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Quantitative Analysis of 30 Drugs in Whole Blood by SPE and UHPLC-TOF-MS

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Abstract

An Ultra-High Pressure Liquid Chromatography Time-of-Flight Mass Spectrometry (UHPLC-TOF-MS) method for quantitative analysis of 30 drugs in whole blood was developed and validated. The method was used for screening and quantification of common drugs and drugs of abuse in whole blood received from autopsy cases and living persons. The compounds included: alprazolam, amphetamine, benzoylecgonine, bromazepam, cathine, cathinone, chlordiazepoxide, cocaine, codeine, clonazepam, 7-aminoclonazepam, diazepam, nordiazepam, flunitrazepam, 7-aminoflunitrazepam, ketamine, ketobemidone, 3,4-Methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, methadone, morphine, 6-monoacetylmorphine, nitrazepam, 7-aminonitrazepam, oxazepam, temazepam, tramadol, O-desmethyltramadol, and zolpidem. Blood samples (200 µL) were subjected to Solid Phase Extraction (SPE). Target drugs were quantified using a Waters ACQUITY UPLC system coupled to a Waters SYNAPT G2 TOF-MS apparatus. Extraction recoveries ranged from 41% (7-aminoclonazepam) to 111% (ketamine) and matrix effects ranged from -13% (temazepam) to 50% (7-aminonitrazepam). For all compounds, a quadratic polynomial was applied for fitting the calibration curves. Lower Limits of Quantification (LOQ) ranged from 0.005 to 0.05 mg/kg. Satisfactory precisions below 15% and accuracies within 85-115% were obtained for all compounds at concentrations exceeding the LOQ. In conclusion, we present a validated UHPLC-TOF-MS method for simultaneous quantification of 30 drugs in whole blood with a run time of 15 min using 200 µL of whole blood.

Keywords: Drugs of abuse; UHPLC-TOF-MS; Whole blood; SPE; Quantification

Introduction

In toxicology, the usual approach is to screen for the presence of drugs and subsequently carry out a quantification of positive findings. Screening procedures may be based on immunoassay techniques, GC-MS, HPLC-DAD, LC-tandem mass spectrometry (LC-MS/MS), LC-ion trap mass spectrometry or LC-high resolution mass spectrometry as Time-of-Flight (TOF) or Orbitrap Fourier- transform mass spectrometry [1-10]. Quantitative determination is usually based on GC-MS or LC- MS/MS approaches [11]. Generally, if screening and quantification can be combined, a more efficient approach is attained. For optimal quantification, deuterated internal standards are usually required, so in practice it is not possible to combine general screening for several hundred compounds with quantification of all compounds that might be found in one analytical run. However, it might be possible to quantify a limited number of frequently occurring compounds, say about 30 compounds. In the present study we were interested in investigating the ability of a LC-TOF system to simultaneously carry out screening for a wide range of compounds as previously described and quantification of a more limited, frequently occurring subset of compounds [12]. Samples were prepared using fully automated Solid Phase Extraction (SPE) [12-14], and the compounds were separated using Ultra-High Pressure Liquid Chromatographic (UHPLC) chromatographic conditions as previously described [15-17]. Focus was on a comparison of Lower Limits of Quantification (LLOQ), precision, accuracy and dynamic range with those of the standard technique of LC-MS/MS for quantitative analysis. The results were primarily related to those recently reported by Bjork, et al. [13]. based on a UHPLC-MS/MS method for 31 commonly occurring medicinal and illicit drugs.

Methods

Chemicals and reagents

The following compounds were purchased as certified

reference solutions from Lipomed GmbH (Bad Sackingen, Germany): alprazolam, amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), morphine, 6-acetylmorphine (6-AM), codeine, cocaine, benzoylecgonine, methadone, bromazepam, clonazepam, flunitrazepam, nordiazepam, 7-aminonitrazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminoflunitrazepam-d3 and flunitrazepam-d3. From Cerilliant (Round Rock, Texas, USA) we obtained the following substances: cathine, cathinone, ketamine, amphetamine-d5, codeine-d6, cocaine-d3, chlordiazepoxide-d5, benzoylecgonine-d8, ephedrine-d3, 6-AM-d6, ketamine-d4, methadone-d3, methamphetamine-d5, morphine-d6, MDA-d5, zolpidem-d6, MDMA-d5, tramadol-C13-d3, O-desmethyltramadol-d6, diazepam-d5, nordiazepam-d5, nitrazepam-d5, oxazepam-d5, alprazolam-d5, clonazepam-d4, 7-aminoclonazepam-d4 and temazepam-d5. We obtained 7-aminonitrazepam-d5 and bromazepam-d4 from Toronto Research Chemicals (Toronto, Canada). All the reference standards were of ≥98% purity. Methanol and acetonitrile (LC-MS grade) were obtained from Fisher Scientific (Leicestershire, UK). Aqueous ammonia (25%) solution was obtained from Merck (Darmstadt, Germany). Purified water was obtained with a Milli-Q system (Millipore, Denmark). The mobile phase used for the LC system was prepared weekly. Mobile phases were solvent A (5 mM ammonium formate, adjusted to pH 3 using formic acid) and B (acetonitrile containing 0.1% (v/v) formic acid).

Samples

We performed the analyses on whole blood stabilized with sodium fluoride and potassium oxalate. Investigations of matrix effects and extraction efficiency were based on negative authentic samples (pre-screened for all kinds of licit and illicit drugs received by the laboratory, either from autopsy cases or from living persons). The whole blood was stored at -20°C until use.

Preparation of standard solutions, calibrators and quality control (QC) samples

A stock solution containing a mixture of all non-deuterated standards at a concentration of 20 mg/L was prepared in methanol. From this stock, three working solutions were prepared in water, at concentrations of 5 mg/L, 0.5 mg/L and 0.05 mg/L, respectively. The stock solution was stored at -20°C; working solutions were stored at -80°C. A single Internal Standard solution (IS), containing the deuterated analogues in methanol at a concentration of 0.4 mg/L, was prepared, divided into aliquots of 1000 µL and kept at -80°C. Calibrators were made by spiking 0.200 g of pooled whole blood with 20 µL of standard working solutions, yielding final calibration concentrations of 0.0050, 0.05, and 0.5 mg/kg. Two levels of quality control (QC) samples containing all compounds were prepared in pooled whole blood at concentrations of 0.02 and 0.5 mg/kg and stored at −20°C. An aliquot (20 µL) of IS was added to all calibrators, QCs, and samples.

Solid Phase Extraction

SPE was performed on a Tecan Freedom EVO 200 robotic platform (Tecan, Mannedorf, Switzerland) fitted with a vacuum station for solid phase extraction [14]. Polymeric mixed-mode cation exchange resin Strata-X-C SPE cartridges (30 mg, 2 mL) were from Phenomenex (Torrance, CA). An aliquot (20 µL) of IS was added to 0.200 g of each whole blood sample following dilution of the samples with 800 µL of 1% formic acid in water. The samples were centrifuged at 1000 g for 10 min. The supernatant was loaded onto pre-conditioned SPE columns (pre-conditioned and equilibrated with methanol followed by water). Columns were washed consecutively with 900 µL water, followed by 2% formic acid in 5% methanol and finally with 250 µL of methanol. Elution was performed by the addition of 500 µL of freshly prepared acetonitrile containing 8% (v/v) ammonia solution. A portion (400 µL) of the eluate was evaporated to dryness by a gentle flow of nitrogen heated to 40°C. Reconstitution was achieved by the addition of 40 µL of acidified methanol (25% methanol in water containing 0.1% formic acid) to the dry residue.

LC chromatographic conditions

The chromatography was performed using an ACQUITY UPLC system (Waters Corporation, Milford, USA) [15-17]. The column used was a 150 mm × 2.1 mm, 1.8 µm ACQUITY UPLC HSS C18, which was maintained at a column temperature of 50°C and eluted at a constant flow rate of 0.4 mL/min. The gradient was 13-13% solvent B (0-0.5 min), 13-50% solvent B (0.5-10 min), 50-95% solvent B (10-10.75 min); the column was then flushed with 95% solvent B (10.75-12.25 min). The total run time was 15 min. The injection volume was 15 µL. A methodology overview is shown in Table 1, and a chromatogram of all 30 analytes is presented in Figure 1.

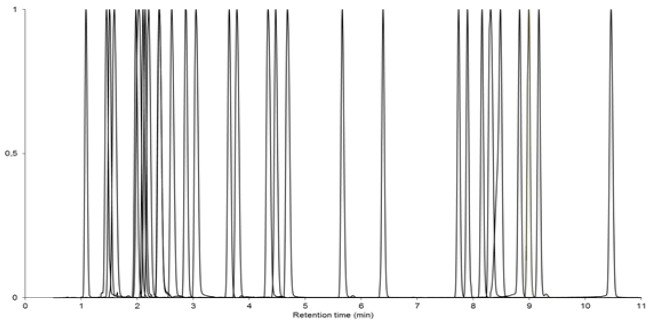

Figure 1: Chromatogram of 30 compounds.

Mass spectrometry

Mass spectrometry was performed using a SYNAPT G2 (Waters MS Technologies, Manchester, UK) orthogonal acceleration Quadrupole Time-of-Flight mass spectrometer. The mass spectrometer was operated in positive ion mode with electrospray ionisation (Z-spray). The nebulisation gas was set to 800 L/h at a temperature of 550°C. The cone gas was set to 10 L/h and the source temperature to 120°C. The capillary voltage and the cone voltage were set to 300 and 20 V, respectively. Argon was used as the collision gas. For the MS^E

experiment, two acquisition functions with different collision energies were used in the trap collision cell. The low energy function (Function 1) was set to 4 eV, and the high energy function (Function 2) was set to use a collision energy ramp from 10-40 eV. The SYNAPT G2 was operated in V optics mode (resolution mode) with >18,000 at full width at half maximum at *m/z* 556. The data acquisition rate was 0.15 sec/scan in both functions, with 0.024 sec interscan delay; data was collected from 0.5 min to 13 min. The mass spectrometer was calibrated to <2 mDa mass error prior to each batch. 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 400 ng/mL and a flow rate of 50 µL/min. Data were collected in centroid mode from *m/z* 50-950. A methodology overview is shown in Table 1.

Chromatography	
Liquid chromatography system:	Waters ACQUITY UPLC
Column:	Waters ACQUITY® HSS C18 (2.1 x 150 mm, 1.8 µm)
Column temperature:	50 °C
Injection volume:	15 µL
Solvent A:	5 mM ammonium formate, adjusted to pH 3 using formic acid
Solvent B:	Acetonitrile containing 0.1% (v/v) formic acid
Gradient:	13-13% solvent B (0-0.5 min)
	13-50% solvent B (0.5-10 min)
	50-95% solvent B (10-10.75 min)
	95-95% solvent B (10.75-12.25 min)
Flow rate:	0.4 mL/min
Mass Spectrometry	
Mass spectrometer:	Waters SYNAPT G2
Ionisation mode:	Electrospray +ve
Capillary voltage:	300 V
Cone voltage:	20 V
Desolvation temperature:	550°C
Desolvation gas:	800 L/h
Source temperature:	120°C
Data acquisition:	MS^E centroid (data independent acquisition)
Function 1:	4 eV
Function 2:	Ramp 10-40 eV
Mass ange:	50-950 Da
Resolution:	>18,000 @ 556 m/z (resolution mode)
Lock Spray:	Leucine encephalin $[M+H]^+$= m/z 556.2771

Table 1: Methodology overview.

TargetLynx software was used for quantitative data processing (Waters Corporation, Milford, USA). The analytes were identified by the exact mass of the precursor ion and the retention time. Tolerance was set to 5 mDa for the precursor ion and +/- 0.2 min for the retention time. Quantification was performed by integration of the peak area under the curve from the specific precursor ions of the analytes and their internal standards. The response (the ratio of the integrated area of the analyte and the corresponding IS) was compared to the calibration curve. The IS chosen for each analyte, retention times, and precursor ions are shown in Table 2.

For screening, the data were assessed by the screening software ChromaLynx XS (Waters Corporation, Milford, USA). In this software the fragments in Function 2 play an important role for the identification of compounds compared to a larger compound library/database. This will not be discussed further in this paper, but is presented by Pedersen, et al. [12].

Calibration curve

Calibration in whole blood was evaluated. The concentration points were 0, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 mg/kg. Calibrators were prepared in 200 µL of whole blood spiked with 25 µL standard and 20 µL IS. Three examples of calibration curves are shown in Figure 2.

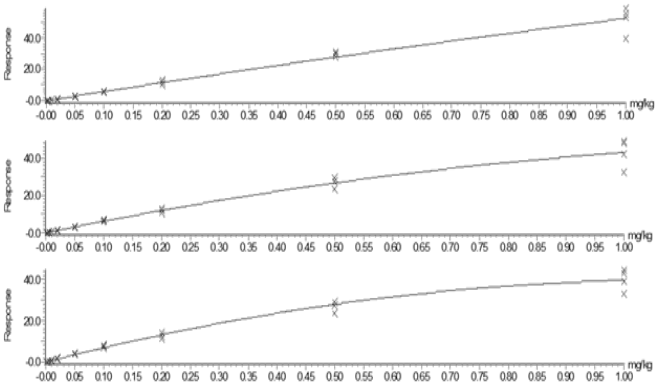

Figure 2: Examples of calibration curves. 7-aminonitrazepam (top), diazepam (middle) and ketobemidone (bottom).

Matrix effects and extraction recoveries

Matrix effects (ME) and extraction recoveries for whole blood were evaluated on the basis of peak area responses [18,19]. Seven authentic case samples (four autopsy cases and three traffic cases) and two blank blood samples (blood bank samples) were used. All samples were screened negative for a broad variety of drugs including drugs of abuse. Two sets of the nine whole blood samples were extracted according to the SPE procedure. The first set was spiked with 30 analytes after the SPE extraction (B), and the second set was spiked before extraction (C) to a corresponding concentration in whole blood. The B and C experiments were performed in duplicate for all nine samples. Thus, nine replicates of a reference solution (A) also prepared in acidified methanol were analyzed directly with the UPLC-TOF-MS system. The ME results obtained in this study were calculated as follows:

Eq. 1: ME (%) = (1− (B/A)) × 100%,

where A equals the peak area of standards in mobile phase and B is the peak area obtained for whole blood samples spiked with analytes after extraction. An ME value >0 indicates ionization suppression and a value <0 indicates ionization

Analyte	Internal standard	[M+H]1+ 1*1	RT	Range	LOD	LOQ			>LOQ		RE	ME
		m/z	Min	mg/kg	mg/kg	mg/kg	Precision	Accuracy	Precision	Accuracy	%	%
6-MAM	6-MAM D6	328.1549	2.03	0.02-1.0	0.0026	0.02	5	85	2-7	87-115	89	15
7-aminoclonazepam	7-aminoclonazepam D4	286.0747	3.67	0.02-0.5	0.0049	0.02	9	88	7-15	89-106	41	-1
7-aminoflunitrazepam	7-aminoflunitrazepam D3	284.1199	4.50	0.02-1.0	0.0041	0.02	8	84	4-8	85-114	47	-4
7-aminonitrazepam	7-aminonitrazepam D5	252.1137	2.08	0.05-1.0	0.0232	0.05	16	99	6-11	94-113	64	50
Alprazolam	Alprazolam D5	309.0907	8.53	0.02-0.5	0.0059	0.02	12	82	4-10	91-108	91	-7
Amphetamine	Amphetamine D5	136.1126	2.15	0.005-1.0	0.0016	0.005	12	86	7-12	91-103	91	13
Benzoylecgonine	Benzoylecgonine D8	290.1392	2.89	0.02-1.0	0.0010	0.02	2	90	5-10	103-112	102	-5
Bromazepam	Bromazepam D4	316.0085	6.42	0.02-1.0	0.0064	0.02	12	87	1-11	97-103	97	-5
Cathine	Ephedrine D3	152.1075	1.44	0.005-1.0	0.0020	0.005	15	97	9-13	95-112	100	28
Cathinone	Ephedrine D3	150.0919	1.52	0.02-1.0	0.0093	0.02	15	101	11-15	98-107	109	39
Chlordiazepoxide	Chlordiazepoxide D5	300.0904	5.69	0.05-1.0	0.0033	0.02	7	81	4-10	90-113	105	1
Clonazepam	Clonazepam D4	316.0489	8.20	0.05-1.0	0.0263	0.02	18	98	10-15	96-103	73	-7
Cocaine	Cocaine D3	304.1549	4.38	0.02-1.0	0.0058	0.02	11	88	3-13	90-114	109	1
Codeine	Codeine D6	300.1600	1.62	0.02-1.0	0.0021	0.02	4	83	2-6	89-113	109	18
Diazepam	Diazepam D5	285.0795	10.50	0.02-0.5	0.0035	0.02	7	82	5-10	92-111	89	-3
Flunitrazepam	Flunitrazepam D3	314.0941	8.86	0.05-1.0	0.0130	0.05	9	95	5-10	88-111	77	-7
Ketamine	Ketamine D4	238.0999	3.08	0.02-1.0	0.0017	0.02	3	86	2-5	98-109	111	3
Ketobemidone	Benzoylecgonine D8	248.1651	2.65	0.02-0.5	0.0014	0.02	3	96	2-12	104-109	109	11
MDA	MDA D5	163.0759*2	2.20	0.02-0.5	0.0039	0.02	7	98	11-14	92-104	97	-8
MDMA	MDMA D5	194.1181	2.43	0.02-1.0	0.0021	0.02	4	86	5-11	93-113	66	5
Methamphetamine	Methamphetamine D5	150.1283	2.42	0.02-1.0	0.0020	0.02	4	87	6-8	89-112	47	3
Methadone	Methadone D3	310.2171	8.37	0.02-1.0	0.0105	0.02	6	106	3-7	89-110	103	-12
Morphine	Morphine D6	286.1443	1.12	0.02-1.0	0.0068	0.02	11	100	5-14	94-115	78	27
Nitrazepam	Nitrazepam D5	282.0879	7.77	0.05-1.0	0.0177	0.05	15	82	3-10	90-92	83	1
Nordiazepam	Nordiazepam D5	271.0638	9.03	0.02-0.5	0.0036	0.02	7	88	5-11	102-115	94	-1
Oxazepam	Oxazepam D5	287.0587	7.93	0.02-1.0	0.0079	0.02	13	100	2-13	93-106	67	-11
Temazepam	Temazepam D5	301.0744	9.21	0.02-0.5	0.0096	0.02	17	93	11-13	85-109	81	-13
Tramadol	Tramadol D3	264.1964	3.82	0.02-1.0	0.0060	0.02	11	94	4-10	91-112	108	2
O-Desmethyltramadol	O-Desmethyltramadol D6	250.1807	2.24	0.02-1.0	0.0022	0.02	4	84	2-5	91-112	108	6
Zolpidem	Zolpidem D6	308.1763	4.74	0.02-0.5	0.0025	0.02	5	91	3-11	104-105	105	-1

*1 There is a difference of around 0.5 mDa between the theoretical mass and the calculated mass, as the software adds the mass of a hydrogen atom instead of the mass of a proton
*2 product ion i Function 2

Table 2: Identification and validation parameters.

enhancement.

Extraction recoveries (RE) were calculated as:

Eq. 2: RE (%) = (C/B) × 100%

Precision and accuracy

Evaluation of precision and accuracy were based on the ISO 17025 guideline [20]. To evaluate precision and accuracy, we analyzed four replicates at seven concentration levels on two different days (n=8). The seven concentration levels analyzed were: 0.002, 0.005, 0.02, 0.05, 0.2, 0.5, and 1.0 mg/kg. A calibrator series was freshly prepared for every run, based on 0.200 g of whole blood spiked with all analytes, yielding the concentration points: 0.005, 0.05, and 0.5 mg/kg. Prior to analysis, seven different stock samples (5 g each) representing the seven concentration levels were prepared by spiking pooled whole blank blood with all of the analytes. On day one of analysis, four samples (0.200 g of blood) were taken from each of the seven stock samples. All 16 samples (four replicates for each concentration level) and the calibrators were spiked with 20 µL of IS, as described above, and subjected to SPE. The procedure was repeated on day two of analysis. Another spiked sample at a concentration level of 0.001 mg/kg was prepared and used for the determination of limit of detection (LOD). Four replicates were analyzed. This procedure was repeated the next day, and LOD was calculated from the eight results as 3 standard deviations.

Results and discussion

Quantification by UHPLC-TOF-MS

The chromatographic separation method used in this study was originally developed by Humbert, et al. [15]. and has been successfully used by us and others [15-17]. Setting up a quantification method on a TOF instrument is relatively easy as compared to a quadrupole MS/MS instrument. The lower resolving power of the MS/MS apparatus requires that the compounds of interest be 'tuned' to determine the specific MRM transitions by optimizing the cone voltage and the collision energy. In contrast, with TOF in data-independent acquisition (MS^E) mode, universal values for cone and collision energy are applied. We found that the optimal capillary voltage should be set at a very low voltage (0.3 kV). A low capillary voltage optimizes the sensitivity for low molecular mass compounds like amphetamine, MDA, MDMA, cathine and cathinone, without losing too much sensitivity for the rest of the target compounds. Also the cone voltage was set to a low voltage (20 V) because of the low molecular mass compounds.

A significant benefit of TOF instrumentation is that all of the data is collected. In contrast, with a MS/MS instrument operated in MRM mode, quantitative information that is acquired during an analytical run is derived from only restricted channels of data. Thus, the original dataset from TOF analysis also offers the opportunity of performing an expanded screen, against a larger database using suitable software. In our laboratory the screening software ChromaLynx XS is used to screen for more than 850 compounds. In a previous study we demonstrated how the fragment data (Function 2) can be used for screening of 256 drugs [12].

Validation

Concerning calibration, we investigated the analyte/IS peak area response ratio in whole blood (Table 2). The calibration curves were slightly curved and fitted to a quadratic regression curve using weighting (1/X), where the coefficient for the quadratic term deviated significantly from zero ($P< 0.01$) for all compounds. The calibration range obtained for all analytes in blood started at 0.02 mg/kg except for amphetamine and cathine, which started at 0.005 mg/kg (Table 2), and 7- aminonitrazepam, chlordiazepoxide, clonazepam, flunitrazepam and nitrazepam, which started at 0.05 mg/kg. The calibration range for all analytes extended to 1.0 mg/kg, except for 7- aminoclonazepam, alprazolam, diazepam, ketobemidone, MDA, nordiazepam, temazepam and zolpidem, where 0.5 mg/kg was the upper limit. Fig. 2 displays examples of calibration curves. The curve of 7-aminonitrazepam was only slightly curved, and the upper measurement limit without dilution was 1 mg/kg. The curves for diazepam and ketobemidone were somewhat more curved, and the upper measurement limits were 0.5 mg/kg.

Matrix effects and extraction recoveries for the 30 tested analytes are listed in Table 2 as the mean of the nine samples. MEs were higher for early eluting compounds (RT<3 min). The MEs were within ±50%, and so we concluded that ME was of minor significance because of the use of internal standards. Extraction recoveries were estimated to 41%-111%, which are better or correspond to those of recently published methods for drugs of abuse in whole blood [21,22]. The LOQ was determined as the lowest concentration yielding precision (CV) of ≤20% and bias of ±20% with fulfillment of retention time and 5 mDa mass tolerances. The CV and accuracy were determined for at least four concentration levels. The precision and accuracy were generally accepted at a maximum of 15% (LOQ 20%). All analytes fulfilled the precision criteria at all concentration levels. The validation procedure in the present study was primarily based on the ISO17025 guideline, because our laboratory is accredited, and the present work was in progress, when the new SWGTOX guideline was published [23].

Comparison of UHPLC-TOF-MS quantitative performance with UHPLC-MS/MS performance

UHPLC-MS/MS is generally the method of choice for quantitative determination, characterized by high sensitivity, large dynamic range and good precision [11]. Although the TOF technique as shown above provided good quantitative determination of the compounds, it is of interest to relate the specifications to that of UHPLC-MS/MS. We recently published an UHPLC-MS/MS method for about the same 30 compounds considered here [13]. For most compounds, the LOQ is about ten times lower for the UHPLC-MS/MS method than for the present UHPLC-TOF-MS method, and the dynamic range is correspondingly wider. Precision and accuracy were about similar for the two methods. Thus, it is no doubt that UHPLC-MS/MS is the optimal approach with regard to quantification, but the TOF approach still performs reasonable and at the same time has a screening potential outperforming the UHPLC-MS/MS approach.

Conclusion

The present study showed that the UHPLC-TOF-MS technique is useful for quantitative determination of common drugs of abuse in blood, which can be carried out simultaneously with a comprehensive general screening for drugs. Thus, it is possible to combine screening with quantitative determination of the main drugs of interest providing a very efficient approach for screening and quantification in toxicology.

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