood was used for negative controls, blanks with internal standard, and spiked samples. Authentic forensic samples of whole blood were preserved with 1% sodium fluoride.

Preparation of standard solutions

The deuterated internal standard and all other reference standards that were supplied as solid material were initially dissolved according to the recommendations of the manufacturer at concentrations of either 100 mg/l or 1000 mg/l in methanol or

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Table 1. Lowest identification concentration (μg/kg) and retention time (min) for compounds in spiked whole blood samples

Compound	Conc	RT	Compound	Conc	RT	Compound	Conc	RT	Compound	Conc	RT	Compound	Conc	RT
2C-B	5	4.52	Chlorprothixene	50	6.18	Flunitrazepam	5	6.16	Medazepam	5	5.45	PMA	50	2.91
2C-I	10	4.92	Cinnarizine	100	6.57	Flunitrazepam, 7-amino-	5	4.02	MeOPP	50	2.59	PMMA	5	3.14
A29	5	5.72	Citalopram	5	5.53	Fluoxetine	5	6.08	Mepivacaine	5	3.46	Prazepam	5	7.25
Acebutolol	5	4.07	Citalopram, demethyl-	5	5.47	Fluoxetine, nor-	10	5.99	Mepyramine	100	4.57	Procyclidine	5	5.78
Acepromazine	10	5.67	Clobazam	5	6.28	Flurazepam	5	5.53	Mescaline	50	2.77	Promethazine	10	5.70
Acrivastine	5	5.64	Clobazam, nor-	50	5.62	Flurazepam, N-dealkyl-	5	6.19	Metamphetamine	10	2.86	Propafenone	5	5.88
Alfentanil	5	5.33	Clomipramine	10	6.22	Fluvoxamine	5	5.91	Methadone	5	6.03	Propranolol	10	5.28
Alprazolam	5	6.09	Clonazepam	10	5.99	Formoterol	50	4.06	Methaqualone	5	6.02	Pseudoephedrine	50	2.28
Amfepramone	5	3.05	Clonazepam, 7-amino-	50	3.39	Gabapentin	100	2.41	Methylphenidate	5	4.16	Quazepam	5	7.59
Amisulpride	5	3.40	Clonidine	10	2.44	Halazepam	5	7.22	Metoclopramide	100	3.64	Quetiapine	10	5.35
Amitriptyline	5	5.86	Clozapine	100	5.10	Haloperidol	10	5.57	Mianserine	5	5.48	Reboxetine	5	5.60
Amlodipine	10	5.92	Cocaine	5	4.46	Hydrocodone	50	2.96	Midazolam	5	5.43	Remifentanil	5	4.71
Amphetamine	50	2.60	Codeine	50	2.50	Hydromorphone	100	1.84	Mirtazapine	50	3.91	Salmeterol	10	6.14
Aripiprazole	50	5.90	Cyclizine	10	5.52	Hydroxizine	5	5.91	Moclobemide	10	3.39	Scopolamine	50	2.60
Atenolol	100	2.10	Demoxepam	50	5.62	Ibogaine	5	4.94	Morphine	50	1.67	Selegiline	10	3.55
Atorvastatin	10	7.06	Dextromethorphan	10	5.38	Imipramine	5	5.81	Naloxone	50	2.46	Sertindol	5	6.30
Atropine	10	3.46	Dextropropoxyphene	5	5.98	Indapamid	100	5.79	Naltrexone	50	2.81	Sertraline	5	6.04
Bambuterol	5	4.84	Dextrorphan	10	3.82	Isradipine	10	7.00	Nifedipine	50	6.40	Sildenafil	50	5.54
Benzphetamine	5	5.24	Diazepam	5	6.56	Ketamine	10	3.57	Nimodipine	10	7.14	Sotalol	100	2.02
Benzocaine	50	5.15	Diazepam, demethyl-	5	6.11	Ketobemidone	10	3.22	Nitrazepam	10	5.80	STP	5	4.67
Benzoylecgonine	100	3.48	Diltiazem	5	5.63	Labetalol	10	4.95	Nitrazepam, 7-amino-	10	2.26	Sulpiride	50	2.21
Biperiden	10	5.78	Diphenoxylate	5	6.56	Lamotrigine	50	3.78	Nortriptyline	10	5.92	Temazepam	5	6.28
Bisoprolol	5	5.06	DMA	5	3.54	Lercanidipine	5	6.8	Noscapine	5	4.99	Terbutaline	100	1.93
Bromazepam	50	5.33	DMT	10	2.89	Levomepromazine	10	5.98	Orphenadrine	5	5.75	TFMPP	10	4.90
Brotizolam	5	6.27	Doxazosin	5	5.43	Levorphanol	5	3.82	Oxazepam	5	5.92	Tolbutamide	50	6.16
Bupivacaine	5	5.08	Doxepine	5	5.59	Loperamide	5	6.37	Oxcarbazepine	100	5.29	Tramadol	5	4.09
Buprenorphine	5	5.59	Eletriptan	5	5.21	Loratadine	5	6.32	Oxycodone	50	2.67	Triazolam	5	6.15
Buspirone	5	5.14	Enalapril	10	5.23	Lorazepam	10	6.02	Oxymorphone	100	1.78	Trimipramine	5	6.05
Carvedilol	5	5.71	Ephedrine	50	2.28	Lormetazepam	5	6.41	Paroxetine	10	5.66	Vardenafil	100	5.17
Cathine	50	2.05	Estazolam	5	5.95	Losartan	10	6.09	Pethidine	5	4.68	Venlafaxine	5	5.06
Cathinone	50	2.09	Felodipine	50	7.44	LSD	5	4.88	Phenazepam	10	6.45	Verapamil	5	5.95
Cetirizine	5	5.92	Fentanyl	5	5.38	MBDB	5	3.61	Phencyclidine	5	5.23	Zaleplon	50	5.68
Chlordiazepoxide	5	4.94	Fexofenadine	5	5.93	mCPP	50	4.08	Phentermin	10	3.08	Zolmitriptan	50	2.70
Chlordiazepoxide, demetyl-	50	4.85	Flecainide	5	5.58	MDA	50	2.70	Pimozide	10	6.28	Zolpidem	5	4.78
Chlorpromazine	10	6.12	Flumazenil	10	5.13	MDMA	50	2.89	Pindolol	50	3.00	Zopiclone	50	3.88

acetonitrile. These stock solutions were stored in ampoules at −20 °C until use.

A working solution of the internal standard was prepared, every second month, by dilution in methanol at a concentration of 0.125 mg/l, and stored at −20 °C in 125 µl ampoules.

Individual solutions of 175 reference compounds (Table 1) were prepared at concentrations of 0.2 mg/l in methanol and were used to establish the reference retention times. A working solution of a mixture of the 175 compounds at a concentration of 0.2 mg/l was prepared from two stock solutions (A and B). Stock solution A contained 100 compounds (10 mg/l), and stock solution B contained 75 compounds (10 mg/l).

Stock solution A and stock solution B were also spiked to whole blood samples in four concentrations (5, 10, 50, and 100 µg/kg) in order to approximately estimate the lowest concentration of each compound that could be identified.

Apparatus

A Gilson robot (ASPEC XL4; Gilson Inc., Columbus, OH, USA) equipped with Bond Elut Certify solid-phase extraction (SPE) columns; 130 mg, 3 ml (Varian, Inc., Palo Alto, CA, USA) was used for SPE. Chromatography was performed using an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) with a 100 mm × 2.1 mm, 1.7 µm Acquity UPLC BEH C_{18} analytical column. A Micromass LCT Premier XE apparatus (Waters MS Technologies, Manchester, UK) was used for orthogonal acceleration TOF-MS, and was operated in positive mode with electrospray ionization (Z-spray).

Analytical procedure

The reference mixture containing a mixture of all 175 compounds (0.2 mg/l) was injected and analyzed daily by UPLC-TOF-MS to verify instrument performance (retention time and sensitivity of response).

Biological samples were extracted using SPE columns, which were conditioned with 2 ml methanol and 2 ml purified water. Blood samples (0.2 ml) spiked with 30 µl of internal standard were diluted with 5 ml ammonium acetate (0.1 M, pH 4.1)/methanol (*v*/*v*, 90 : 10) and introduced into the SPE columns at a constant flow rate of 1 ml/min. The columns were washed with 2 ml purified water/methanol (*v*/*v*, 95 : 5), followed by two, 2-ml washes of purified water/methanol (*v*/*v*, 70 : 30), and with 2 ml purified water/methanol (*v*/*v*, 95 : 5). The analytes were eluted with 3 ml freshly prepared acetonitrile with 0.5% aqueous ammonium. The elution was carried out in two steps by eluting twice with 1.5 ml into one collection tube without intermediate drying of the columns. Eluates were evaporated at room temperature under a stream of nitrogen and redissolved in 200 µl of 0.1 M ammonium acetate buffer (pH 4.1)/methanol (*v*/*v*, 90 : 10). Ten microliters of this solution were analyzed by UPLC-TOF-MS.

UPLC separation was achieved using mobile phase of solvents A (0.1% formic acid) and B (100% acetonitrile). The column was maintained at 50 °C and eluted with a gradient of 0–20% solvent B (0–4.0 min), 20–95% solvent B (4.0–9.0 min); the column was then flushed with 100% solvent B (9–11 min). The total run time was 15 min at a flow rate of 0.60 ml/min. The autosampler was maintained at 10 °C. The nebulization gas was set to 750 l/h at a temperature of 350 °C. The cone gas was set to 10 l/h, and the source temperature was set to 120 °C. The capillary and cone voltages were set to 3000 V and 30 V, respectively. The LCT Premier XE was operated in W optics mode with >10 000 resolution. The data acquisition rate was set to 0.15 s/scan, with 0.010 s interscan delay using dynamic range enhancement; data were collected from 0.5–9 min. All analyses were acquired using lock spray to ensure accuracy and reproducibility; leucine-enkephalin was used as the lock mass (*m*/*z* 556.2771) at a concentration of 300 ng/ml and a flow rate of 50 µl/min. Data were collected in centroid mode from *m*/*z* 50–1000.

Data processing

ChromaLynx XS application manager (MassLynx 4.1) was used in targeted mode for identification of compounds. A database with 175 target compounds and internal standard was created as a tablature separated text file (.txt) with compound name, molecular formula, and retention time. The ChromaLynx XS processing method was linked to the text file and was programmed to automatically identify compounds that met the following criteria: exact mass within 5 mDa, RT ±0.2 min of expected, and with a peak-area response exceeding 10 arbitrary units. Compounds fulfilling these three criteria were displayed (Figure 1), and could be further evaluated by also comparing the recorded isotopic pattern to the theoretical isotopic pattern, which can be displayed in the elemental composition window (Figure 2) via the ChromaLynx XS identify browser.

Results and discussion

Spiked samples

Blood-bank samples spiked with compounds at concentrations of 5, 10, 50, and 100 µg/kg were analyzed according to the described method to approximately assess the lowest concentration at which compounds could be identified with a signal to noise greater than 3. Eighty-two compounds were identified at a concentration of 5 µg/kg, 123 compounds at 10 µg/kg, 161 at 50 µg/kg, and all 175 compounds at 100 µg/kg (Table 1). Concerning the common drugs of abuse, cocaine could be identified at 5 µg/kg, whereas amphetamine and morphine required higher levels, i.e. 50 µg/kg. Tetrahydrocannabinol (THC) was not assayed by the UPLC-TOF-MS method. For the therapeutics, many of the benzodiazepines class could be identified at 5 µg/kg, for example, alprazolam, brotizolam, chlordiazepoxide, diazepam, flunitrazepam, and triazolam. Common antidepressants were generally identified at levels (5–10 µg/kg) corresponding to the therapeutic intervals, including amitriptyline, nortriptyline, citalopram, fluoxetine, and venlafaxine. Antipsychotic drugs were identified from 5 µg/kg (sertindole) to 100 µg/kg (clozapine). Some opioids could be identified at low levels, such as 5 µg/kg for buprenorphine and fentanyl, but 50 µg/kg was required for others, for example, codeine, morphine, and oxycodone.

Authentic samples

Assessments were carried out for 119 consecutive authentic blood samples, 32 samples from autopsies, and 87 from living subjects. We did not experience any problems with automated SPE of autopsy blood. All detected drugs were subjected to verification and quantification by in-house developed quadrupole LC-MS/MS procedures. The UPLC-TOF-MS screening method indicated the presence of 302 hits (Table 2) for the 119

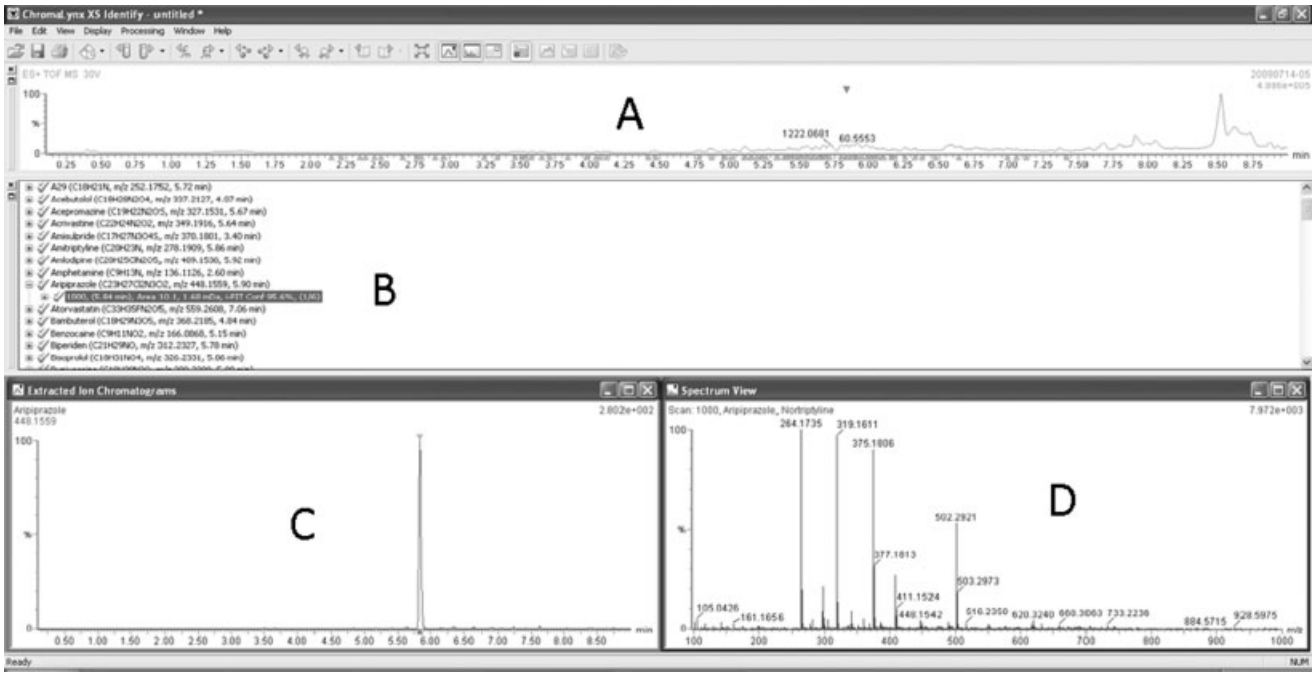

Figure 1. ChromaLynx XS browser showing 175 identified compounds. (**A**) Total ion chromatogram. (**B**) The list of the 175 detected compounds. Aripiprazole is highlighted. (**C**) Extracted ion chromatogram of aripiprazole. (**D**) Spectrum view of aripiprazole.

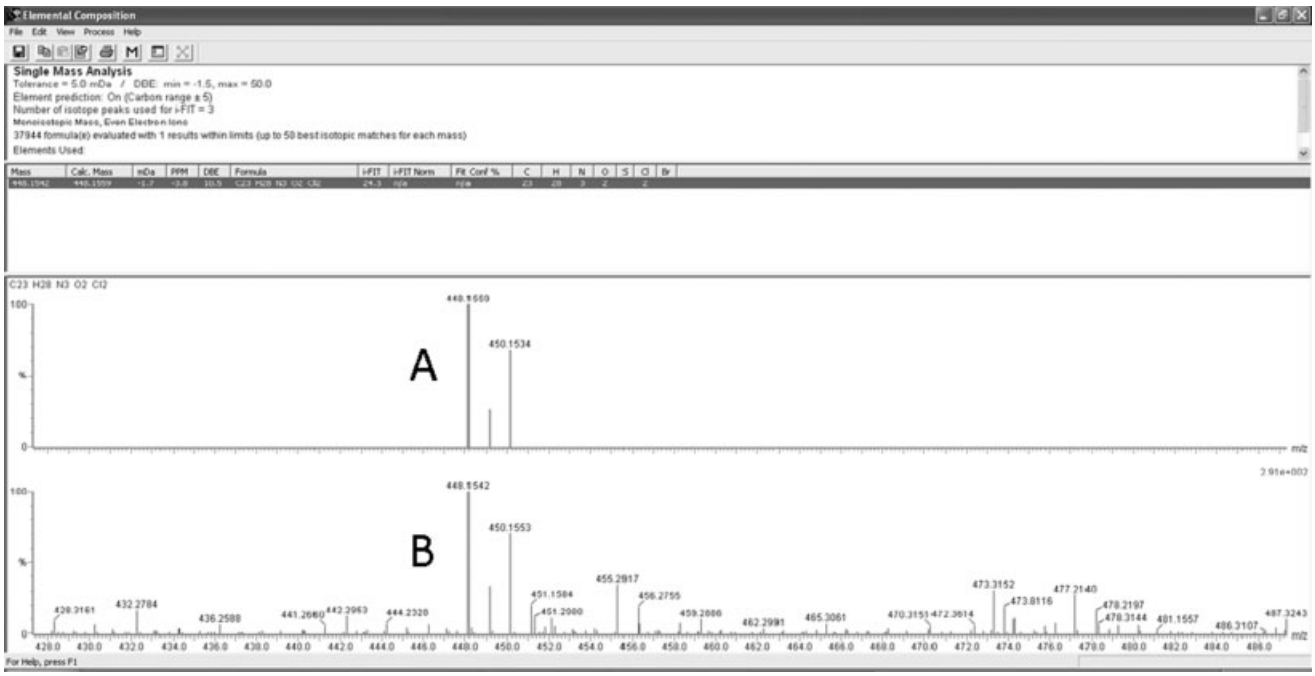

Figure 2. Window for comparing observed isotopic patterns with theoretical isotopic patterns. (**A**) Theoretical isotopic pattern. (**B**) Observed isotopic pattern.

samples. Two hundred and ninety-one, i.e. 96%, of these hits were subsequently verified as true positives by the LC-MS/MS confirmation methods.

The remaining 11 cases that could not be confirmed as true positives are presented in Table 3; seven of these had peak areas that only just exceeded the threshold of 10 (range 10 to 13.5), and as such, were already considered to be borderline cases. Some cases exhibited relatively low areas (18–39.5; Table 3).

Taking the large number of compounds into account, we did not study matrix effects in detail. However, for seven random authentic samples (post-mortem blood and blood from living people), we added nine additional deuterated internal standards for various benzodiazepines being available in our laboratory with retention times distributed over the chromatography run and recorded mean, SD and CV% of the areas. The CV% of the areas ranged from 4 to 13%. The variation can be ascribed to variation in extraction

Table 2. Detected compounds in 119 authentic blood samples

Compound	Number of findings
A29	1
Alfentanil	1
Alprazolam	5
Amitriptyline	1
Amphetamine	12
Aripiprazole	2
Atropine	11
Benzocaine	1
Benzoylecgonine	20
Bromazepam	7
Bupivacaine	1
Buprenorphine	2
Cathine	1
Cathinone	1
Cetirizine	2
Chlordiazepoxide	2
Chlordiazepoxide, demetyl-	1
Chlorprothixene	1
Citalopram	11
Citalopram, demethyl-	12
Clonazepam	8
Clonazepam, 7-amino-	9
Cocaine	16
Codeine	3
Demoxepam	3
Diazepam	17
Diazepam, demethyl-	21
Diltiazem	2
Ephedrine	2
Fentanyl	6
Flunitrazepam	1
Flunitrazepam, 7-amino-	3
Fluoxetine	2
Fluoxetine, nor-	3
Hydroxizine	3
Imipramin	1
Ketamine	2
Ketobemidone	2
Lamotrigine	4
Levomepromazine	2
Lorazepam	1
Metamfetamine	1
Methadone	16
Methylphenidate	2
Metoclopramide	2
Midazolam	1
Mirtazapine	1
Morphine	5
Nitrazepam	8
Nitrazepam, 7-amino-	9
Nortriptyline	1
Noscapine	2
Orphenadrine	5
Oxazepam	15
Oxycodone	2
Propranolol	2
Pseudoephedrine	1
Quetiapine	2
Sildenafil	1
Temazepam	10
Tramadol	2
Venlafaxine	2
Zolpidem	2
Zopiclone	4

Table 3. Compounds suggested by UPLC-TOF that were not verified as present

Sample no.	Compound	UPLC-TOF-MS area (arb. units)	LC-MS/MS
2342	Amphetamine	18.5	NF
2405	Citalopram	11.5	NF
2392	Diazepam	13.4	NF
2343	Diazepam, demethyl-	10.3	NF
2245	Diazepam	11.1	NF
2272	Flunitrazepam	19.1	NF
2244	Flunitrazepam	11.5	NF
2414	Ketobemidone	13.5	NF
2243	Ketobemidone	18.0	NF
2245	Lorazepam	10.0	NF
2382	Tramadol	39.5	NF

NF: Not found.

recovery and possible matrix effects. The rather modest variation suggests that for these compounds there were no signs of major ion suppression in individual blood samples.

General remarks

This UPLC-TOF-MS screening method for basic drugs in blood has been used routinely for approximately six months in our laboratory with stable performance. The retention times of 175 compounds are routinely checked prior to the run of every batch, and diazepam D5 is used to verify extraction efficiency (on average 80%). The UPLC method was optimized to give narrow and sharp peaks as seen in Figure 1C with good separation. UPLC has the advantage of providing a better resolution than HPLC, which especially in the context of screening for many compounds is an advantage.

ChromaLynx XS when used in targeted mode can be described as a reverse target database search, which means the TOF file is searched for specified target masses in a database. ChromaLynx XS presents peaks that fulfill the set criteria (RT $\pm$0.2 min, mass error $\pm$5 mDa, and area $>$10). In a whole blood sample, the browser typically displays 2–20 hits, and some of these hits need to be evaluated by comparing the observed and the theoretically isotopic pattern as shown in Figure 2. Using other types of equipment, an automated isotopic pattern filter has been applied to eliminate false positive hits,[8,9] but this is not possible in the current version of ChromaLynx XS, and therefore a manual evaluation step was performed.

Concerning evaluation, we did not formally determine detection limits and extraction recovery, but took a practical approach by measuring blood spiked with the compounds in concentrations 5,

10, 50, and 100 µg/kg. From initial studies, we found that an area of 10 was a suitable criterion for detection of compounds. The signal-to-noise ratio exceeded 3 at this area for all compounds, and most background signals were below this area limit. The performance on the spiked samples with approximate assessment of the identification levels, and the results for the authentic samples suggested an adequate performance in a forensic context. Generally the expected basic compounds from the case stories were identified, whereas a separate screening for THC was carried out, as well as regards acid/neutral drugs, which were detected by HPLC-DAD. In order to avoid too many false positive signals, the retention time should be included as an identification criterion. Under daily use, it is important to monitor the validity of the retention time intervals for the compounds in order to assure the reliability of the method.

As mentioned previously, several other studies have demonstrated the suitability of LC-TOF-based screening of drugs in urine.[7–11, 13,14] The lower limits of identification reported in these studies were of the same magnitude as observed here. For some methods, libraries of up to 800 compounds were used.[7,8] However, only about half the compounds had their retention times recorded and the lower limits of identification were not specified.

In a recently published study by Broecker *et al*., the accurate mass CID spectra of more than 2500 compounds were measured with a hybrid quadrupole time-of-flight mass spectrometer (QTOF-MS) at three different collision energies and included in a TOF-MS database with molecular formulas of more than 7500 substances from which the accurate masses and isotope pattern were calculated.[10] This approach was tested on authentic blood samples and could identify compounds without depending on retention time, but primarily using accurate mass and database spectra.

We were not able to handle the screening of drugs without relating to their retention times, because too many false positive signals occurred. However, in special cases, where some specific compound is expected that is not included in the set of 175 standards, it is possible to enter the exact mass in the ChromaLynx XS program and search for the compound, even without having a reference compound available. For example, we had a case where lysergamide (LSA) was expected from the case history. The exact mass was entered, and a peak was noticed with a plausible isotopic pattern suggesting the presence of the compound. Later on, a reference standard was attained, and the presence of the compound was verified.[15] We are now working on extending the number of basic drugs used in the procedure, with a goal of approximately 400 compounds, which is judged suitable for our demands. Other authors have performed screening by the TOF-MS principle with reference to large libraries comprising more than 50 000 compounds.[16,17] In this context there will be problems with identifying compounds with identical masses. Whether such an approach is feasible in a daily routine may depend on the local conditions.

Although we have focused on detection in blood, the LC-TOF-MS principle is also suitable for other matrices, including hair and vitreous humor.[12,18,19]

Conclusion

The developed UPLC-TOF-MS method was able to identify 175 common compounds and drugs in concentrations from 5–100 µg/kg. A good performance on authentic samples was achieved. The present study demonstrates that the combination of retention time and accurate mass provides good selectivity, indicating that the developed UPLC-TOF-MS method is useful and effective for the screening of drugs in whole blood. Inclusion of more compounds in the procedure is planned.

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