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Single-step procedure for gas chromatography–mass spectrometry screening and quantitative determination of amphetamine-type stimulants and related drugs in blood, serum, oral fluid and urine samples

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Abstract

We describe a rapid GC/MS assay for amphetamine-type stimulant drugs (ATSs) and structurally related common medicaments in blood, serum, oral fluid and urine samples. The drugs were extracted from their matrices and derivatized with heptafluorobutyric anhydride (HFBA) in a single step, using the following procedure: 100 μl (oral fluid) or 200 μl (blood, serum, urine) of the sample were mixed with 50 μl of alkaline buffer and 500 μl of extraction–derivatization reagent (toluene + HFBA + internal standard), centrifuged, and injected into a GC/MS apparatus. As revealed by the validation data this procedure, with its limit of quantitation being set at 20 ng/ml for oral fluid, 25 ng/ml for blood or 200 ng/ml for urine, is suitable for screening, identification and quantitative determination of the ATSs and related drugs in all the matrices examined. Thus, time-consuming and expensive multiple analyses are not needed, unless specifically required.

Keyword: Amphetamine-type stimulant drugs

1. Introduction

The expanding market for synthetic amphetamine-type stimulant drugs (ATSs), including amphetamine, methamphetamine (MA), and 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy'), is currently believed to be one of the world's most severe future drug problems [1]. A particular challenge to laboratories performing drug tests is the fact that the ATS market is also changing, partly in response to the efforts of drug control authorities and partly as a result of the dynamics of abuse patterns. This trend is evidenced, for example, by the recent reports of fatalities related to the thus far less common ATSs such as 3,4-methylenedioxyethylamphetamine (MDEA), *para*-methoxyamphetamine (PMA) and 4-methylthioamphetamine (4-MTA, also called *para*-methylthioamphetamine) [2–9].

Routine analyses of biological samples for ATSs are performed on a daily basis in professional clinical, forensic and toxicological laboratories using high-quality mass spectrometric (MS) methods. However, preliminary screening to determine whether the sample is subject to confirmation analysis is frequently carried out using procedures based on immunological identification. In the case of ATSs, this practice has two major drawbacks: firstly, the capability of immunoassays to detect the wide variety of ATSs and related substances is limited, and secondly, certain common medicaments, or

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their metabolites, may produce false-positive results [10–15]. Given that positive cases are (or should be) always confirmed with MS analysis, thus identifying the cause of the positive immunological result, the false-positives are usually not a problem. However, the apparently negative results may be left unconfirmed, leading to false-negative results even when high concentrations of ATSs are present.

MS confirmation analysis should be sensitive enough to provide the highest level of confidence. To date, the most widely used method is gas chromatography/mass spectrometry (GC/MS) [16]. The compounds with the amphetamine core structure, however, have base peaks at low masses, resulting in interference from biological background. This can be overcome by the use of derivatization, a step necessary for improving the GC properties of the compounds as well [17]. The plenitude of existing GC/MS procedures for determination of ATSs and related drugs has been reviewed recently [16]. Most of these procedures consisted of liquid–liquid or solid-phase extraction, followed by a separate derivatization step to yield, e.g. heptafluorobutyrated or acetylated derivates [16]. Regarding a routine laboratory with a large number of samples to be analyzed in a short time, a major drawback of derivatization is that the procedure becomes laborious and time-consuming.

Traditionally, urine has been the most common sample matrix to be screened for recent drug use in living subjects, but along with the advances in analytic techniques, blood (whole blood, serum, plasma), which reflects systemic drug concentrations more accurately, has become the matrix of choice [18]. Recently, these conventional matrices have been supplemented by oral fluid (saliva), which is believed to reflect systemic drug concentration–time profiles comparably with blood [19]. In addition, the non-invasive sampling of oral fluid enables on-site testing applications such as roadside drug testing, which may increase the future importance of this matrix.

Despite the large number of publications describing sophisticated procedures for ATS analysis, there is still need for an assay as rapid and labour saving as the immunoassays and with the versatility and accuracy of confirmation analyses. The aim here was to develop a single-step GC/MS procedure capable of screening, identifying and quantitating a wide variety of ATSs as well as structurally related medicinal drugs in whole blood, serum, oral fluid and urine samples. The basis for development of the method was heptafluorobutyric anhydride (HFBA) derivatization, which has proven highly reliable in our lab during two decades and which is probably the most widely accepted derivatization reagent for the ATSs [16,17]. The following drugs were included in the study: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), MDMA, MDEA, 4-MTA, PMA, ephedrine, norephedrine (phenylpropanolamine), pseudoephedrine, cathinone, 1-(1′,3′-benzodioxol-5′-yl)-2-butanamine (BDB), *N*-methyl-1-(1′,3′-benzodioxol-5′-yl)-2-butanamine (MBDB), 4-bromo-2,5-dimethoxyphenethylamine (2C-B, “nexus”), and 1-benzylpiperazine.

2. Experimental

2.1. Chemicals and reagents

Amphetamine sulphate and pseudoephedrine hydrochloride were purchased from Sigma (St. Louis, MO, USA). MDA hydrochloride, MDEA hydrochloride, MDMA hydrochloride, BDB hydrochloride and MBDB hydrochloride were obtained from RBI (Natick, MA, USA), the last two as 1 mg/ml of free base (w/v in methanol). Cathinone hydrochloride and 2C-B hydrochloride were purchased from Radian Corporation (Austin, TX, USA) as 1 mg/ml of free base in methanol. Ephedrine hydrochloride was obtained from the University Pharmacy (Helsinki, Finland), 4-MTA hydrochloride from the Scientific Institute of Public Health—Louis Pasteur (Brussels, Belgium) and 1-benzylpiperazine from Fluka Chemie GmbH (Buchs, Switzerland). MA hydrochloride and PMA hydrochloride were donated by the UN Narcotics Laboratory (Vienna, Austria), norpseudoephedrine hydrochloride by the National Institute on Drug Abuse (NIDA; Bethesda, MD, USA) and norephedrine hydrochloride by Orion Corporation (Espoo, Finland). Methylmexiletine, which was used as the internal standard (IS), was obtained from Boehringer Ingelheim GmbH (Ingelheim am Rhein, Germany). Sheep blood was obtained from the Department of Internal Services, National Public Health Institute, Helsinki, Finland. Drug-free human blood, serum, urine and oral fluid were collected from the laboratory staff. Oral fluid was collected by spitting in a test tube.

The alkaline extraction buffer was prepared by mixing 1.5 ml of 10 M KOH with 8.5 ml of saturated $NaHCO_3$. Derivatives of the psychoactive amines were formed using HFBA (Fluka). The extraction–derivatization reagent was a mixture containing, as calculated per sample, 485 μl of toluene, 0.500 μg of methylmexiletine as IS and 15 μl of HFBA. All the reagents used were of the highest quality.

2.2. Extraction–derivatization and GC/MS determination

The 15 psychoactive amines were extracted and derivatized in a single step by mixing 100 μl (oral fluid) or 200 μl of sample (blood, serum or urine) with 50 μl of buffer and 500 μl of extraction–derivatization reagent. To achieve a complete and stabile derivatization reaction, the extraction–derivatization reagent was added while vortexing the combined sample and buffer solution in 75 mm × 12 mm round bottom soda-lime-glass test tubes. The mixture of extraction–derivatization reagent, sample and buffer was vortexed for 15 s. After centrifugation (3000 rpm, 5 min), the toluene layer was transferred to an autosampler vial and injected into the GC/MS apparatus in a volume of 3 μl.

The analysis was performed with an apparatus consisting of a Hewlett-Packard (Agilent; Agilent Technologies, Palo Alto, CA, USA) 5890 Series II gas chromatograph, a Hewlett-Packard (Agilent) 5971 A mass selective detector (EI, pos-

itive ions, 70 eV) and a Hewlett-Packard (Agilent) ChemStation data system. The pentafluorotributylamine (PFTBA) ionic ratio targets were set as follows: *m*/*z* 69, 100%; *m*/*z* 219, 120%; *m*/*z* 502, 10%. The system was operated in the splitless injector mode. The GC column was a DB-5MS of length 30 m, internal diameter 0.32 mm and film thickness 1 μm (J&W Scientific Inc., Folsom, CA, USA). Helium was used as the carrier gas. The inlet and detector temperatures were maintained at 250 and 280 °C, respectively. The column temperature was initially 130 °C with a hold time of 2.0 min, and was increased 15 °C/min to 320 °C, with a final hold time of 3.0 min. After initial establishment of peak location and MS spectra for the HFB derivates of each analyte in full scan mode (scanning range 50–550 amu), MS detection was performed in selected ion monitoring (SIM) mode. The monitored ions are printed in bold in Fig. 1. All the ions were monitored from 5 min post-injection until the end of the analytical run.

2.3. Validation experiments

The standard samples used in the validation experiments were prepared from methanolic stock solutions containing all the analytes (for blood, serum or urine assay) or the most common analytes (for oral fluid) at a concentration of 1 mg/ml free base. The pools of standard samples were prepared from these stock solutions in volumetric flasks at the following concentration levels: blood, 25, 50, 500 and 1000 ng/ml; serum, 25, 500 and 1000 ng/ml; urine, 200, 500 and 2000 ng/ml; oral fluid, 20, 500, and 1000 ng/ml.

The presence of interfering peaks was evaluated by analyzing blank blood, serum, oral fluid, or urine samples from 10 different sources. In addition, as high concentrations of ephedrine may interfere with quantitation of MA, blank blood samples were spiked with ephedrine and MA to concentrations of 5000 and 25–200 ng/ml, respectively. Concentrations of MA were then calculated against standards containing both ephedrine and MA, and standards containing MA only.

Linearity experiments were performed at two levels: initially, the wide-ranging linearity was estimated with standard samples spiked over a wide concentration range (10 concentrations evenly distributed over the concentration range of 10–5000 ng/ml in duplicate), and linearity of calibration was investigated using 8–10 replicate samples at low (25 ng/ml, blood and serum; 200 ng/ml, urine), intermediate (500 ng/ml, blood, serum and urine) and high concentrations (1000 ng/ml, blood and serum; 2000 ng/ml, urine) over the calibration range. The wide-ranging linearity experiments were performed as overall estimation of the range of concentrations where the method may be used with appropriate set of calibration standards. Then, the routine working range (or calibration range) was estimated as a range of concentrations that could be calibrated from the lower LOQ to high concentrations in a single calibration batch without additional recalibration steps. The detector response was measured as peak height ratio (analyte/IS). The regression line was calculated using a least-squares regression model without any weighing. The estimation of wide-ranging linearity was based on the coefficient of correlation (r^2); it should equal or exceed 0.98. The limits of acceptability for the linearity of calibration were as follows: the deviation of back-calculated values from the theoretical values should not exceed ±15% (20% at the lower LOQ), and the coefficient of correlation (r^2) should equal or exceed 0.99.

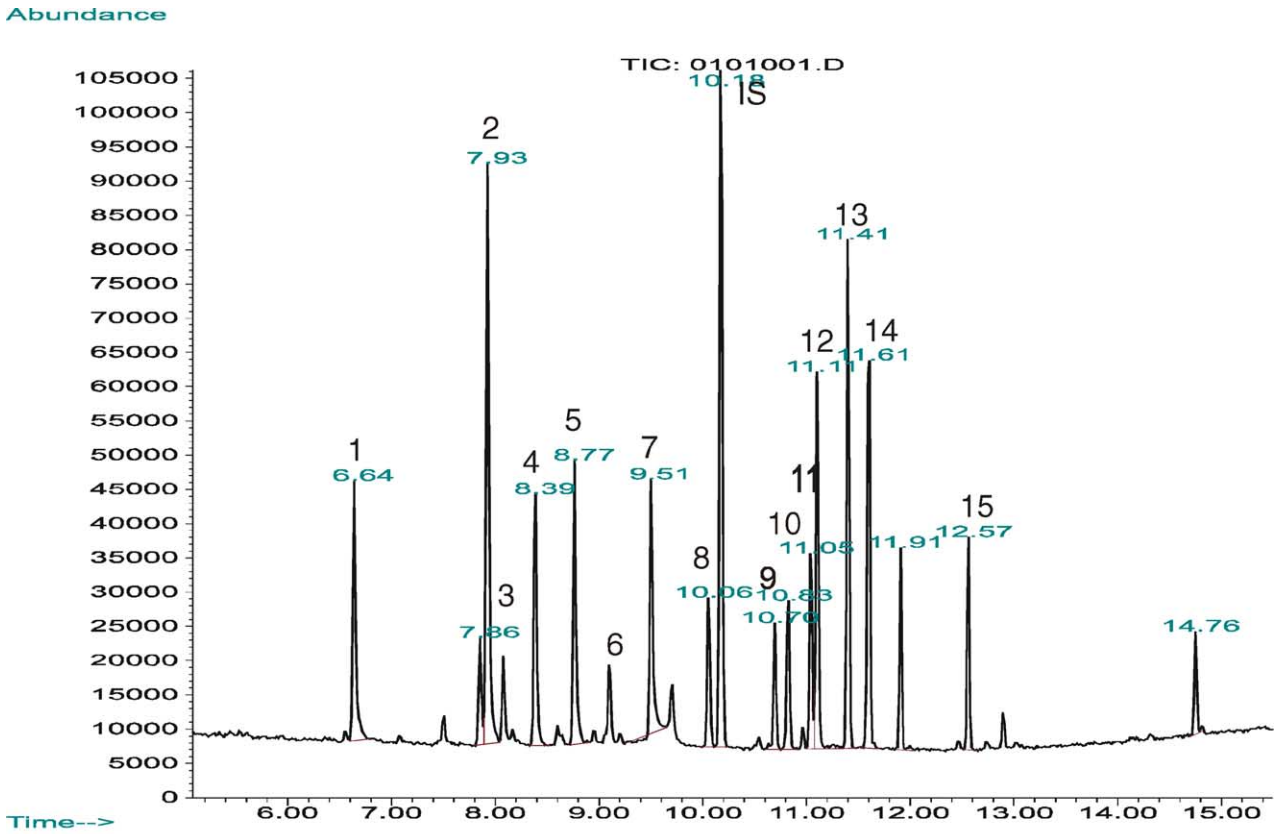
Repeatability (relative standard deviation, R.S.D.%) and accuracy (bias%) were determined by analyzing 10 individually prepared spiked standard samples consecutively, at low, intermediate and high concentrations over the calibration range. Intermediate precision (R.S.D.%) was determined by analyzing individually prepared spiked samples in 10 consecutive days. The concentrations of the samples were always calculated against daily calibration curves. The limits of acceptability were set according to widely approved guidelines [20], and were thus for both precision and accuracy 15% (20% at the lower LOQ).

Relative recovery was determined by comparing the results from samples spiked in quadruple in each matrix and extracted using the method described, with “total samples” which were prepared by adding drugs directly to the extraction solvent and mixing with buffer and water (in place of the sample matrix). The mean relative recovery in each matrix was calculated as percentage of the result of the “total sample” which was considered as 100%. Further experiments were conducted with different types of blood matrices in order to evaluate whether haemolysis affects the relative recovery, or whether sheep blood could be use as standard matrix in place of human blood. The relative recoveries were calculated as described above, and then the data was subjected to one-way analysis of variance (ANOVA) followed by Bonferroni’s test. The concentrations of all the analytes were spiked at a concentration level of 500 ng/ml.

3. Results and discussion

3.1. Sample preparation

The ATSs and related drugs were extracted and derivatized in a single step by mixing 100 μl (oral fluid) or 200 μl (blood, serum, or urine) of sample with 50 μl of buffer (saturated $NaHCO_3$ + 10 M KOH) and 500 μl of extraction–derivatization reagent (toluene + HFBA + IS). The ratio of sample to extraction solvent volume was optimized in such a way that an aliquot of the solvent phase could be directly injected into the GC, without losing the sensitivity necessary for acceptable limits of quantitation (LOQs). Thus, we were able to omit the concentration step from the sample preparation procedure, which is an advantage, because evaporation after extraction or derivatization, the most common means of concentrating samples, may lead to the loss of some volatile compounds [16], especially amphetamine and MA. Others have also avoided this situation, e.g. by addi-



Drug		Rt (min)	Target and Qualifier Ions *m/z* (relative response in parentheses)
1.	Amphetamine	6.64	**240** (100), **169** (7), **118** (15)
2.	MA	7.93	**254** (100), **210** (26), **118** (5)
3.	Cathinone	8.09	**240** (100), **105** (158), **77** (110)
4.	Pseudoephedrine	8.39	**254** (100), **210** (18), **344** (5), *no 118 & 91*
5.	Norephedrine	8.77	**241** (100), **169** (7), **107** (5), *no 118 & 91*
6.	PMA	8.90	**240** (100), **121** (98), **361** (50)
7.	Ephedrine	9.51	**254** (100), **210** (37), **169** (15), *no 118 & 91*
8.	MDA	10.06	**162** (100), **375** (40), **135** (110), *no 91*
9.	BDB	10.70	**176** (100), **254** (65), **389** (65), *no 118 & 91*
10.	4-MTA	10.83	**377**(100), **164** (74), **240** (80)
11.	BZP	11.05	**281** (100), **372** (104), **91** (110)
12.	MDMA	11.11	**254** (100), **210** (40), **162** (40), *no 118 & 91*
13.	MDEA	11.41	**268** (100), **240** (44), **162** (40), *no 118 & 91*
14.	MBDB	11.61	**268** (100), **210** (22), **176** (16), *no 118 & 91*
15.	2C-B	12.57	**242** (100), **244** (100), **229** (74)
IS		10.18	**254** (100), **136** (29)

Fig. 1. Chromatographic and mass spectral characteristics of the 15 HFBA-derivatized ATSs and related drugs.

tion of hydrochloric acid to transform the compounds to less volatile forms, or by introducing further extraction and back-extraction steps [21]. However, these procedures may lead to drawbacks, e.g. traces of acid can interfere with the detection of basic drugs [16] or procedures may become labor-intensive when used at large scales.

Derivatization with HFBA has many advantageous properties, one of which is that no standing or heating period is necessary following the derivatization [21]. However, elimination of excess reagent and the reaction product heptafluorobutyric acid is considered necessary to prevent degradation of the GC column [17]. Peters et al. [22] extracted the HFB derivatives into hexane and washed the organic phase with sodium phosphate solution to remove the excess reagent. In the procedure described here, the aqueous alkaline conditions present during the extraction–derivatization step ensure that no anhydride is present and that the hydrolysis product heptafluorobutyric acid is in ionized form and consequently trapped in the aqueous phase. Thus, no separate washing steps were needed.

Due to the rapidity of the pre-treatment procedure described, a large number of samples can be processed within a working day. Consequently, this method can be considered as a true alternative for immunological screening procedures, especially if the prevalence of positive samples is high. With regard to ATSs and related drugs, the most important benefit of GC/MS screening is that the variety of illicit drugs detected is manifold, compared with the results obtained in immunological tests, which are capable of detecting only a few ATSs at pharmacologically relevant concentrations. The detection of a compound by an immunochemical reaction depends solely on the specificity of the antibodies used, which in turn depends on the molecule against which the antibody has originally been elicited. In case of the ATSs, the antibodies are usually targeted against *S*-(+)-amphetamine and *S*-(+)-MA, which leads to a situation where there is likely to be lower reactivity with other ATS compounds, as well as the *R*-(−) enantiomers of amphetamine and MA [23]. According to the published reports, the ATSs with lower reactivity may include compounds as common as MDMA or MDA, as well as MDEA, BDB, MBDB, and PMA, depending on the assay used [24–27]. However, along with the increasing popularity of MDMA, manufacturers have brought to market assays more sensitive to MDMA [24,25], but there are still many compounds with lower reactivity, and compounds of which no published data are available.

With regard to blood samples, the applicability of GC/MS screening is further indicated by the fact that many of the commercially available immunoassays are of homogenous type, and require additional pre-treatment steps to make them suitable for use with turbid body fluids. These adaptations include procedures such as protein precipitation [28] and in many cases also other modifications to attain the lower cut-off levels needed when analyzing blood samples. This problem is largely solved by recent introduction of microtiterplate (inhomogenous) immunoassays in the market, because these procedures allow the use of whole blood or serum without any pre-treatment [29–31]. To achieve full benefit of the microtiterplate procedures, however, one should invest in a fully automated system. Thus, the advantages of immunoassays, designed to minimize labour costs and analysis time, may not be evident when screening for ATSs from blood samples, unless a fully automated microtiterplate assay system is available in the laboratory.

Use of this GC/MS procedure eliminates false-positive results caused by ephedrine-containing common medicinal drugs. Thus, the qualitative accuracy of the screening procedure is improved. However, it must be noted that the false-positives caused by precursor drugs, which are metabolised to amphetamine or MA, present a risk of misinterpretation of amphetamine and MA results even when using GC/MS [32–34]. Precursor drugs are used therapeutically as sympathomimetics, anorectics, analgesics, antiparkinsonian agents, or vasodilators, and they include amphetaminil, benzphetamine, clobenzorex, deprenyl, dimethylamphetamine, ethylamphetamine, famprofazone, fencamfamine, fenethylline, fenproporex, furfenorex, mefenorex, mesocarb, prenylamine, selegiline and mesocarb [32–34]. Using full scan MS mode facilitates identification as long as parent compounds and/or specific metabolites are present, but in a late phase of excretion differentiation from amphetamine or MA intake may not be possible [16]. Nevertheless, the possibility that a positive amphetamine or MA case can be result of intake of a variety of therapeutic drugs should be kept in mind when interpreting analytical results. In addition, the laboratories should have the best available means to differentiate the use these substances from that of amphetamine and MA, at a minimum when specifically requested.

The present method was designed for detecting recent drug use in living subjects. Consequently, the sample matrices included were limited to blood (whole blood and serum), oral fluid and urine. The advantages of blood as a matrix for clinical and forensic drug analysis are evident: screening and quantitation of the drugs can be performed in one sample, the unchanged drug is usually present, the matrix is homogenous, the relationship between drug concentration and human psychomotor performance or clinical condition can be established more accurately etc. [18,35]. The heterogeneity of urine as a matrix has been well demonstrated, e.g. by the study of Poklis et al. [36] in which healthy volunteers were given single oral doses of D-amphetamine at doses ranging from 5 to 20 mg. At a dose of 5 mg, the peak urinary amphetamine ranged from 620 to 3160 ng/ml, occurring from 2 to 8 h post-administration. At a dose of 20 mg, the variability in the time to peak was even greater, ranging from 2 to 18 h. At all doses, amphetamine excretion increased with increasing urine flow and decreasing urine pH.

Oral fluid is considered as an alternative for conventional matrices, i.e. blood or urine [19]. Drug concentration–time profiles measured in oral fluid are generally believed to be similar to those measured in blood, although oral fluid/blood

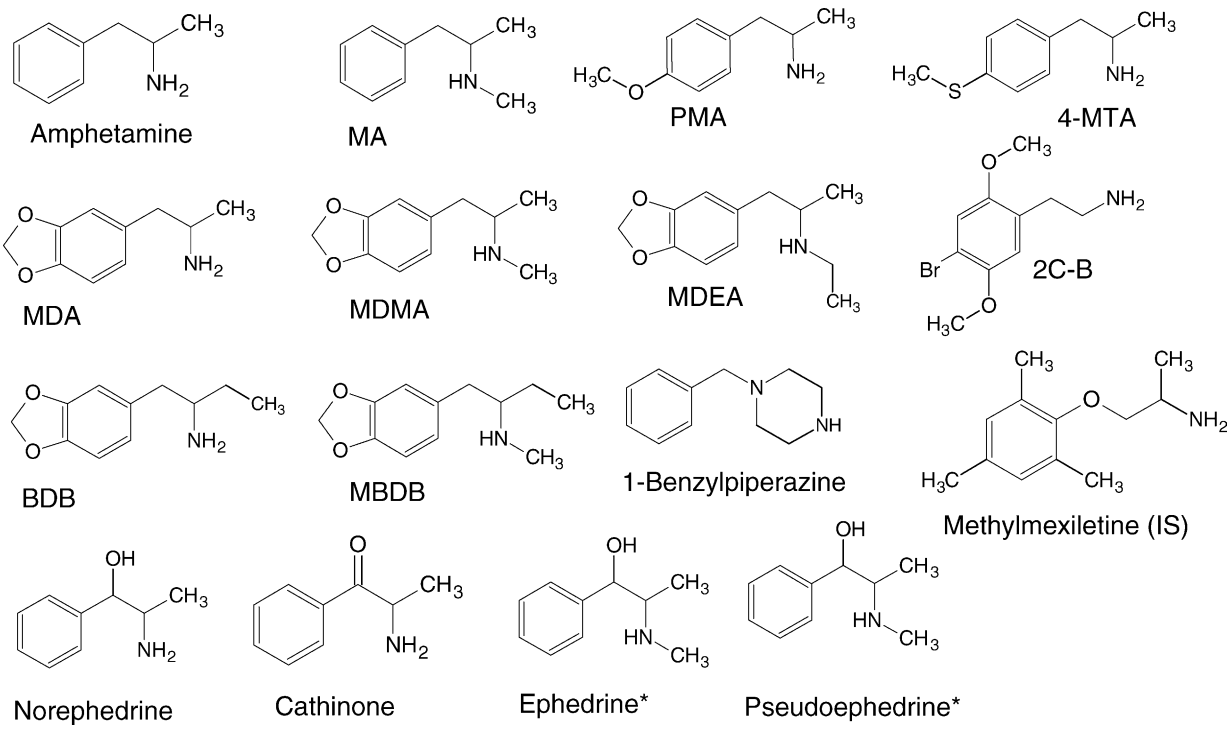


Fig. 2. Molecular structures of selected ATSs and related drugs. Asterisk refers to diastereomeric relationship of ephedrine and pseudoephedrine.

concentration ratios (S/B ratios) may vary a lot depending on substance [37,38]. In addition, many important topics remain poorly understood. For example, pharmacological effects corresponding to certain drug concentrations in oral fluid and the effects of sampling method on drug concentration of different drugs in oral fluid [39,40] need further investigation. Nevertheless, the future importance of oral fluid as a matrix to be screened for drugs may increase, particularly since supervised and non-invasive sampling enables on-site testing applications such as roadside drug testing [41]. On the other hand, the shorter detection times and lower concentrations of the compounds observed compared with those seen in urine complicate the identification of drugs in oral fluid [19]. With the method developed, the ATSs are simultaneously, rapidly and reliably screened and quantitated at low concentrations from a small sample volume that is usually the only one available.

3.2. GC/MS analysis

The 15 ATSs and related drugs were separated by their retention times, as shown in Fig. 1, thus presenting the total ion chromatogram of a standard serum sample containing 500 ng/ml of each HFBA-derivatized amine drug. Due to the structural similarity of many of the analyzed substances (Fig. 2), their separation by GC is vitally important, even during the process of identification by MS. We found that the bonded, cross-linked non-polar DB-5MS column (5% phenyl–95% dimethyl arylene siloxane) of length 30 m, internal diameter 0.32 mm and film thickness 1 μm (J&W) yielded satisfactory separation of all the substances. The use of thicker (1 μm) film enhanced the chromatographic properties of the drugs compared with a similar column with film thickness of 0.25 μm (data not shown). The tailing of amphetamine and MA peaks also appears to have diminished, in comparison to a chromatogram obtained with a DB-5MS with a narrower bore (0.25 mm) and thinner film (0.25 μm) [22]. We also tested a third column, the mid-polar DB-35MS (35% phenyl–65% dimethyl arylene siloxane; J&W) of length 30 m, internal diameter 0.32 mm and film thickness 0.25 μm, but found it less suitable and consequently omitted this column from further testing (data not shown).

One of the most common problems encountered in GC/MS analyses of ATSs and related drugs concerns MA and ephedrine. These substances, the former of which is a drug of abuse, and the latter a constituent of common medicaments, may be difficult to distinguish due to their close structural resemblance (see Fig. 2). Under the chromatographic conditions applied here, ephedrine forms two peaks, with retention times of 7.86 and 9.51 min (main peak) in the sample chromatogram, the former peak eluting adjacent to MA. Most of the ephedrine is eluted in the main peak, the presence (and size) of which provides chromatographic means to distinguish one drug from the other. The experiment with blood samples spiked with high concentrations of ephedrine (5000 ng/ml) and low concentrations of MA (25, 100 and 200 ng/ml) confirmed that MA can be accurately quantitated even in the presence of high ephedrine concentrations; the results obtained against standards containing both ephedrine and MA equaled those obtained with standards containing

MA only. The ion chromatograms obtained from samples containing 25 ng/ml of MA (plus 5000 ng/ml ephedrine), however, had to be integrated manually, while those containing 100 or 200 ng/ml of MA were integrated automatically by the ChemStation (Agilent) software.

The reason why ephedrine forms two peaks is that the molecule contains two active groups—secondary amine and hydroxyl—that are able to react with HFBA. Thus, the first peak corresponds to the mono-(*N*)-HFB, whilst the latter corresponds to the bis-(*NO*)-HFB derivative. Same phenomenon can be observed in case of ephedrine's diastereomer pseudoephedrine, whose two peaks elute at 8.39 and 9.70 min in the sample chromatogram (Fig. 1). Similarly, norephedrine also tends to form two peaks, although the first peak (at 7.10 min) is very small as compared to the second peak at 8.77 min in the sample chromatogram.

Another problematic group of ATSs, as pointed out by Aalberg et al. [42], are the regioisomeric 3,4-methylenedioxyphenethylamines, whose identification is dependent to a great extent on chromatographic separation. The separation of MDEA and MBDB is satisfactory under the chromatographic conditions applied here, as shown in Fig. 1.

The HFB derivatives of MA, MDMA, ephedrine and pseudoephedrine share the *m/z* 254 ion at their base peak, consisting of the HFBA reagent, a nitrogen atom, three carbon atoms and seven hydrogen atoms $[CH_3CHN(CH_3)COCF_2CF_2CF_3]^+$ [22]. The mass spectral identification of adjacently eluting MA and ephedrine, especially, is critically important; both drugs share the *m/z* 210 ion, although its relative abundance differs. As pointed out by Thurman et al. [43], a better means of differentiation between the two drugs are the low-molecular weight ions *m/z* 91 (tropylium) and *m/z* 118, which are present in the mass spectra of MA-HFB, but not ephedrine-HFB. Amphetamine has a base peak at *m/z* 240, assigned to the fragment $[CH_3CHN(H)COCF_2CF_2CF_3]^+$, and may thus be interfered with substances such as norephedrine (phenylpropanolamine), cathinone, PMA, MDA and 4-MTA, which share the same ion. As shown in the sample chromatogram, these substances elute much later than amphetamine, eliminating the risk of interference. It should be noted that the *m/z* 169 and 69 ions correspond to the $[CF_2CF_2CF_3]^+$ and $[CF_3]^+$ fragments, at least one of which is present in all HFBA-derivatized amine drugs [44].

3.3. Validation

No interfering peaks were detected in blank blood, serum, oral fluid, or urine samples. Validation was performed in each matrix, using drug-free samples spiked with the analytes. The wide-ranging linearity experiments showed that the response was linear ($r^2 > 0.98$) for all the analyzed substances in the concentration range from 20 to 5000 ng/ml in blood, oral fluid and urine, with the exception of PMA, whose lower limit of linearity was 50 ng/ml. The more thorough investigation (8–10 replicates per concentration level) at the calibration range confirmed the requirements of linearity ($r^2 > 0.99$; relative deviation from theoretical value less than 15%) were fulfilled. There were no matrix-specific differences in linearity. The lower LOQs, 20 ng/ml for oral fluid, 25 ng/ml (PMA: 50 ng/ml) for blood and serum and 200 ng/ml for urine, which also serve as the cut-off concentrations, were set in compliance with the suggested limits of acceptability [20] and cut-off concentrations [45].

Repeatability measured as relative standard deviation (R.S.D.%), time-different intermediate precision (R.S.D.%) and accuracy (bias%) are listed in Table 1 (blood, serum, urine) and Table 2 (oral fluid). With very few exceptions, the precision—both repeatability and intermediate precision—did not exceed 15% R.S.D. (20% R.S.D. at the lower LOQ) and the mean value was within ±15% of the theoretical value (±20% at the lower LOQ), which indicate that the measures of precision and accuracy are acceptable [20]. The reasons for the poor precision and accuracy observed for some of the analyzed substances are likely to be due to the structural features of the molecules, such as hydroxyl group in ephedrine-like compounds, which complicate both the derivatization reaction and chromatography. In addition, compromises made during optimization of a multisubstance assay may worsen the analytical performance of individual substances. The present method was optimized for the most commonly encountered illegal ATSs, and consequently the minor aberrations observed in analytical performance of some of the more uncommon ATSs or constituents of legal medications were considered to be of minor importance.

The relative recovery was determined by comparing the results from samples prepared by spiking the drugs in the matrix and extracted using the method described, with total samples prepared by adding drugs directly to the extraction solvent and mixing with buffer and water (in place of the sample matrix). As seen in Table 1, the recovery of most ATSs from all the matrices was acceptable. In case of ephedrines, however, we were not able to determine the relative recovery using this experimental design. As discussed earlier, reaction of ephedrines with HFBA produces mono-(*N*)-HFB and bis-(*NO*-)HFB derivatives, whose relative abundances may change along with changes in reaction conditions. When ephedrines were added directly to the solvent phase (total samples), the equilibrium of the mono- and bis-derivatives were shifted in such way that the relative recoveries were either enormous (hundreds of percents) or negligible, depending on the derivative used for calculation. Nevertheless, recovery per se is not very important; rather it is essential to attain a *reproducible* recovery, which is high enough to satisfy the requirements for quantifying low-concentration substances [46]. The moderate differences between recoveries from different matrices, however, emphasize the importance of diluting the working standards in the sample matrix.

In addition to the type of matrix, the quality of the matrix may affect analyses, especially when dealing with whole blood samples. For example, haemolytic whole blood samples are generally considered problematic for drug analyses

Table 1
Validation results: extraction recovery, repeatability, intermediate precision and accuracy

Analyte	Rec (%)	Repeatability (R.S.D.%)			Intermediate precision (R.S.D.%)		Accuracy (bias%)		
	B_{500}	B_{1000}	B_{500}	B_{25}	B_{500}	B_{25}	B_{1000}	B_{500}	B_{25}
Blood									
AM	85	5.1	6.9	7.4	5.0	19.2	7.3	1.5	−2.1
MA	93	3.3	5.0	6.7	4.2	11.2	9.0	−1.7	0.3
CATH[a]	77	3.7	9.8	9.9	13.6	32.4	9.4	5.2	6.0
PSEPH	§	10.0	13.4	10.4	9.1	16.2	−3.8	−7.8	8.5
NEPH	§	5.7	4.1	2.9	14.0	10.4	15.8	0.6	8.4
PMA[a]	90	6.2	6.0	7.4	6.2	21.6	4.2	−6.4	−7.2
EPH	§	8.3	7.1	4.9	10.6	20.6	2.8	−0.4	19.7
MDA	83	4.6	5.7	10.0	7.6	19.7	2.4	0.2	8.1
BDB	86	5.1	8.6	9.3	7.4	19.8	7.8	−3.3	1.8
MTA	78	2.5	7.2	7.1	6.8	16.7	15.7	−14.1	11.1
BZP	88	4.8	7.0	11.4	7.0	21.6	6.8	5.9	8.3
MDMA	93	4.6	9.4	5.7	6.2	18.2	10.1	8.1	10.2
MDEA	105	6.4	10.3	6.0	7.3	16.7	14.6	3.6	−1.2
MBDB	88	4.4	6.4	4.2	6.2	21.6	13.7	−5.5	13.4
2C-B	72	7.7	6.0	11.8	10.2	8.7	13.2	9.0	8.8

Analyte	Rec (%)	Repeatability (R.S.D.%)			Intermediate precision (R.S.D.%)	Accuracy (bias%)		
	S_{500}	S_{1000}	S_{500}	S_{25}	S_{500}	S_{1000}	S_{500}	S_{25}
Serum								
AM	85	4.0	4.1	8.5	6.6	3.9	−10.3	7.8
MA	93	4.0	3.6	7.9	7.6	7.0	−9.4	10.9
CATH	71	5.4	4.3	5.5	9.2	−2.3	−4.9	−6.5
PSEPH	§	5.7	8.5	12.9	10.5	3.5	−9.8	−1.1
NEPH	§	4.3	7.2	8.7	6.5	9.2	−9.4	−8.9
PMA	93	6.7	8.1	10.1	7.7	−3.2	−18.8	4.7
EPH	§	8.5	6.1	5.7	6.5	11.6	−2.8	12.2
MDA	76	2.9	5.9	9.6	7.7	5.8	−10.3	7.8
BDB	83	3.4	5.8	7.5	8.2	6.8	−13.8	9.6
MTA	76	5.2	5.8	12.3	7.4	14.4	−17.5	−0.3
BZP	81	2.6	4.6	12.2	6.1	3.1	−9.2	3.4
MDMA	95	7.7	4.3	8.0	8.0	2.7	−13.5	7.3
MDEA	108	4.9	8.0	12.3	5.6	9.5	−14.3	−4.8
MBDB	93	2.4	7.3	8.7	5.9	7.8	−16.9	1.2
2C-B	76	5.8	5.0	12.0	11.0	9.5	5.1	−5.1

Analyte	Rec (%)	Repeatability (R.S.D.%)			Intermediate precision (R.S.D.%)		Accuracy		
	U_{500}	U_{2000}	U_{500}	U_{200}	U_{500}	U_{200}	U_{2000}	U_{500}	U_{200}
Urine									
AM	99	6.4	5.5	3.6	9.1	5.4	−9.4	5.4	0.7
MA	90	7.4	10.4	5.3	10.5	6.3	−2.7	−4.5	−1.5
CATH	88	6.2	5.7	5.8	12.6	5.0	3.9	−0.7	0.9
PSEPH	§	10.9	14.3	7.8	14.3	9.2	−13.3	−5.1	−3.7
NEPH	§	12.2	8.4	6.2	9.7	8.2	4.3	−0.8	6.9
PMA	103	9.8	7.5	5.7	6.3	6.6	−8.6	−13.2	−3.3
EPH	§	14.4	15.4	0.1	16.1	10.1	9.2	−5.1	0.8
MDA	98	4.1	5.6	5.5	12.8	5.3	−11.1	1.1	1.0
BDB	101	6.9	4.4	5.4	13.1	6.0	−7.9	−3.2	−2.3
MTA	93	6.6	3.3	4.7	10.1	14.9	8.9	−5.9	1.9
BZP	88	5.7	3.8	7.5	8.9	8.8	9.8	−4.6	−3.2
MDMA	89	11.9	10.3	5.8	7.6	7.9	−2.7	−4.5	−1.9
MDEA	79	14.3	8.6	2.9	5.4	7.4	9.7	−4.4	−6.6
MBDB	83	5.3	5.9	4.1	11.0	6.2	−3.4	−0.9	−4.7
2C-B	104	5.6	4.8	3.9	24.1	7.8	7.3	0.6	−1.9

Relative recovery was determined by comparing the results from samples spiked in each matrix and extracted using the method described, with total samples which were prepared by adding drugs directly to the extraction solvent and mixing with buffer and water (in place of the sample matrix). Repeatability was determined by analyzing 10 individually prepared spiked samples consecutively. Intermediate precision was determined by analyzing individually prepared spiked samples in 10 consecutive days. All the validation was carried out with samples which were spiked with all the analytes. *Nonstandard abbreviations*: Rec, relative recovery; AM, amphetamine; CATH, cathinone; PSEPH, pseudoephedrine; NEPH, norephedrine; EPH, ephedrine; §, study design used not applicable.

[a] Lower limit of quantitation 50 ng/ml.

Table 2
Validation results: repeatability, intermediate precision and accuracy (oral fluid)

Analyte	Repeatability (R.S.D.%)			Intermediate precision (R.S.D.%)			Accuracy (bias%)			LOD[a] (ng/ml)
	20_{LOQ}	500	1000	20_{LOQ}	500	1000	20_{LOQ}	500	1000	
AM	4.3	4.8	3.8	3.7	3.9	8.6	−0.8	−2.8	−5.5	5
MA	7.2	6.4	7.0	3.6	3.1	8.0	3.0	−3.1	−4.3	5
PSEPH	16.4	20.1	7.9	14.6	26.8	19.7	−1.9	17.7	4.1	10
NEPH	5.0	8.0	7.4	10.9	7.6	17.3	−2.5	−9.3	−5.6	10
EPH	9.4	7.2	6.9	2.4	6.8	14.4	−8.2	−15.0	−11.4	10
MDA	4.0	4.5	4.5	4.1	4.1	7.1	−1.4	−2.7	−4.2	5
BDB	6.7	4.1	5.1	4.4	4.2	6.8	0.5	−3.0	−4.9	5
MDMA	8.5	7.6	8.0	4.9	1.8	7.1	0.8	−3.0	−3.5	5
MDEA	9.2	8.4	9.6	3.4	4.5	9.0	6.4	−5.8	−4.5	5
MBDB	9.2	7.4	8.0	8.1	4.6	11.4	3.1	−4.3	−4.7	5

Repeatability was determined by analyzing eight individually prepared spiked oral fluid samples. Intermediate precision was determined by analyzing individually prepared spiked samples in five consecutive days. All the validation was tested with samples, which were spiked with the most common analytes. *Nonstandard abbreviations*: AM, amphetamine; MA, methamphetamine; PSEPH, pseudoephedrine; NEPH, norephedrine; EPH, ephedrine.

[a] Signal-to-noise (S/N) ratio of 3 was used for target ions. However, authors recommend using LOQ values (20 ng/ml) for the reliable identification and quantitation.

[18,35]. This problem is frequently encountered in forensic laboratories, which are obliged to perform drug analyses in haemolytic blood samples taken into grey-capped Vacutainers (NaF + K_2Ox as preservative) for alcohol determination. To evaluate the effect of blood quality on extraction recovery, we spiked a mixture of selected ATSs (see Fig. 3) in quadruple in fresh (<1 h old) blood, blood stored in grey-capped Vacutainers for more than 2 months and in sheep blood. The results of this experiment showed that the relative recovery of all the analytes studied, except cathinone ($p = 0.005$, ANOVA), BDB ($p = 0.031$, ANOVA) and MDEA ($p < 0.001$, ANOVA), did not differ significantly between the blood sample types. The pair-wise comparisons revealed that the significant differences occurred without exception between fresh human blood and other types of blood, i.e. the matrix effect caused by stored haemolytic human blood appears to resemble that of stored sheep blood more closely than that of fresh human blood (Fig. 3). However, it must be emphasized that majority of the ATSs studied behaved similarly in different types of blood samples, which suggests that haemolysis does not interfere with analysis of most of the ATSs. Furthermore, less expensive sheep blood can be safely used as a standard matrix in place of human blood, at least when analyzing these ATSs and related drugs. It must be noted, however, that the

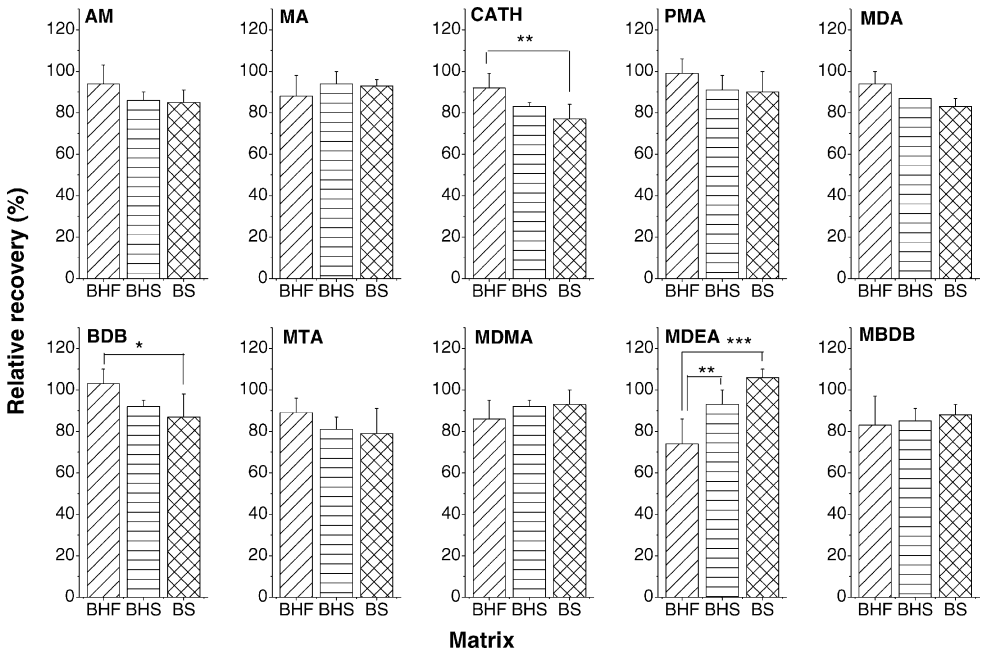


Fig. 3. A comparison of relative recovery from fresh human whole blood, stored human whole blood preserved with NaF + K_2Ox, and sheep blood. Blood samples were spiked to concentrations of 500 ng/ml in quadruple. *Nonstandard abbreviations*: BHF, fresh human blood; BHS, stored human blood; BS, sheep blood; AM, amphetamine; CATH, cathinone. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with group indicated, Bonferroni's test.

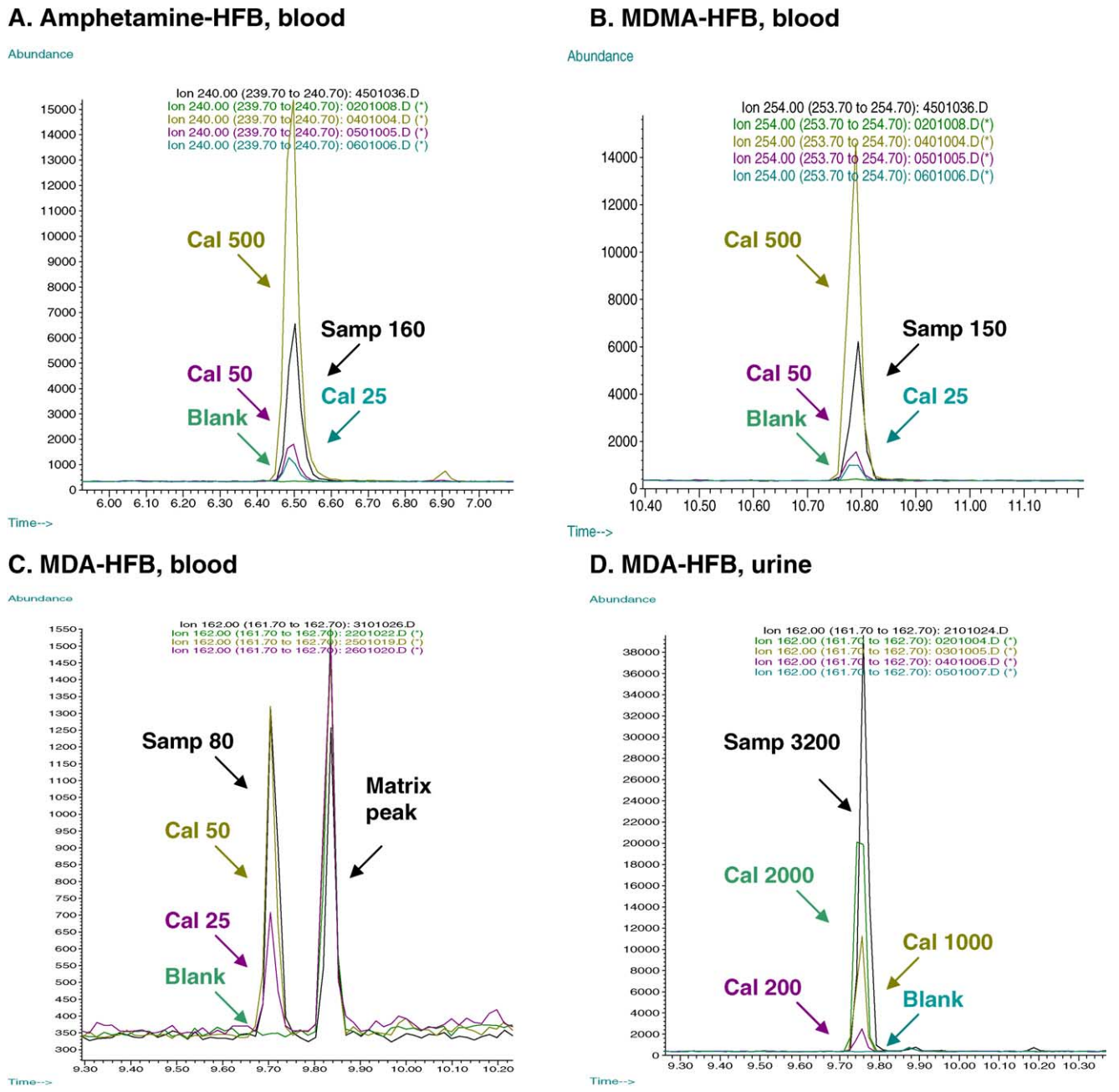


Fig. 4. Merged target ion chromatograms of authentic samples and spiked calibration standards. Panels A–D show target ion chromatograms of authentic samples (Samp) plotted together with some of their calibration standards from same calibration batch (Cal). Numbers after abbreviations refer to concentrations of the calibration standards or authentic samples in ng/ml. The concentrations were calculated as peak height ratios (analyte/IS). Panels A and B represent an analytical run of a patient sample containing both amphetamine (160 ng/ml) and MDMA (150 ng/ml). Using other chromatographic methods, this sample was also found positive for Δ^9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCC) and alprazolam. Panels C and D show ion chromatograms of blood and urine samples of a patient positive for MDA (80 and 3200 ng/ml, respectively). In addition, his blood sample was also found to contain alprazolam, oxazepam, and fluoxetine, while THCC, oxazepam, temazepam, alprazolam, α-OH-alprazolam and α-OH-midazolam were detected in urine.

stability of the ATSs in haemolyzed blood during storage was not evaluated in this study.

3.4. Routine performance

The method described here has been used as the primary method for ATS screening and quantitative determination for more than 1 year in the Laboratory of Substance Abuse/Drug Research Unit, National Public Health Institute. The laboratory is accredited and its technical competence is assessed against the European EN 45000 and international ISO 17000 Standards and respective ISO/IEC Guides by the Finnish Accreditation Service (FINAS).

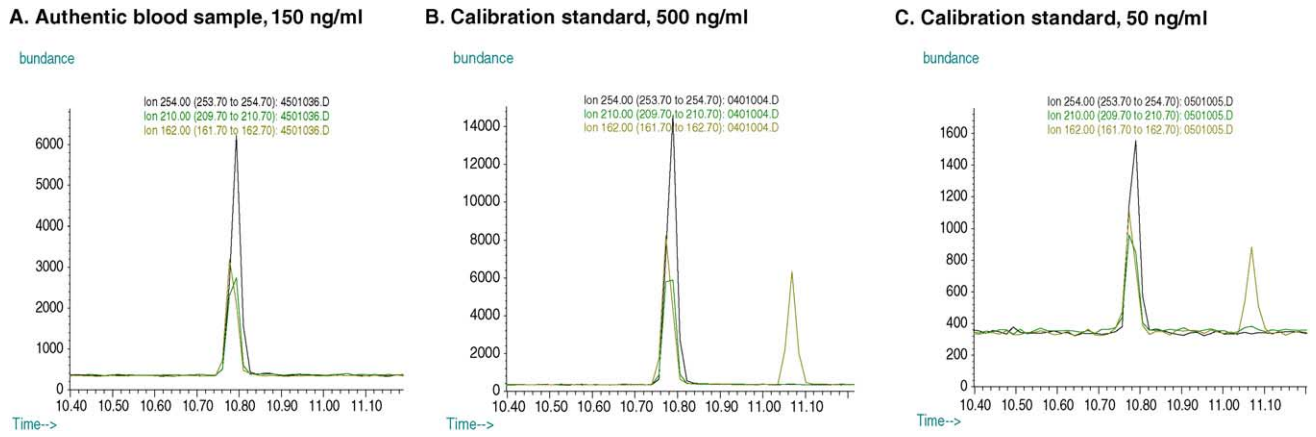


Fig. 5. Merged selected ion chromatograms of MDMA-HFB target and qualifier ions in authentic blood sample and spiked calibration standards. Panel A shows merged target and qualifier ion chromatograms of MDMA-HFB in an authentic blood sample, which was also found to contain amphetamine, Δ^9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THCC) and alprazolam. Merged ion chromatograms of calibration standards from same calibration batch are shown in panels B and C. The concentrations were calculated as peak height ratios (analyte/IS).

The performance of the method is assessed in proficiency tests (external quality assessments; see Table 3). So far, no false-positive or false-negative results have been obtained and the quantitative results have been acceptable, with the exception of one aberrantly low MBDB result. The ATSs present in the external quality control samples are listed in Table 3.

Table 3
Performance in proficiency testing programs (06/02–06/04)

Program	Matrix	Mode	ATSs present	Results
Nordquant	Blood	Quant	Amphetamine	OK
Labquality[a]	Urine	Quant	Amphetamine	OK
			Methamphetamine	OK
			MDMA	OK
			Pseudoephedrine	OK
PPT[a]	Urine	Quant	Amphetamine	OK
			Methamphetamine	OK
			MDA	OK
			MDMA	OK
			MDEA	OK
			MBDB	Low outlier
			Ephedrine	OK
UKNEQAS	Urine	Qual	Amphetamine	OK
			Methamphetamine	OK
			MDA	OK
			MDMA	OK
ICE	Urine	Qual	MDMA	OK
			Norephedrine	OK
			Ephedrine	OK

Nonstandard abbreviations: Norquant, Nordic Control of Quantification; PPT, Programma di Proficiency Testing, U.O. Tossicologia Forense e Antidoping, Universita di Padova, Italy; ICE, United Nations International Collaborative Exercises, Cardiff Bioanalytical Services Ltd., Cardiff, UK; UKNEQAS, United Kingdom National External Quality Assessment UKNEQAS for Drugs of Abuse in Urine, Cardiff Bioanalytical Services Ltd., Cardiff, UK; Labquality, Labquality Urine Confirmation Tests, Labquality Oy, Helsinki, Finland.

[a] Reference laboratory, reports quantitative results.

The number of samples analyzed for ATSs and related compounds in our laboratory is on average 400 per month, which means that since the introduction of this method in routine use, roughly 7000 analyses have been carried out. Majority of patient samples are blood or urine samples from multi-drug users, whose abuse profile typically includes drugs of different classes, most often ATSs, cannabis, benzodiazepines and/or opiates. Examples of ion chromatograms of authentic patient samples, together with calibration standards from the same analytical runs are shown in Figs. 4 and 5. So far, we have not observed any interference from other drugs or metabolites in our ATS analyses.

As the derivatization reagent HFBA is known to damage GC columns a question may arise whether our one step extraction–derivatization procedure allows residual derivatization reagent to be injected into the column. During the 18 months we have used this protocol, we have not suffered from injector or column problems. On the contrary, the apparatuses reserved for the ATS analyses require less maintenance than those used for other analyses. On average, the preventive maintenance, which includes cleaning of the injector and ion source, and column cutting, has been performed every 2–3 months. The lifetime of a column has been more than 6 months. This supports our theory that the alkaline conditions during extraction–derivatization step hydrolyses the anhydride and keeps the ionic hydrolysis product in the aqueous phase.

4. Conclusion

Along with the variety of ATSs available in the expanding market, it is becoming increasingly important to include as wide as possible a selection of these substances in drug analyses from different matrices. With its rapid pre-treatment procedure, the present GC/MS method may be used for preliminary screening of large numbers of samples, especially

if the prevalence of positive samples is high. This would markedly improve the qualitative accuracy of ATS screening by detecting a wider variety of substances and by complying better with the low cut-off values required for drug analyses in blood. With its low LOQ, this fully validated method provides a means for rapid quantitative determination of a variety of ATSs and related drugs as well. Thus, both screening and quantitation can be performed sensitively and accurately using this method.

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