Screening and semiquantitative analysis of drugs and drugs of abuse in human serum samples using gas chromatography-mass spectrometry

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Abstract

OBJECTIVES: The purpose of this study is to develop the gas chromatographic-mass spectrometric method (GC-MS) for screening and semiquantification of drugs and drugs of abuse in human serum. **METHOD**: GC-MS method after liquid-liquid extraction (LLE) and derivatization with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) is presented for screening as well as identification and semiquantification of the most frequently used drugs and drugs of abuse in human serum. **RESULTS**: Bovine serum spiked with ephedrine (EPHE), 3,4-methylenedioxymethamphetamine (MDMA), guaifenesin (GUAIF), tramadol (TRAM), phenobarbital (PHENO), amitriptyline (AMITR), cocaine (COCA), mirtazapine (MIRTA), dothiepin (DOTH), citalopram (CITAL), clomipramine (CLOMI), bromazepam (BMZPM), diazepam (DZPM), codeine (COD), morphine (MORPH), levomepromazine (LEVO), zolpidem (ZOLP), clozapine (CLOZP), alprazolam (ALPZM) was used for the recovery and repeatability study and for preparation of calibration curves of individual compounds or their TMS derivatives. Recoveries were tested on concentration levels 0.05, 0.1 and 0.5 μg/mL (n=6) and established in range 72.0–98.0%. Repeatabilities expressed as relative standard deviations (RSDs) measured at concentration levels 0.05, 0.1 and 0.5 μg/mL (n=6) were lower than 10.0%. The calibration curves for analytes or their TMS derivatives were linear in concentration range 0.025–2.000 μg/mL (except EPHE 2TMS, MDMA TMS, MORPH 2TMS, BMZPM TMS, ALPZM) with correlation coefficients exceeding 0.99. The limit of quantification (LOQ) for analytes used for evaluation study was 0.025 μg/mL (except analytes mentioned above). **CONCLUSIONS**: The GC-MS method presented here is allowing screening, identification and semiquantification of the most commonly encountered drugs and drugs of abuse in human serum and can be successfully applied to analysis of real samples from clinical and forensic toxicology cases.

ORIGINAL ARTICLE

Abbreviations

EI	- electron ionization
GC-MS	- gas chromatography-mass spectrometry
HEXO	- hexobarbital IS
IS	- internal standard
LLE	- liquid-liquid extraction
LOD	- limit of detection
LOQ	- limit of quantification
LC-MS	- liquid chromatography-mass spectrometry
MSTFA	- N-methyl-N-trimethylsilyltrifluoroacetamide
R	- coefficient of determination
Rt	- retention time
RSD	- relative standard deviation
SD	- standard deviation
S/N	- signal to noise
SPE	- solid phase extraction
SPI	- septum equipped programmable injector
TIC	- total ion chromatogram
TMS	- trimethylsilyl
TRIMI-d_3	- deuterated trimipramine-d_3 IS

INTRODUCTION

At present, gas chromatography coupled with mass spectrometry (GC-MS) in the full scan mode is still the most frequently used technique for screening of a wide range of analytes in biological samples in clinical and forensic toxicology and in doping control [1,3–7,9–15,17–21,23,24,28]. The separation power of capillary GC as well as the selectivity of the detection of MS, make GC MS the technique of choice for systematic toxicological analysis [9]. Matching both the retention time and full scan mass spectra of an unknown peak with a standard is proof of identification [18]. MS detection is obviously carried out using electron impact (EI) ionisation mode in order to obtain mass spectra of compounds comparable those contained in commercially available reference libraries. Liquid chromatography coupled with a diode-array detector (LC-DAD) [22] is also often used for screening, but its separation power and its specificity are lower then those of GC-MS. Today, liquid chromatography-mass spectrometry (LC-MS) [1,2,8,10–11,13–16,21,23,25–27] is applied for this purposes and after reducing current disadvantages like irreproducibility of fragmentation, reduction of ionization by matrix, etc. LC-MS may become the standard in clinical and forensic toxicology and in doping control [14]. In general, urine is used as a primary specimen in screening analysis of unknown drugs or poisons, owing to the higher concentrations and longer detection windows of compounds of interest, compared to blood. Nevertheless, acute toxicity correlates with the concentration of substances that occur in blood, not in urine. Isolation of analytes is usually carried out by liquid-liquid (LLE) extraction or by solid phase extraction (SPE). Universal LLE methods are preferable for general screening procedures of the broadest spectrum of compounds with different physicochemical properties. SPE methods are preferable for selective isolations of particular substances or drug classes.

MATERIAL AND METHODS

Drugs free bovine serum was obtained from the Institute of Physiology, Czech Academy of Sciences, Praque. Authentic human serum samples were submitted to laboratory for toxicological analysis. This study was approved by a medical ethics committee and the subjects gave their informed consent to participate. Standards of drugs and drugs of abuse and internal standards were purchased from Sigma Aldrich (Steinheim, Germany), chemicals from Lachema (Brno, Czech Republic) and solvents from Merck (Darmstadt, Germany).

Liquid-liquid extraction for the purposes of validation of the method: 4 mL of ethylacetate:1-chlorbutane:cyclohexane (3:1:1 v/v/v) [6] were added to 2 mL bovine serum spiked with 19 commonly used drugs, drugs of abuse, IS trimipramine-d_3 and hexobarbital (0.5 µg/mL), whose pH value was adjusted to 8.0–9.0 by addition of 0.5 mL of 2 mol/L Tris buffer. After extraction 3 mL of organic layer were acidified to pH 2.0–3.0 by addition of 0.5 mL 1 mol/L hydrochloric acid, extracted and freezed at –20 °C. Basic and neutral analytes were re-extracted into acidic aqueous layer and after setting pH to 8.0–9.0 by addition of 1.5 mL of 2 mol/L Tris buffer again back extracted to organic solvent mixture. After extraction 3 mL of organic layer were evaporated to dryness under a stream of air at 40 °C. The final extract of basic and neutral analytes was derivatized with 100 µL MSTFA at 80 °C for 30 min and 1 µL was injected to GC. 4 mL of ethylacetate:toluene (4:1 v/v) [5] were added to aqueous layer from the initial extraction which contained acidic and remaining neutral analytes, pH of the layer was adjusted to 2.0–3.0 by addition of 1 mL of 1 mol/L hydrochloric acid and extracted. After extraction 3 mL of organic layer were dried as mentioned previously. The final extract of acidic and neutral analytes was derivatized as describe above and 1 µL was injected to GC.

GC-MS analysis

HP-5 MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) and Finnigan MAT Magnum ion trap GC-MS system (A200S GC autosampler, 3400 gas chromatograph (Varian Instrument Group)) were used. GC conditions were as follows: SPI injection mode programmed from 60 °C to 260 °C, carrier gas helium, flow rate 1 mL/min, column temperature initially 60 °C for 1 min and increased to 280 °C at 10 °C/min and held at this temperature for 20 min. MS conditions were as follows: transfer line heater 270 °C, electron ionization (EI) mode, full scan, ionization energy 70 eV, mass range m/z 35 to 500 amu, scan speed 1scan/s.

The presence of analytes was screened for by extraction of characteristic fragment ions from the total ion chromatograms (*m/z*): 130 for EPHE 2 TMS (retention time Rt=13.45 min), 130 for MDMA TMS (Rt=15.35 min), 342 for GUAIF 2 TMS (Rt=16.33 min), 58 for TRAM TMS (Rt=18.37 min), 146 for PHENO 2

TMS (Rt=19.30 min), 58 for AMITR (Rt=20.73 min), 182 for COCA (Rt=20.83 min), 195 for MIRTA (Rt=21.33 min), 58 for DOTH (Rt=22.53 min), for CITAL (Rt=22.68 min), for CLOMI (Rt=22.75 min), 387 for BMZPM TMS (Rt=22.86 min), 256 for DZPM (Rt=23.12 min), 371 for COD TMS (Rt=23.13 min), 429 for MORPH 2TMS (Rt=23.58 min), 58 for LEVO (Rt=23.83 min), 235 for ZOLP (Rt=27.35 min), 256 for CLOZP (Rt=28.20 min), 273 for ALPZM (Rt=30.55 min), 249 for TRIMI-d_3 IS for basic and neutral analytes (Rt=20.95 min), 221 for HEXO IS for acidic and neutral analytes (Rt=15.68 min).

Quantification was carried out by comparison of peak area ratios (analyte versus IS) with calibration curves in which peak area ratios of spiked calibrators were plotted against their concentrations.

Assay evaluation for serum analysis

Three stock solutions were prepared containing each of the drugs listed bellow at concentration of 0.5 mg/mL in methanol. Solution 1 contained EPHE, MDMA, GUAIF, TRAM, PHENO, DOTH, COD, MORPH, BMZPM. Solution 2 contained COCA, MIRTA, CLOMI, ALPZM, ZOLP, CLOZP. Solution 3 contained AMITR, CITAL, DZPM, LEVO.

Aliquots 2 mL of drugs free bovine serum were spiked with 50 μL (0.020 μg/μl) IS TRIMI-d_3 (HEXO for solution 1) and the corresponding analytical standard solutions to obtain calibration samples at concentrations of 0, 0.025, 0.050, 0.100, 0.500, 1.000 and 2.000 μg/mL of each analyte. Samples were analyzed in doublets. The regression line was calculated using a weighted [1/(concentration)^2] least-squares regression model. The means and standard deviations (SD) of slopes and intercepts as well as the coefficients of determination (R^2) were calculated. Samples for solution 1 were analyzed after silylation.

Samples for repeatability study (n=6) at concentrations 0.050, 0.100 and 0.500 μg/mL were prepared by spiking of 2 mL bovine serum with the corresponding analytical standard solutions. After LLE extraction 50 μL (0.020 μg/μL) IS TRIMI-d_3 (HEXO for solution 1) were added to each eluate, extracts were evaporated, (the residues for solution 1 were derivatized) and analyzed as described above. Repeatability was calculated (as RSDs).

Samples for recovery study (n=6) at concentrations 0.050, 0.100 and 0.500 μg/mL were prepared as described above. The control samples (n=6) were prepared at the same concentration levels by adding the analytical standard solutions with 50 μl (0.020 μg/μL) IS TRIMI-d_3 (HEXO for solution 1) into vials and evaporated. After evaporation, the residues of extraction and control samples were analyzed as described above. Recoveries (means and SDs) were estimated by comparison of the peak area ratios (analyte vs IS) from extraction samples and control samples for each analyte at each concentration.

The LOD and LOQ values were based on a signal-to-noise (S/N) ratios. Bovine serum samples spiked by mixtures of standards used for determination of calibration parameters were assayed to determine LOD (S/N greater than 3:1) and the LOQ (S/N greater than 10:1).

RESULTS AND DISCUSSION

Liquid-liquid extraction was used as universal technique for screening of a broad spectrum of in advance unknown compounds in human serum samples from clinical and forensic toxicology cases. This method used variability in composition of extraction mixtures, at pH 8.0–9.0 basic and neutral analytes were extracted with ethylacetate:1-chlorbutane:cyclohexane (3:1:1 v/v/v), at pH 2.0–3.0 acidic and remainder neutral analytes were extracted with ethylacetate:toluene (4:1 v/v).

GC-MS analysis

The presence of tested analytes or their TMS derivatives were screened for by characteristic fragment ions from the corresponding full-scan mass spectra of LLE extracts. Peaks, that apeared in the extracted fragmentograms of the screening ions were checked.

Assay evaluation for serum analysis

Doublets of matrix calibrators at six different concentrations from 0.025 to 2.000 μg/mL were analyzed. A weighted linear regression model was used to account for unequal variances across the calibration range. In Table 1., slopes, intercepts (means± SDs), coefficients of determination of calibration curves, LOD and LOQ are shown. The SDs of the slopes corresponded to RSDs values for all analytes, showing, that callibration curves were reproducible. The calibration curves for most of tested analytes or their TMS derivatives were linear in the concentration range from 0.025 to 2.000 μg/mL with correlation coefficients exceeding 0.99. Detection limits LOD were detected below 0.025 μg/mL and quantification limits LOQ were established at 0.025 μg/mL except MORPH 2TMS, EPHE 2TMS, MDMA TMS, BMZPM TMS and ALPZM.

Repeatability was tested at concentration levels 0.050, 0.100 and 0.500 μg/mL (n=6). The criteria for repeatability (within-day precision) were filled for all tested analytes or their TMS derivatives (<10% RSD). Recoveries were tested at concentration levels 0.050, 0.100 and 500 μg/mL (n=6) and established in range 72.0–98.0%. Data for repeatability and data for extraction efficiency are shown in Table 2.

CONCLUSION

GC-MS method after liquid-liquid extraction and derivatization with MSTFA is presented for screening as well as identification and simultaneous quantification of drugs and drugs of abuse in serum and can be suc-

Table 1. Slopes, y-intercepts and correlation coefficients of calibration curves done in doublet of the GC-MS assay for 19 commonly abused drugs and drugs of abuse, LOD and LOQ

			Linearity of calibration (in doublet)				
Analyte	**Derivative**	**IS**	**Slope (mean ± SD)**	**y-intercept (mean ± SD)**	**R^2**	**LOD µg/mL**	**LOQ µg/mL**
Ephedrine	2TMS	trimi-d_3	0.0004 ± 0.0001	0.0021 ± 0.0015	0.9962	0.050	0.100
MDMA	TMS	trimi-d_3	0.0004 ± 0.0001	0.0047 ± 0.0040	0.9953	0.050	0.100
Guaifenezin	2TMS	hexo	0.0011 ± 0.0001	0.0785 ± 0.0235	0.9970	<0.025	0.025
Tramadol	TMS	trimi-d_3	0.0062 ± 0.0018	–0.1556 ± 0.0214	0.9971	<0.025	0.025
Phenobarbital	2TMS	hexo	0.0004 ± 0.0005	0.0005 ± 0.0001	0.9930	<0.025	0.025
Amitriptyline		trimi-d_3	0.0040 ± 0.0001	0.0670 ± 0.0083	0.9995	<0.025	0.025
Cocaine		trimi-d_3	0.0019 ± 0.0002	0.0101 ± 0.0044	0.9974	<0.025	0.025
Mirtazapine		trimi-d_3	0.0029 ± 0.0003	0.0524 ± 0.0189	0.9918	<0.025	0.025
Dothiepin		trimi-d_3	0.0035 ± 0.0005	–0.0775 ± 0.0575	0.9932	<0.025	0.025
Citalopram		trimi-d_3	0.0044 ± 0.0001	0.1986 ± 0.0527	0.9913	<0.025	0.025
Clomipramine		trimi-d_3	0.0006 ± 0.0001	–0.0186 ± 0.0011	0.9978	<0.025	0.025
Diazepam		trimi-d_3	0.0020 ± 0.0004	0.0394 ± 0.0101	0.9979	<0.025	0.025
Codeine	TMS	trimi-d_3	0.0015 ± 0.0005	0.0165 ± 0.0035	0.9935	<0.025	0.025
Morphine	2TMS	trimi-d_3	0.0003 ± 0.0001	0.0035 ± 0.0012	0.9940	0.025	0.050
Levomepromazine		trimi-d_3	0.0014 ± 0.0001	0.0169 ± 0.0040	0.9994	<0.025	0.025
Bromazepam	TMS	trimi-d_3	0.0003 ± 0.0001	0.0119 ± 0.0007	0.9912	0.025	0.050
Zolpidem		trimi-d_3	0.0024 ± 0.0002	–0.0689 ± 0.0176	0.9983	<0.025	0.025
Clozapine		trimi-d_3	0.0012 ± 0.0001	0.0212 ± 0.0131	0.9994	<0.025	0.025
Alprazolam		trimi-d_3	0.0006 ± 0.0001	–0.0211 ± 0.0054	0.9958	0.050	0.100

cessfully applied to analysis of real samples in clinical and forensic toxicology. Bovine serum spiked with a mixture of the most commonly encountered drugs and drugs of abuse was used for determination of validation parameters as linearity, LOQ, LOD, recovery and repeatability. The calibration curves were linear in the range 0.025–2.000 µg/mL and LOQ was 0.025 µg/mL for most of all analytes or their TMS derivatives. Recoveries tested at concentration levels 0.050, 0.100 and 0.500 µg/mL were established in range 72.0–98.0%, repeatabilities measured at 0.050, 0.100 and 0.500 µg/mL were lower than 10.0%.

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Table 2. Repeatability and recovery of the GC-MS assay for 19 commonly abused drugs and drugs of abuse at the concentration levels 0.050, 0.100 and 0.500 μg/mL

			Repeatability RSD (%) n = 6			Recovery (%) n = 6		
Analyte	**Derivative**	**IS**	**0.050 μg/mL**	**0.100 μg/mL**	**0.500 μg/mL**	**0.050 μg/mL**	**0.100 μg/mL**	**0.500 μg/mL**
Ephedrine	2TMS	trimi-d_3	10.6	8.6	7.8	71.9	72.2	74.8
MDMA	TMS	trimi-d_3	9.4	8.2	6.1	79.2	84.3	86.5
Guaifenezin	2TMS	hexo	6.6	5.9	5.2	86.8	84.6	92.4
Tramadol	TMS	trimi-d_3	6.5	6.1	3.1	81.8	87.2	88.6
Phenobarbital	2TMS	hexo	7.1	5.5	4.1	78.4	79.4	80.4
Amitriptyline		trimi-d_3	7.7	6.8	5.7	89.1	90.8	90.3
Cocaine		trimi-d_3	7.5	5.4	4.2	99.8	98.7	96.6
Mirtazapine		trimi-d_3	8.8	6.6	4.3	73.5	75.7	79.1
Dothiepin		trimi-d_3	6.5	5.1	5.4	84.5	90.1	91.2
Citalopram		trimi-d_3	5.5	7.9	6.7	92.9	99.8	98.0
Clomipramine		trimi-d_3	6.4	5.7	3.9	82.2	79.8	86.4
Diazepam		trimi-d_3	8.8	5.0	4.3	96.2	97.3	97.5
Codeine	TMS	trimi-d_3	7.8	7.5	5.3	78.1	76.2	87.4
Morphine	2TMS	trimi-d_3	7.5	6.8	6.0	79.4	74.3	84.8
Levomepromazine		trimi-d_3	8.7	8.4	7.2	81.4	86.5	86.2
Bromazepam	TMS	trimi-d_3	9.4	7.9	5.2	74.6	77.1	83.0
Zolpidem		trimi-d_3	8.6	6.1	5.3	85.1	82.5	84.7
Clozapine		trimi-d_3	6.4	9.2	8.3	79.8	82.8	80.4
Alprazolam		trimi-d_3	7.5	6.9	5.9	76.6	78.8	79.5

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