Screening for and validated quantification of amphetamines and of amphetamine- and piperazine-derived designer drugs in human blood plasma by gas chromatography/mass spectrometry

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INTRODUCTION

The classical stimulants amphetamine, methamphetamine, ethylamphetamine and the amphetamine-derived designer drugs MDA, MDMA (‘ecstasy’), MDEA, BDB and MBDB have been widely abused for a relatively long time. In recent years, a number of newer designer drugs have entered the illicit drug market. 4-Methylthioamphetamine (MTA), p-methoxyamphetamine (PMA) and p-methoxymethamphetamine (PMMA) are also derived from amphetamine. Other designer drugs are derived from piperazine, such as benzylpiperazine (BZP), methylenedioxybenzylpiperazine (MDBP), trifluoromethylphenylpiperazine (TFMPP), m-chlorophenylpiperazine (mCPP) and p-methoxyphenylpiperazine (MeOPP). A number of severe or even fatal intoxications involving these newer substances, especially PMA, have been reported. This paper describes a method for screening for and simultaneous quantification of the above-mentioned compounds and the metabolites p-hydroxyamphetamine and p-hydroxymethamphetamine (pholedrine) in human blood plasma. The analytes were analyzed by gas chromatography/mass spectrometry in the selected-ion monitoring mode after mixed-mode solid-phase extraction (HCX) and derivatization with heptafluorobutyric anhydride. The method was fully validated according to international guidelines. It was linear from 5 to 1000 µg l⁻¹ for all analytes. Data for accuracy and precision were within required limits with the exception of those for MDBP. The limit of quantification was 5 µg l⁻¹ for all analytes. The applicability of the assay was proven by analysis of authentic plasma samples and of a certified reference sample. This procedure should also be suitable for confirmation of immunoassay results positive for amphetamines and/or designer drugs of the ecstasy type. Copyright © 2003 John Wiley & Sons, Ltd.

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MDEA especially popular among young people as so-called rave drugs of the ecstasy type. However, abuse of these substances is not without risk. A great number of severe and even fatal intoxications have been reported in literature. 2–9 Furthermore, it is well documented that these substances can cause irreversible damage to serotonin and dopamine nerve terminals in the central nervous system. 3–5,7,10–12 In addition to these classical compounds, a number of newer designer drugs have turned up on the illicit drug market in the last few years. Among them are the amphetamine-derived compounds 4-methylthioamphetamine (MTA), 13–17 p-methoxymethamphetamine (PMA), 9,18–25 and p-methoxymethamphetamine (PMMA). 9,24–27 Only little information exists on the pharmacology and toxicology of these compounds in humans, but all three have led to severe and fatal intoxications. 9,13,15–27 Most of these fatalities were attributable to PMA.

The other class of new designer drugs is derived from piperazine. They have also entered the illicit drug market in...
the last few years and can be divided into two groups: the benzylpiperazines 1-benzylpiperazine (BZP) itself and its methylenedioxymethamphetamine (MDMA) and also the phenylpiperazines 1-(3-trifluoromethylphenyl)piperazine (TFMPP), 1-(3-chlorophenyl)piperazine (mCPP) and 1-(4-methoxyphenyl)piperazine (MeOPP). These drugs have been mentioned in scene books and on so-called drug information sites on the internet (e.g. http://www.ewrowid.org, http://www.lycaeum.org) as psychoactive chemicals. There, the effects of BZP have been claimed to be comparable to those of AM and the effects of TFMPP to those of MDMA. Some pharmacological studies on BZP, mCPP and TFMPP could partly support the above-mentioned comparisons. However, little information exists on MDBP and MeOPP. Especially the two most important derivatives BZP and TFMPP are touted, e.g. as an alternative to amphetamine-derived designer drugs or as combinations with these. They are still legally sold in some countries. One fatality after ingestion of BZP and MDMA has already been reported and the increasing abuse of BZP and TFMPP in the USA led to the temporary placement of these two compounds in Schedule I of the Controlled Substance Act in September 2002.

In addition, designer drugs have not only led to more or less severe intoxications, they have also been described to cause driving impairment. For both reasons, screening procedures are needed in forensic and clinical toxicology, which allow reliable detection of these substances in biological matrices. Immunoassays (IA) of various types have often been used for this purpose. However, not all amphetamine IAs were suitable for detection of the amphetamine-derived designer drugs and especially not for the new piperazine-derived substances. In any case, positive IA results must be confirmed by a second independent method owing to their preliminary character. Gas chromatography/mass spectrometry (GC/MS) is the most widely used method for confirmation of positive IA screening tests. It not only allows discrimination of different designer drugs but also differentiation from non-scheduled therapeutics, which may also lead to positive IA results.

Numerous methods, mostly based on GC or liquid chromatography (LC) coupled to various detectors, have been published for the determination of amphetamines and classical designer drugs in biological matrices. They have been extensively reviewed in recent years. Unfortunately, the situation is very different for the newer drugs. Most analytical data are only available from case reports, in which little information is given on the objective performance (validation data) of the methods used. However, such information would be necessary to assess the reliability and usefulness of these methods in routine analysis. Validated assays have only been published for the determination of MTA and PMA in various biological matrices. These methods also allowed the determination of AM, MDA and MDMA, taking into account that designer drugs are often taken in combinations. Concerning PMMA and the piperazine-derived drugs, screening procedures have been reported for their detection in urine. Only for mCPP, a pharmacologically active metabolite of the antidepressants trazodone and nefazodone, has determination in plasma by GC been described. Owing to the growing importance of blood in analytical toxicology in recent years, especially in the context of driving under the influence of drugs, reliable methods for screening for and quantification/confirmation of the above-mentioned substances in this body fluid are needed, especially for the newer ones.

In addition, quantification of PMA, PMMA and their main metabolites 4-hydroxymethamphetamine (HO-AM) and 4-hydroxymethamphetamine (pholedrine, PHOL) in plasma may help to overcome the problems concerning differentiation between ingestion of these illicit drugs and ingestion of the antihypotonic medicament PHOL encountered in urinalysis.

In this paper, a GC/MS method after mixed-mode solid-phase extraction (SPE) and derivatization with heptafluorobutyric anhydride (HFBA) is presented for screening and simultaneous quantification of AM, MA, EA, MDA, MDMA, MDEA, BDB, MBDB, PMA, PMMA, MTA, BZP, TFMPP, mCPP, MeOPP and MDBP. The metabolites HO-AM and PHOL were also included for future studies on the differentiation of ingestion of PMA, PMMA and/or PHOL. The method was finally validated according to international criteria and its applicability was proven by analyzing authentic blood plasma samples and a certified reference sample.

**EXPERIMENTAL**

**Chemicals and reagents**

Methanolic solutions (1000 mg l\(^{-1}\)) of AM-\(d_5\) and MA-\(d_5\) and EA and methanolic solutions (100 mg l\(^{-1}\)) of MDMA-\(d_5\), MDA-\(d_5\) and MDEA-\(d_5\) were obtained from Promochem (Wesel, Germany). Hydrochlorides of AM, MA, MDA, MDMA, MDEA, BDB, MBDB, PMA, PMMA, MTA, BZP, TFMPP, mCPP, MeOPP and MDBP were also obtained from future studies on the differentiation of ingestion of AM, MA, MDA, MDMA, MDEA, BDB, MBDB, PMA, PMMA, MTA, BZP, TFMPP, mCPP, MeOPP and MDBP. The metabolites HO-AM and PHOL were also included for future studies on the differentiation of ingestion of PMA, PMMA and/or PHOL. The method was finally validated according to international criteria and its applicability was proven by analyzing authentic blood plasma samples and a certified reference sample.

**Plasma samples**

Pooled blank human plasma samples were used for validation of the procedure and were obtained from a local blood bank. Authentic human blood samples were submitted to the authors’ laboratory for toxicological analysis. A certified reference sample (Medidrug BTMF 2/99-B S-plus)
was obtained from Medichem (Steinenbronn, Germany). All samples were stored at −20°C.

Sample preparation
Plasma samples (1 ml) were diluted with 2 ml of purified water. After addition of 0.1 ml of a methanolic solution of the internal standards (IS) containing 1.0 mg l−1 each of AM-d5, MA-d5, MDA-d5, MDMA-d5, MDEA-d5 and pTP, the samples were briefly mixed (15 s) on a rotary shaker, centrifuged for 3 min at 1000 g and loaded on mixed-mode (HXC) SPE cartridges previously conditioned with 1 ml of methanol and 1 ml of purified water. After extraction, the cartridges were washed with 1 ml of purified water, 1 ml of 0.01 M aqueous hydrochloric acid and 2 ml of methanol. Reduced pressure was applied until the cartridges were dry, and the analytes were eluted with 1 ml of methanol–aqueous ammonia (98:2, v/v) into 1.5 ml polypropylene reaction vials. The eluates were washed down to dryness under a stream of nitrogen at 56°C. After addition of 20 µl of HFBA, the reaction vials were sealed and left on a rotary shaker (15 s). Derivatization was carried out under microwave irradiation (440 V, 5 min). After cooling, the derivatized extracts were briefly mixed (15 s) with 0.1 ml of hexane, and the mixtures were centrifuged for 15 s at 10,000 g to avoid spilling when opening the reaction vials. Then, 0.2 ml of an aqueous 0.5 M Na2PO4 solution was added and the vials were shaken on a rotary shaker for 3 min and centrifuged for 2 min at 10,000 g. The organic layers were transferred to autosampler vials and 2 µl were injected into the GC/MS system.

GC/MS analysis
Apparatus
The samples were analyzed using a Hewlett-Packard HP 6890 Series GC system combined with an HP 5972 Series mass-selective detector, an HP 6890 Series injector and an HP ChemStation G1701AA version A.03.00 (Agilent, Waldbronn, Germany).

GC conditions
The GC conditions were as follows: splitless injection mode; column, HP-5MS capillary (30 m × 0.25 mm i.d.), 5% phenylmethylsilsioxane, 250 nm film thickness; injection port temperature, 280°C; carrier gas, helium; flow-rate, 0.6 ml min−1; column temperature, initially 100°C, increased to 250°C at 10°C min−1 and to 310°C at 30°C min−1, and 310°C for 1 min.

MS conditions
The MS conditions were as follows: transfer line heater, 280°C; source temperature, 140°C; electron ionization (EI) mode; ionization energy, 70 eV; selected-ion monitoring (SIM) with the following program: solvent delay, 4 min; time window A, 4.00–6.20 min, m/z 92, 122, 244 (target ion, t) for AM-d5 and m/z 91, 118, 240 (t) for AM; time window B, 6.20–6.76 min, m/z 120, 213, 258 (t) for MA-d5 and m/z 118, 210, 254 (t) for MA; time window C, 6.76–7.60 min, m/z 254 (t) for MA, m/z 258 (t) for MA-d5 and m/z 118, 240, 268 (t) for EA; time window D, 7.60–8.50 min, m/z 240 (t), 303, 330 for HO-AM and m/z 121 (t), 148, 361 for PMA; time window E, 8.50–9.03 min, m/z 210, 254 (t), 330 for PHOL and m/z 136, 167 (t), 380 for MDA-d5 and m/z 135, 162 (t), 375 for MDA; time window F, 9.03–9.20 min, m/z 162 (t) for MDA, m/z 167 (t) for MDA-d5 and m/z 121, 148, 254 (t) for PMMA; time window G, 9.20–9.90 min, m/z 135 (t), 176, 389 for BDB, m/z 137 (t), 164, 377 for MTA and m/z 372 (t) for BZP; time window H, 9.90–10.35 min, m/z 91, 281, 372 (t) for BZP, m/z 200 (t), 229, 426 for TFMPP, m/z 164, 213, 258 (t) for MDMA-d5 and m/z 162, 210, 254 (t) for MDMA; time window I, 10.35–10.64 min, m/z 241, 273 (t), 408 for MDEA-d5, m/z 240, 268 (t), 403 for MDEA; time window J, 10.64–12.00 min, m/z 135, 176, 268 (t) for MBDB and m/z 91, 175 (t), 372 for pTP; time window K, 12.00–13.30 min, m/z 195, 392 (t), 394 for mCPP and m/z 135, 191, 388 (t) for MeOPP; time window L, 13.30–18.00 min, m/z 277, 105, 135 (t) for MBDB. The electron multiplier voltage (EMV) offset was set as follows: 4.00–7.60 min, 250 V; 7.60–8.50 min, 150 V; 8.50–9.20 min, 0 V; 9.20–10.35 min, 100 V; 10.35–13.30 min, 50 V; 13.30–18.00 min, 150 V.

Screening procedure
The presence of analytes was screened for by extraction of characteristic fragment ions from the summarized mass fragmentograms. Two groups of ions were used for this purpose, one for the amphetamines, the amphetamine-derived substances and their respective Is, m/z 121, 135, 137, 167, 240, 244, 258, 268 and 273, and the other for the piperazine-derived substances and their Is, m/z 135, 175, 200, 237, 200, 388 and 392. Positive peaks were checked for the presence of the three ions (target and qualifiers) each for the suspected analyte and the corresponding IS. For convenience, both operations were carried out by user-defined macros. For identification of the analytes, the peak area ratios (target vs qualifier ions) were required to be within ±20% of those obtained for the respective reference substance.

Quantification procedure
Quantification was carried out by comparison of peak area ratios (analyte vs IS) with calibration curves in which peak area ratios of spiked calibrators were plotted against their concentrations. The Is and analytes used for the calculation of peak area ratios were as follows: AM-d5 for AM and HO-AM; MA-d5 for MA and PHOL; MDA-d5 for MDA, BDB and MTA; MDMA-d5 for PMMA, MDMA, MBDB, MDEA-d5 for EA and MDEA; pTP for BZP, mCPP, MBDB, MeOPP and TFMPP.

Assay validation for plasma analysis
The GC/MS assay was fully validated according to international guidelines.69,70 The experimental design for the validation experiments was based on that proposed by Wieling et al.71 with modifications introduced by Peters et al.72

Preparation of solutions
The following stock solutions were prepared (concentrations of free bases): separate aqueous solutions (100 mg l−1) of AM, MA, MDA, MDMA, MDEA, BDB, MBDB and HO-AM; an aqueous solution of EA, PMA, PMMA, MTA, BZP, TFMPP,
mCPP, MeOPP, PHOL and MDBP (100 mg l\(^{-1}\) each, to avoid combined spiking volumes exceeding the final volumes); a methanolic stock solution (100 mg l\(^{-1}\) each) containing AM, MA, HO-AM, MDA, MDMA, BDB, MDBB and MDEA and a methanolic stock solution (100 mg l\(^{-1}\) each) containing EA, PMA, PMMA, MTA, BZP, TFMP, mCPP, MeOPP, PHOL and MDBP; a methanolic solution of AM-\(d_5\) and MA-\(d_5\) (100 mg l\(^{-1}\) each) prepared from commercially available methanolic solutions (1000 mg l\(^{-1}\)); a methanolic solution (100 mg l\(^{-1}\)) of pTP. From aqueous stock solutions aqueous analytical standard solutions containing AM, MA, MDA, MDMA, MDEA, BDB, MDBB, HO-AM, EA, PMA, PMMA, MTA, BZP, TFMP, mCPP, MeOPP, PHOL and MDBP (0.05, 0.1, 1.0, 2.5, 5.0, 7.5, 10.0 mg l\(^{-1}\), each) were prepared. Aqueous spiking solutions for the preparation of quality control (QC) samples containing the above-mentioned analytes (1.0, 2.5, 10.0 and 9.0 mg l\(^{-1}\), each) were also prepared from the stock solutions. Methanolic spiking solutions (0.25 and 9.0 mg l\(^{-1}\)) containing all analytes were prepared from the stock solutions for addition to samples used in extraction efficiency experiments. A methanolic working solution of the ISs (AM-\(d_5\), MA-\(d_5\), MDA-\(d_5\), MDMA-\(d_5\), MDEA-\(d_5\) and pTP; 1.0 mg l\(^{-1}\), each) was prepared from the stock solutions and commercially available solutions in methanol. All solutions were stored at 4 °C.

**Preparation of QC samples**

Pools of QC samples containing the above-mentioned analytes were prepared at four different concentrations: 10 µg l\(^{-1}\), limit of quantification QC sample (LQS); 25 µg l\(^{-1}\), low QC sample (LOW); 500 µg l\(^{-1}\), medium QC sample (MED); 900 µg l\(^{-1}\), high QC sample (HIGH). Each pool was prepared by transferring a defined volume of the corresponding spiking solution to volumetric flasks to which blank plasma was then added stepwise until the final volume had been reached. Before each addition step and after the final volume had been reached, the samples were thoroughly mixed to obtain homogeneous sample pools. The following volumes of spiking solutions were used at the given concentrations and final volumes (volume of spiking solution, concentration of spiking solution, final volume): LQS (0.25 ml, 1.0 mg l\(^{-1}\), 25.0 ml); LOW (0.5 ml, 2.5 mg l\(^{-1}\), 50.0 ml); MED (1.25 ml, 10.0 mg l\(^{-1}\), 25.0 ml) and HIGH (5.0 ml, 9.0 mg l\(^{-1}\), 50.0 ml).

**Peak purity and selectivity**

Blank plasma samples from 10 different sources were prepared as described above to check for peaks that might interfere with the detection of the analytes or the ISs. A zero sample (blank sample plus ISs) was analyzed to check for the absence of the ions of the ISs in the respective peaks of the analytes.

**Linearity of calibration**

Aliquots of blank plasma (1 ml) were spiked with 0.1 ml of the corresponding analytical standard solutions to obtain calibration samples at concentrations of 5, 10, 100, 250, 500, 750, and 1000 µg l\(^{-1}\) of each analyte (free bases). Replicates (\(n = 6\)) at each concentration were analyzed as described above. The regression line was calculated using a weighted \([1/(\text{concentration})^2]\) least-squares regression model. A weighted second-order model with the same weighting factors was also calculated. Daily calibration curves using the same concentrations (single measurement per concentration) were prepared with each batch of validation samples. The back-calculated concentrations of all calibration samples were compared with their respective nominal values. Calibrators whose back-calculated concentrations deviated more than ±15% (±20% near the LOQ) from their respective nominal values were excluded from the calculations of the daily calibration curves. If more than two calibrators were outside these limits, the whole calibration curve and the corresponding results were excluded from the calculations. Furthermore, the means and standard deviations (SD) of slopes and intercepts as well as the coefficients of determination \((R^2)\) of the daily calibration curves from the precision and accuracy experiments were calculated.

**Accuracy and precision**

QC samples (LQS, LOW, MED, HIGH) were analyzed as described above in duplicate on each of eight days. The concentrations in the QC samples were calculated based on the daily calibration curves. Accuracy was calculated in terms of bias as the percent deviation of the mean calculated concentration at each concentration level from the corresponding theoretical concentration. Repeatability (within-day precision) and time-different intermediate precision were calculated (as relative standard deviation, RSD) according to Massart et al.,\(^\text{73}\) using one-way ANOVA with the grouping variable 'day.'

**Processed sample stability**

For estimation of the stability of processed samples under the conditions of GC/MS analysis, LOW and HIGH QC samples (\(n = 10\) each) were extracted and derivatized as described above. The extracts obtained at each concentration were pooled. Aliquots of these pooled extracts at each concentration were transferred to autosampler vials and injected under the conditions of a regular analytical run at 3.3 h intervals over a total run time of 30 h. The stability of the derivatives was tested by regression analysis in which the absolute peak areas of each analyte at each concentration were plotted versus injection time. Instability of processed samples would be indicated by a negative slope significantly different from zero \((p < 0.05)\).

**Freeze–thaw stability**

For evaluation of freeze–thaw stability, QC samples (LOW and HIGH) were analyzed before (control samples; \(n = 6\) at each level) and after three freeze–thaw cycles (stability samples; \(n = 6\) at each level). For each freeze–thaw cycle, the samples were frozen at −20 °C for 21 h, thawed, and kept at ambient temperature for 3 h. The concentrations of the QC samples were calculated based on the daily calibration curves. Stability was tested against an acceptance interval of 90–110% for the ratio of the means (stability samples vs control samples) and an acceptance interval of 80–120% from the control samples mean for the 90% confidence interval (CI) of stability samples.
Extraction samples \( (n = 5) \) at low (25 \( \mu \)g l\(^{-1} \)) and high (900 \( \mu \)g l\(^{-1} \)) concentrations were prepared by spiking blank plasma (1 ml, previously diluted with 2 ml of purified water) with 0.1 ml each of the methanolic spiking solutions (0.25 and 9.0 mg l\(^{-1} \)) for the low and high extraction samples, respectively. Samples were loaded on SPE columns and extracted. Before evaporation, 0.1 ml of the IS solution was added to each eluate. For the control samples \( (n = 5) \), 1 ml of blank plasma was diluted with 2 ml of purified water, loaded on SPE columns, and extracted. Before evaporation, 0.1 ml each of the methanolic spiking solutions (0.25 and 9.0 mg l\(^{-1} \)) for the low and high control samples, respectively) and 0.1 ml of IS solution were added to each eluate. After evaporation, the residues were derivatized and analyzed as described above. Extraction efficiencies (mean and SD) were estimated by comparison of the peak area ratios (analyte vs IS) from extraction samples and control samples for each analyte at each concentration.

Limits
The lowest point of the calibration curve was the limit of quantification (LOQ) of the method (5 \( \mu \)g l\(^{-1} \) for each analyte). It was tested whether the signal-to-noise ratios (S/N) of all analytes was greater than 10. Furthermore, the accuracy and precision data of the LQS QC sample were compared with the criteria for the parameters at the LOQ established by Shah et al., accuracy within ±20% of the nominal value and a RSD <20%. The limit of detection (LOD) was not systematically evaluated. However, a spiked blank plasma sample containing 1 \( \mu \)g l\(^{-1} \) of all analytes was analyzed to check, whether this concentration would still be detectable (S/N > 3).

Proof of applicability
Plasma samples from authentic cases and a certified reference sample were assayed with the described method. The calculated concentrations of the reference sample were compared with the certified confidence intervals.

RESULTS AND DISCUSSION
Sample preparation
The analytes were isolated from 1 ml of plasma using a mixed-mode SPE procedure, previously reported to be versatile for the extraction of AM, MA, MDA, MDMA and MDEA and also neuroleptics from plasma.72,74,75 This sample volume was necessary to quantify the analytes reliably even at low concentrations. In the authors’ experience, no significant loss even of the most volatile analytes AM and MA occurred under the mild conditions used for evaporation.72 Therefore, addition of hydrochloric acid to the extracts prior to evaporation, which had been proposed to avoid evaporation losses,61,76 was not necessary. Derivatization with HFBA was used as it had proven to be useful for GC/MS analysis of amphetamines and amphetamine- and piperazine-derived compounds.61,68,76–80 Microwave irradiation allowed the derivatization time to be reduced to only 5 min, which is in accordance with the findings of Thompson and Dasgupta.81 However, the use of HFBA for derivatization can cause high background levels and degradation of the stationary phase of the GC column, if excess reagent is not completely removed before injection into the GC/MS system.77,80 In the authors’ experience, removal of excess HFBA and its reaction by-product heptafluorobutyric acid by evaporation is problematic owing to the low volatility of the excess reagent. This makes it almost impossible to evaporate all excess reagent without losing considerable amounts of the heptafluorobutyryl (HFB)-derivatized analytes, especially of AM and MA. Therefore, the HFB derivatives were extracted into hexane and any excess reagent was removed from the organic phase by washing with the sodium phosphate solution. This solution effectively neutralized heptafluorobutyric acid formed by hydrolysis of HFBA. The resulting phosphate buffer kept the pH value of the aqueous phase in the neutral range. At these pH values heptafluorobutyric acid was present in the ionized form and thus was effectively washed out of the organic phase. Therefore, evaporation with the risk of analyte losses was no longer necessary. In addition, the presented procedure was simpler than the double wash step procedures described previously.76,77

GC/MS analysis
The analytes were separated by GC and detected by MS operated in the SIM mode to enhance sensitivity. Modifications of the EMV offset were necessary to obtain comparable linear dynamic ranges of the apparatus for all analytes.

Three characteristic ions, one target and two qualifier ions, were chosen from the corresponding full-scan mass spectrum. The spectra, structures and postulated predominant fragmentation patterns are shown in Fig. 1 in order to demonstrate the selection of the target and qualifier ions. In some cases, less characteristic ions had to be chosen owing to interferences in the fragmentograms of the more characteristic alternatives.

A long temperature program of 18 min was necessary to obtain sufficient separation. Baseline separation was obtained for all analytes except for the pairs HO-AM–PMA, MDMA-d5–TFMPP and mCPP–MeOPP, which partly co-chromatographed. Both analytes of the first pair contained the fragment ion of m/z 240, but they could be differentiated via the target and respective qualifier ions. Furthermore, they were separated almost down to the baseline, allowing quantification of HO-AM via its base peak at m/z 240. The second and third pair had no common fragment ions and could therefore be easily differentiated from each other.

The determination of time windows for SIM analysis was a critical task, as some peaks eluted close to each other. Therefore, the time windows had to be re-adjusted, e.g. after shortening of the column during routine maintenance of the apparatus. Furthermore, the target ions of compounds eluting close of the end of a time window were monitored also in the following time windows (m/z 254, 258 in C and m/z 162, 167 in F). In analogy, target ions of compounds eluting close to the beginning of a time window were also monitored in the respective preceding time window (m/z 372...
Figure 1. El mass spectra, structures and postulated predominant fragmentation patterns of HFB derivatives of AM-d$_5$ (1), AM (2), MA-d$_5$ (3), MA (4), EA (5), HO-AM (6), PMA (7), PHOL (8), MDA-d$_5$ (9), MDA (10), PMMA (11), BDB (12), MTA (13), BZP (14), TFMPP (15), MDMA-d$_5$ (16), MDMA (17), MDEA-d$_5$ (18), MDEA (19), MBDB (20), pTP (21), mCPP (22), MeOPP (23) and MDBP (24). The fragment ions selected for SIM are underlined.
Figure 1. (Continued).
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in G). This allowed quantification of the respective analytes even if the separation line of the time windows was not situated exactly between the peaks. In Fig. 2, merged mass fragmentograms after SPE and derivatization with HFBA of a blank sample (top) and a spiked calibration sample containing 100 µg 1⁻¹ of each analyte and IS (bottom) are shown. The numbers of the peaks correspond to those of the mass spectra in Fig. 1.

**Screening procedure**

The presence of analytes was screened for via characteristic fragment ions. User-defined macros extracting these fragment ions from the summarized mass fragmentograms were used to facilitate this procedure. As the number of ions in these macros was limited to 10, two screening macros, one for the amphetamines and amphetamine-derived drugs and the other for the piperazine-derived drugs, were used. For all substances, the target ion was used for screening, with the exception of MDA. Owing to the above-mentioned limitation of 10 ions per macro, this analyte had to be screened via its base peak m/z 135, which was also the target ion of BDB. Peaks that appeared in the extracted fragmentograms of the screening ions were checked for the simultaneous presence of the target and the two qualifier ions. If this criterion was
Figure 2. Merged mass fragmentograms with the given ions of plasma samples after SPE and derivatization with HFBA. Top: blank plasma sample. Bottom: spiked calibration sample containing 100 µg l⁻¹ of each analyte and IS. The peaks indicate AM-d₅ (1), AM (2), MA-d₅ (3), MA (4), EA (5), HO-AM (6), PMA (7), PHOL (8), MDA-d₅ (9), MDA (10), PMMA (11), BDB (12), MTA (13), BZP (14), TFMPP (15), MDMA-d₅ (16), MDMA (17), MDEA-d₅ (18), MDEA (19), MBDB (20), pTP (21), mCPP (22), MeOPP (23) and MDBP (24).

The numbers of the peaks correspond to those of the mass spectra in Fig. 1.

fulfilled and if the peak area ratios (target vs qualifier ions) were further within ±20% of those obtained for the respective reference substance, the sample was judged positive for this particular analyte. Again, user-defined macros were used to facilitate this task. Inclusion of the corresponding characteristic ions of the ISs in each analyte-specific macro provided an additional identification criterion, as the retention times of the analytes and ISs could be compared. This was especially useful if deuterated analogues were used as IS. A closer look at the mass spectra of the deuterated ISs AM-d₅, MA-d₅, MDA-d₅, MDMA-d₅ and MDEA-d₅ showed that they were not entirely free of fragments used as qualifiers for the corresponding analytes (m/z 91 for AM, m/z 118 for AM and MA, m/z 135 for MDA and MDMA, m/z 162 for MDA and MDMA). Similar findings have been reported by Urry et al.82 Unfortunately, more selective fragments which could have been used as alternative qualifiers were not present in the mass spectra of these analytes. In cases of
doubt, especially at low analyte concentrations, it is recommended to re-analyze the sample without addition of the ISs. Anyway, no such problem occurred with any of the chosen target ions, so that quantification was not affected.

At this point, it must be mentioned that some of the analytes are also metabolites of other structurally related compounds, which must be considered in clinical or forensic interpretation of the result. For example, AM may be a metabolite of amphetamine, clofenoxone, OA, famprofazone, fenetyline, fenproporex, mefenorex, MA, prenylamine and selegiline; MA of famprofazone and selegiline; HO-AM of clofenoxone, EA, fenproporex, MA, PMA, PMMA and selegiline; PHOL of famprofazone, MA, PMMA and selegiline; mCPP of nefazodone and trazodone.

Quantification procedure and the choice of suitable ISs
Quantification was carried out by comparison of peak area ratios (analyte vs IS) with calibration curves obtained with spiked calibrators. Unfortunately, deuterated analogues as ISs were not available for all analytes. Therefore, it was tested which of the used ISs was most applicable for which analyte. The following combinations proved to be most useful: AM-d5 for AM and HO-AM; MA-d3 for MA and PHOL; MDA-d5 for MDA, BD, PMA and MTA; MDMA-d5 for MDMA, MBDB and PMMA; MDEA-d5 for MDEA and EA; pTP for BZP, mCPP, MDPB, MeOPP and TFMP. As none of the deuterated analogues had been found to be applicable as IS for the pipеразине-derived compounds, the structurally related IS pTP was chosen for these analytes. These results show that, if no deuterated analogue is available, the similarity of the side-chain is obviously a better predictor for the suitability of the IS than, e.g., retention time or substitution of the aromatic ring. This hypothesis is supported by the fact that MA-d5 also proved to be applicable as an IS for MBDB, whereas the much closer eluting MDEA-d5 did not. A similar observation was made for EA. Here, the closer eluting MA-d5 led to worse results than MDEA-d5, which has a side-chain similar to that of EA. Furthermore, the use of 4-(1-aminoethyl)phenol, which was tested as an alternative IS for HO-AM and PHOL because of its combined 4-hydroxy and amino moieties, did not lead to useful results.

Assay validation
The GC/MS assay was validated according to international guidelines. The experimental design employed had proved to be useful.

Peak purity and selectivity
No peaks interfering with the analytes or the ISs were detected in blank plasma samples from 10 different sources. Owing to the above-mentioned presence of qualifier fragments of some analytes in the mass spectra of their deuterated analogues, minor peaks were observed in the zero sample (blank + ISs). However, this problem was not observed for any of the target ions so that quantification was not affected.

Linearity of calibration
Replicates (n = 6) of matrix calibrators at seven different concentrations from 5 to 1000 µg l⁻¹ were analyzed. This range covers the concentrations to be expected in plasma

| Table 1. Slopes, y-intercepts and coefficients of determination of daily calibration curves and extraction efficiencies at low and high concentrations of the GC/MS assay for amphetamines and amphetamine- and piperazine-derived designer drugs |
|---|---|---|---|
| Analyte | IS | Slope (mean ± SD) (n = 8) | y-Intercept (mean ± SD) (n = 8) | R² (range) (n = 8) | Extraction efficiency (mean ± SD) (%) |
| | | | | | Nominal concentration (µg l⁻¹) |
| AM | AM-d5 | 0.009 ± 0.0001 | 0.017 ± 0.0009 | 0.994–1.000 | 100 ± 4.9 | 89 ± 1.7 |
| MA | MA-d5 | 0.009 ± 0.0001 | –0.002±0.0027 | 0.997–1.000 | 102 ± 6.1 | 90 ± 1.3 |
| EA | MDEA-d5 | 0.055 ± 0.0047 | –0.016±0.0181 | 0.995–1.000 | 105 ± 6.5 | 94 ± 4.6 |
| HO-AM | AM-d5 | 0.003 ± 0.0004 | 0.001 ± 0.0012 | 0.976–0.997 | 38 ± 5.6 | 30 ± 1.7 |
| PMA | MDA-d5 | 0.026 ± 0.0016 | –0.009±0.0088 | 0.993–1.000 | 104 ± 3.7 | 89 ± 1.7 |
| PHOL | MA-d5 | 0.012 ± 0.0016a | 0.006 ± 0.0074a | 0.983–0.998a | 88 ± 11.3 | 80 ± 10.6 |
| MDA | MDA-d5 | 0.016 ± 0.0002 | 0.005 ± 0.0031 | 0.998–1.000 | 102 ± 2.5 | 88 ± 1.7 |
| PMMA | MDMA-d5 | 0.004 ± 0.0002 | –0.002±0.0009 | 0.995–0.999 | 103 ± 7.3 | 98 ± 6.6 |
| BDB | MDA-d5 | 0.015 ± 0.0008 | 0.000 ± 0.0023 | 0.997–1.000 | 94 ± 2.7 | 86 ± 4.4 |
| MTA | MDA-d5 | 0.011 ± 0.0010 | –0.004±0.0053 | 0.991–0.997 | 110 ± 6.1 | 94 ± 7.6 |
| BZP | pTP | 0.006 ± 0.0008 | –0.011±0.0029 | 0.981–0.999 | 98 ± 17.8 | 93 ± 6.3 |
| TFMP | pTP | 0.012 ± 0.0010 | –0.001±0.0014 | 0.996–1.000 | 96 ± 3.7 | 92 ± 2.3 |
| MDMA | MDMA-d5 | 0.009 ± 0.0002 | –0.002±0.0020 | 0.998–1.000 | 101 ± 1.2 | 90 ± 0.4 |
| MDEA | MDMA-d5 | 0.010 ± 0.0002 | –0.003±0.0020 | 0.998–1.000 | 103 ± 6.8 | 93 ± 1.1 |
| MBDB | MDMA-d5 | 0.006 ± 0.0001 | –0.001±0.0016 | 0.996–1.000 | 100 ± 2.7 | 95 ± 3.8 |
| mCPP | pTP | 0.004 ± 0.0006 | –0.002±0.0009 | 0.998–1.000 | 69 ± 0.9 | 63 ± 1.8 |
| MeOPP | pTP | 0.009 ± 0.0011 | –0.006±0.0035 | 0.993–0.999 | 106 ± 7.6 | 96 ± 3.1 |
| MDBP | pTP | 0.053 ± 0.0060b | –0.044±0.0389b | 0.983–0.998b | 107 ± 7.8 | 82 ± 12.4 |

a n = 7 (one day excluded).
b n = 6 (two days excluded).
samples after recreational use of AM, MA, MDA, MDMA and MDEA. For the other compounds, only little information on plasma concentrations in non-fatal cases is available. However, one would expect the plasma concentrations of these compounds to lie in the same range as for the classical compounds. A weighted linear regression model was used to account for unequal variances (heteroscedasticity) across the calibration range. The inverse of the squared concentration was found to be an appropriate weighting factor. A weighted second-order model was also evaluated to check for a curvature in the data. Only for PMA, MDA and MDBP was a slight curvature detected. However, as proposed by Hartmann et al., the simpler linear model should be used if the data for accuracy and precision are within required limits. As this was the case for all of these three analytes, the slight curvatures were negligible and a weighted linear model was used for all 18 analytes.

For all following experiments, daily calibration curves were prepared with each batch of validation samples. The back-calculated concentrations of all calibration samples were compared with their respective nominal values. As proposed by Shah et al., calibrators whose back-calculated concentrations deviated more than ±15% (±20% near the LOQ) of their respective nominal values were excluded from the calculations. However, such observations were only made for BZP, MDBP, HO-AM and PHOL. In the case of PHOL, one complete calibration curve had to be discarded because of two outlying calibrators; in the case of MDBP, the same applied to two calibration curves. The corresponding data were excluded from the accuracy and precision calculations. In Table 1, slopes, intercepts (means ± SDs) and coefficients of determination of all daily calibration curves from the accuracy and precision experiments and data for extraction efficiencies are shown. The SDs of the slopes corresponded to RSD values of <15% for all analytes, showing that calibration curves were reproducible. For such analytes, for which a deuterated analogue was available as IS even RSD values of <2.5% were obtained.

**Accuracy and precision**

QC samples (LQS, LOW, MED, HIGH) were analyzed in duplicate on each of eight days as proposed by Hartmann et al. The concentrations in the QC samples were calculated based on the daily calibration curves. Accuracy, repeatability and time-different intermediate precision were calculated as described above. The results are shown in Table 2. The data for accuracy, in terms of bias, were all within the acceptance limits (±15% of the nominal value, ±20% near LOQ) specified by Shah et al. The criteria for repeatability (within-day precision) and time-different intermediate precision (combined within-day and between-day effects) were all within acceptance limits (±15% RSD (<20% at the LOQ), <20% near LOQ) specified by Shah et al. The only exception was the value for intermediate precision of the HIGH QC sample of MDBP. However, at this point it must be mentioned that the data for one day for PHOL and two days for MDBP had been excluded.

### Table 2. Repeatability, intermediate precision and accuracy data of the GC/MS assay for amphetamines and amphetamine- and piperazine-derived designer drugs [n = 16 (8 days × 2 replicates) at each level]

<table>
<thead>
<tr>
<th>Analyte</th>
<th>IS</th>
<th>Nominal concentration (µg l⁻¹)</th>
<th>Repeatability, RSD (%)</th>
<th>Intermediate precision, RSD (%)</th>
<th>Accuracy, bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>25</td>
<td>500</td>
<td>900</td>
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<tr>
<td>AM</td>
<td>AM-d₅</td>
<td>LQS</td>
<td>LOW</td>
<td>MED</td>
<td>HIGH</td>
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<tr>
<td>18.7</td>
<td>18.7</td>
<td>9.5</td>
<td>0.8</td>
<td>1.5</td>
<td>1.9</td>
</tr>
<tr>
<td>MA</td>
<td>MA-d₅</td>
<td>3.5</td>
<td>5.5</td>
<td>0.7</td>
<td>1.5</td>
</tr>
<tr>
<td>EA</td>
<td>MDEA-d₅</td>
<td>5.6</td>
<td>5.6</td>
<td>3.3</td>
<td>1.2</td>
</tr>
<tr>
<td>OH-AM</td>
<td>AM-d₅</td>
<td>18.6</td>
<td>8.8</td>
<td>11.4</td>
<td>8.0</td>
</tr>
<tr>
<td>PMA</td>
<td>MDA-d₅</td>
<td>5.6</td>
<td>9.0</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td>PHOL</td>
<td>MA-d₅</td>
<td>18.0₅</td>
<td>8.1₅</td>
<td>7.0₅</td>
<td>7.6₅</td>
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<tr>
<td>MDA</td>
<td>MDA-d₅</td>
<td>3.1</td>
<td>4.1</td>
<td>1.2</td>
<td>1.5</td>
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<tr>
<td>PMMA</td>
<td>MDMA-d₅</td>
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<td>5.5</td>
<td>2.4</td>
<td>2.5</td>
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<tr>
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<td>MDA-d₅</td>
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<td>4.3</td>
<td>1.4</td>
<td>2.7</td>
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<tr>
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<td>MDMA-d₅</td>
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<td>5.4</td>
<td>6.2</td>
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<tr>
<td>BZP</td>
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<td>9.9</td>
<td>5.7</td>
<td>10.5</td>
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<tr>
<td>TFMP</td>
<td>pTP</td>
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<td>8.0</td>
<td>3.2</td>
<td>3.6</td>
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<tr>
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<td>4.3</td>
<td>1.5</td>
<td>1.2</td>
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<tr>
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<td>4.4</td>
<td>2.0</td>
<td>1.5</td>
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<td>MBDB</td>
<td>MDMA-d₅</td>
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<td>3.7</td>
<td>2.6</td>
<td>2.0</td>
</tr>
<tr>
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<td>pTP</td>
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<td>9.3</td>
<td>3.3</td>
<td>4.4</td>
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<td>MeOPP</td>
<td>pTP</td>
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<td>8.8₅</td>
<td>6.3₅</td>
<td>6.4₅</td>
</tr>
</tbody>
</table>

*Precision = (SD/mean) × 100.
Accuracy = [(mean calculated concentration − nominal concentration)/actual concentration] × 100.

a n = 14 (one day excluded).
b n = 12 (two days excluded).
from the calculations because of unacceptable calibration curves. Inclusion of these days would have changed the data for PHOL only very little, while the data for MDBP would have been outside the acceptance limits at two more concentration levels. These results show that the assay could not be considered to be valid for the quantification of MDBP. However, MDBP has been of little importance so far and the assay still allows one to obtain semiquantitative data for this analyte. Concerning the analysis of PHOL, the calibration curves should be closely monitored for outliers.

**Processed sample stability**

LOW and HIGH QC samples (n = 10 each) were extracted and derivatized as described above. Aliquots of the pooled extracts at each concentration were transferred to autosampler vials and injected at time intervals of 3.3 h. Regression analysis, plotting absolute peak areas of each analyte at each concentration against injection time, resulted in slopes not significantly different from zero (p > 0.05) for all analytes at both concentration levels. Therefore, there was no indication of instability of processed samples over a time period of 30 h under the conditions of a regular analytical run.

**Freeze–thaw stability**

For evaluation of freeze–thaw stability, QC samples (LOW and HIGH) were analyzed before (control samples) and after three freeze–thaw cycles (stability samples). For each freeze–thaw cycle, the samples were frozen at −20 °C for 21 h, thawed, and kept at ambient temperature for 3 h as proposed previously. This procedure allowed the simultaneous evaluation of freeze–thaw stability and benchtop stability, i.e. stability of the analytes in the matrix at ambient temperature over the expected maximum period of time needed for preparation of a batch of samples. The first criterion, ratio of means (stability vs control samples) within 90–110%, was fulfilled for all analytes at both concentrations. The second criterion, 90% CI for stability samples within 80–120% of control mean, was violated only by MTA at the LOW level and by HO-AM and PHOL at the high level. The lower 90% confidence limit of MTA outside the lower limit
of the acceptance interval might indicate instability of this substance under the applied conditions. Therefore, repeated freezing and thawing of samples suspected to contain MTA should be avoided. No obvious explanation could be found for the upper 90% confidence limits of HO-AM and PHOL lying outside the upper acceptance limits. However, daily differences in the calibration curves and/or the low precision (high RSD values) for these compounds might explain this phenomenon.

**Extraction efficiency**

For the estimation of extraction efficiency, the analytes were spiked into blank plasma samples and into the eluates of blank plasma samples for the preparation of extraction and control samples, respectively. Methanolic solutions containing low and high concentrations of all analytes were used for this purpose because aqueous solutions might have caused losses, especially of AM and MA, during evaporation. The ISs were added to the eluates of both extraction and control samples. This procedure allowed the estimation of extraction efficiency by comparison of the peak area ratios (analyte vs IS) of extraction samples with those of the control samples. The results, including SDs, are listed in Table 1. These data show, that all analytes except HO-AM were extracted effectively. The results of preliminary experiments had indicated that at the native pH of blood plasma (7.4), the reversed-phase part of the mixed mode sorbent was important for the initial retention of the analytes. However, owing to the rather hydrophilic properties of HO-AM, its affinity to this fraction of the sorbent should be low compared with the other analytes. Fortunately, no sensitivity problems occurred with HO-AM, so that its low extraction efficiency was of no practical relevance.

**Limits**

The lowest points of the calibration curves were the practical LOQ. Values of \( S/N > 10 \) were observed at this concentration for the target ions of all analytes. Furthermore, the accuracy and precision of the LQS samples (cf. Table 2) were within the acceptance limits (accuracy within \( \pm 20\% \) of the nominal concentration).

![Figure 4](image-url)

**Figure 4.** Top: merged mass fragmentograms of the target ions of piperazine-derived designer drugs and their IS of an authentic plasma sample after SPE and derivatization with HFBA. Peak 14 indicates the possible presence of BZP, the peak marked by an arrow indicates the possible presence of MDBP. Bottom, left: merged mass fragmentograms of target and qualifier ions BZP and its IS. Peak 14 indicates 100.4 \( \mu g/l \) of BZP and peak 21 indicates 100 \( \mu g/l \) of pTP. Bottom, right: merged mass fragmentograms of target and qualifier ions show no peak containing the corresponding target and qualifier ions of MDBP.
value and a RSD <20%) specified for concentrations near the LOQ by Shah et al.70 The LOD was not systematically evaluated. However, in a spiked sample containing 1 µg l⁻¹ of each analyte all target ions were still detectable (S/N > 3).

Unfortunately, this was not the case for all qualifier ions. Therefore, most analytes could not be identified reliably at these concentrations and the authors recommend the use of the LOQ as a cutoff value.

Proof of applicability

Plasma samples from authentic cases and a certified reference sample were assayed with the described method. In Fig. 3 (top), merged mass fragmentograms of the given characteristic ions of amphetamines, amphetamine-derived designer drugs and their ISs are shown of an authentic plasma sample after SPE and derivatization with HFBA. Peaks 2, 10 and 17 indicate the possible presence of AM, MDA and MDMA and peaks 1, 3, 9, 16 and 18 indicate the ISs. In Fig. 3 (bottom), merged mass fragmentograms of target and qualifier ions of the three analytes in question AM (left), MDA (middle) and MDMA (right) and their respective ISs are shown. The presence of the respective target and qualifier ions in the peaks of these analytes and deviations of their peak area ratios (target vs qualifier ions) from those obtained for the respective reference substances ranging from −7.4 to 2.2% proved their presence in the sample. Their concentrations were determined to be 110.8 µg l⁻¹ for AM, 97.9 µg l⁻¹ for MDA and 460.5 µg l⁻¹ for MDMA.

Figure 4 (top) shows merged mass fragmentograms of the given target ions of piperazine-derived designer drugs and their IS of an authentic plasma sample after SPE and derivatization with HFBA. Peak 14 indicates the possible presence of BZP, the peak marked by an arrow indicates the possible presence of MDPB. Peak 21 indicates the IS. In Fig. 4 (bottom, left), merged mass fragmentograms of target and qualifier ions of BZP and its IS are shown. The presence of the respective target and qualifier ions in the peak of the analyte in question and deviations of their peak area ratios (target vs qualifier ions) from those obtained for the reference substance being −12.1 and −17.9% proved the presence of BZP in the sample. Its concentration was determined to be 100.4 µg l⁻¹. In Fig. 4 (bottom, right), merged mass fragmentograms of target and qualifier ions show that no peak contains the corresponding target and qualifier ions of MBDB, so that its presence in this sample could not be confirmed.

The certified sample assayed was found to contain 81.8 µg l⁻¹ of AM (target concentration, 78.1 µg l⁻¹; certified confidence range, 59.7–96.5 µg l⁻¹), 77.4 µg l⁻¹ of MDA (80.7 µg l⁻¹; 61.8–99.6 µg l⁻¹), 82.1 µg l⁻¹ of MDMA (83.6 µg l⁻¹; 64.1–103.1 µg l⁻¹), 57.1 µg l⁻¹ of MDEA (61.7 µg l⁻¹; 46.6–76.8 µg l⁻¹), 103.0 µg l⁻¹ of MA and 11.3 µg l⁻¹ of MBDB. For the last two, only approximate target values of 105.6 µg l⁻¹ (MA) and 25.0 µg l⁻¹ (MBDB) had been given. The deviation of the measured MBDB concentration from the target value remained unclear, as no certified confidence range had been given.

CONCLUSIONS

The GC/MS assay presented here is the first allowing screening for and simultaneous and reliable quantification of amphetamines, classical and new amphetamine-derived designer drugs and new piperazine-derived designer drugs in human blood plasma. Only for MDPB did the validation data not fulfil the established criteria, thus allowing only semiquantification by means of this assay. This procedure should be of help in acquiring reliable data in plasma, especially for the newer designer drugs needed for the interpretation of toxicological findings in clinical and forensic toxicology. In addition, this procedure should be suitable for the confirmation of immunoassay results positive for amphetamines and/or designer drugs of the ecstasy type.

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REFERENCES