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Quantification of amphetamine plasma concentrations by gas chromatography coupled to mass spectrometry

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Abstract

We developed a fast and sensitive method for identification and quantification of plasma concentrations of amphetamine using gas chromatography with mass spectrometry detection (GC–MS). Amphetamine-d_8 served as internal standard. The method involves a single extraction procedure and an easy treatment of the samples that allowed no losses during the evaporation process. Derivatisation of amphetamine with *N*-methyl-bis(trifluoroacetamide), a potent acylating agent, provides many advantages to the method compared with common derivatisation reactions usually used for amphetamines. The limits of detection and quantification following this method were 0.43 and 1.42 ng/ml, respectively. The assay has been successfully employed in the quantification of amphetamine in plasma samples from healthy volunteers at four different doses.

Keywords: Amphetamine; Gas Chromatography–Mass Spectrometry; Pharmacokinetics

1. Introduction

Amphetamine, β-phenylisopropylamine, is one of the most potent sympathomimetic amines causing stimulation of the central nervous system. The cardiovascular toxicity and the psychological dependence that develops after repeated consumption of this compound limit its use for therapeutic purposes [1,2]. Amphetamine has a place in the field of pharmacological research as prototype and reference agent in trials in which the stimulant effects of other drugs are being evaluated. Analytical methods for the determination of amphetamine in biological fluids include immunoassay techniques [3–6], high-performance liquid chromatography (HPLC) [7–9], capillary electrophoresis [10] gas chromatography (GC) with nitrogen–phosphorus detection (NPD) [11–13], flame-ionisation detection (FID) [14], electron capture detection (ECD) [15], or gas chromatography coupled to mass spectrometry (GC–MS) [16,17]. An extensive review of analytical proce-

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dures for the determination of amphetamine, methamphetamine and amphetamine-derived designer drugs or medicaments has been published recently [18]. Sample preparation and the way of avoiding amine losses during the evaporation process are two issues of relevance in the analysis of amphetamine in plasma. The method developed offers some straightforward approaches for these two aspects of amphetamine analysis that improve procedures described previously [11].

We describe a fast and sensitive method for the determination of plasma concentrations of amphetamine by GC–MS after a single liquid–liquid extraction step using an amphetamine deuterated analogue as internal standard. Under these conditions, low limits of detection and quantification were achieved. This method was validated and applied over a period of 2 months. Results of amphetamine plasma concentrations at four different doses in healthy volunteers are also presented.

2. Materials and methods

2.1. Chemicals and reagents

DL-Amphetamine and DL-amphetamine-d_8 (phenyl-d5,3,3,3) were supplied by Radian (Austin, TX, USA). The gas chromatography grade *N*-methyl-bis(trifluoroacetamide) (MBTFA) was purchased from Macherey-Nagel (Düren, Germany). Analytical grade sodium hydroxide, sodium chloride and *tert*-butyl methyl ether were supplied by Merck (Darmstadt, Germany). Methanol (Carlo Erba, Milan, Italy) was HPLC grade.

2.2. Working standards

Standard solutions (100 μg/ml) of DL-amphetamine and DL-amphetamine-d_8, used as internal standard (ISTD), were prepared in methanol. Working standard solutions at concentrations of 0.1, 1 and 10 μg/ml of amphetamine were prepared by dilution of the stock standard solutions with methanol. ISTD was diluted in methanol to give a working solution at a concentration of 2.5 μg/ml. All solutions were checked chromatographically for purity on a routine basis. Standard solutions were stored at −20°C until analysis.

2.3. Calibration and quality control samples

Calibration standards containing 2.5, 5, 50 ng/ml of amphetamine were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 1 ml of prechecked drug-free plasma, each concentration in duplicate. Samples were then processed as described in Section 2.4. At the beginning of the study, quality control samples of 4 ng/ml (low control) and 40 ng/ml (high control) were prepared once from bulk drug-free plasma samples, aliquoted and stored at −20°C. They were included in each analytical batch to control daily the quality of the analytical process and to check the stability of the samples under storage conditions.

2.4. Sample preparation

Aliquots of 1 ml of plasma were allowed to thaw at room temperature and processed together with a calibration curve after addition of 25 ng of d_8-amphetamine (10 μl of the working solution) as ISTD. Samples were treated by adding 0.2 ml of 0.4 N sodium hydroxide and 0.5 ml of sodium chloride saturated solution. They were then extracted with 5 ml of *tert*-butyl methyl ether by rocking mixing for 20 min. The organic phase was separated and treated with 20 μl of MBTFA to avoid amphetamine losses during the evaporation process. After evaporating to dryness under a nitrogen stream at 40°C, samples were derivatised with 50 μl of MBTFA at 70°C during 20 min, then 2 μl were injected onto the chromatographic system.

2.5. Instrumentation

GC–MS analysis was performed in a gas chromatograph (Hewlett Packard 5890 A, Palo Alto, CA) coupled to a quadrupole mass spectrometer detector (Hewlett Packard 5970). The gas chromatograph was fitted with an autosampler injec-

tor (HP 7673). Samples were injected in splitless mode into a 25 m × 0.2 mm i.d., 0.11-μm film thickness methylsilicone column (Ultra1, Hewlett Packard) connected to a 1 m × 0.32 mm i.d. glass retention gap. The injector block and the GC–MS interface were operated at 280°C. The oven temperature was initially programmed at 80°C for 2 min and increased until 280°C at a rate of 20°C per min. Helium was used as carrier gas at a flow rate of 0.7 ml/min. The mass spectrometer was operated by electron impact ionisation and in the selected ion monitoring acquisition mode (SIM). Ions *m/z* 91, *m/z* 118, *m/z* 140 and *m/z* 96, *m/z* 126, *m/z* 143 were selected for the identification of

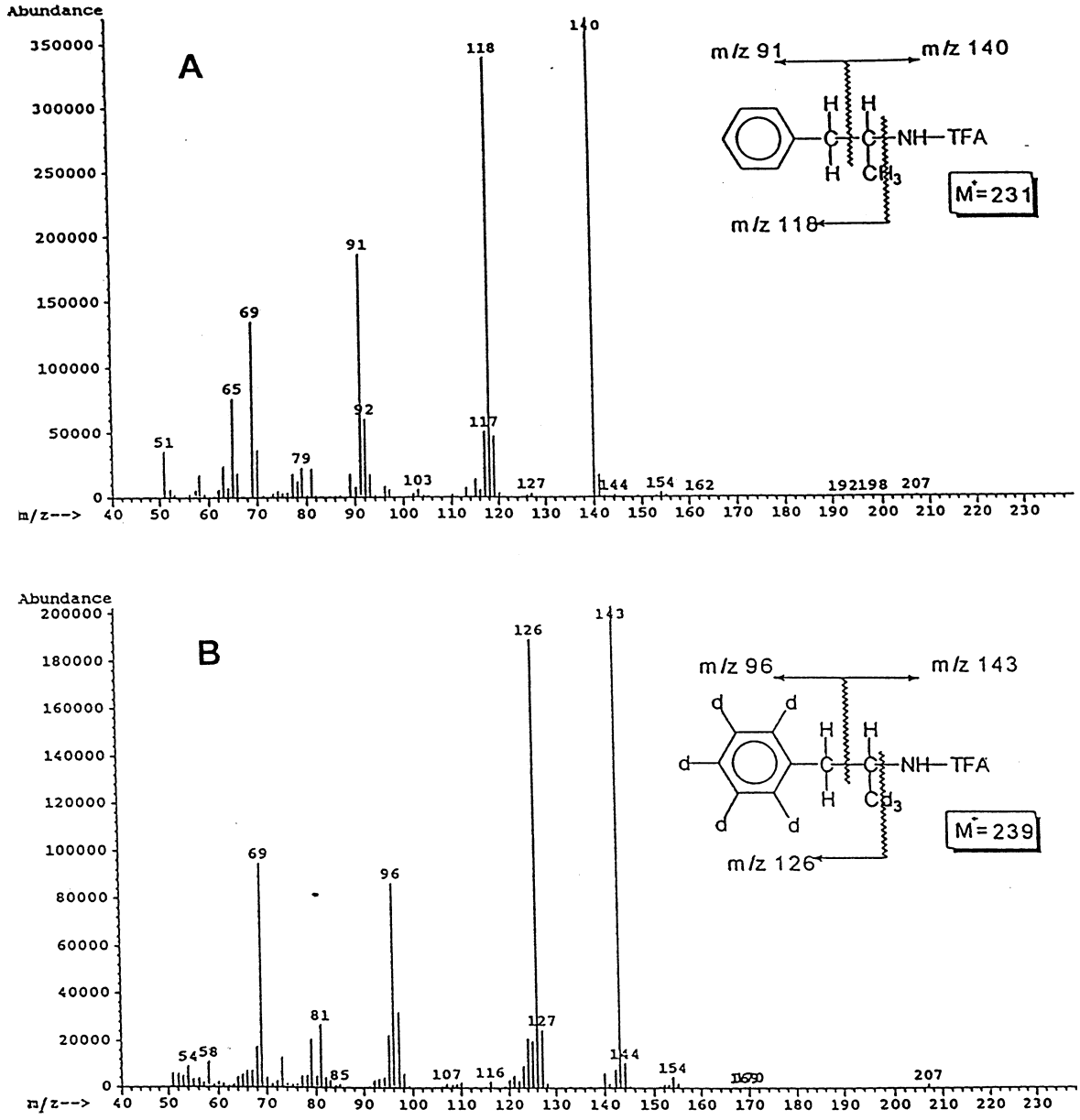

Fig. 1. Mass spectra and proposed fragmentation pattern of (A) amphetamine-*N*-TFA, and (B) d_8-amphetamine-*N*-TFA.

amphetamine-*N*-TFA and d_8-amphetamine-*N*-TFA respectively (see Fig. 1 for full mass spectrum of both compounds). Ions m/z 118 of amphetamine-*N*-TFA and m/z 143 of d_8-amphetamine-*N*-TFA, were selected for amphetamine quantification because they show no interferents in their ion traces as compared with minor ones observed with alternative ions like m/z 126 and m/z 140.

2.6. Clinical studies design

Healthy, male volunteers familiar with the recreational use of amphetamine derivatives took part in the study, which was approved by the local Ethical Committee and the Spanish Ministry of Health (Ref. DGFPS 95/297). Subjects received single doses of 20 mg ($n=2$), 30 mg ($n=2$), 35 mg ($n=2$) and 40 mg ($n=11$) of DL-amphetamine (Centramina, Miquel SA, Barcelona, Spain). Blood samples were drawn before the administration of the compound and at 15, 30, 45, 60 and 90 min and 2, 3, 4, 6, 8, 10 and 24 h after drug administration. The heparinized blood was centrifuged for 10 min at 3000 rev./min and plasma was transferred into polypropylene tubes and stored at -20°C until analysis.

3. Results

Quantitative results were calculated with ions m/z 118 and 143 for amphetamine and amphetamine-d_8, respectively. No interfering peaks were observed at the elution time of analytes for the ions monitored (Fig. 2).

3.1. Recovery

Extraction efficiencies for amphetamine and d_8-amphetamine were calculated by comparing the areas of the chromatographic peaks of equal concentrations of drug extracted and non-extracted. The experiment was carried out with concentrations of analytes (in duplicate) identical to those used for calibration. Ten aliquots of drug-free plasma ($n=10$) were extracted following the standard sample preparation procedure. Final extracts were spiked with the corresponding concentrations of reference substances. A calibration curve was extracted in parallel. Recoveries for amphetamine in the range of concentrations studied and for d_8-amphetamine at the concentration used as ISTD were 89.7 ± 4.3 and 85.6 ± 4.3%, respectively.

3.2. Linearity

Five concentrations ranging from 2.5 to 50 ng/ml were used to assess the linearity of the method. Regression analysis using the least squares method of the theoretic concentrations versus peak-area ratios (between amphetamine and d_8-amphetamine) gave a slope of 0.0357 ± 0.0012 ($n=3$), an intercept of 0.01539 ± 0.0040 ($n=3$) and a determination coefficient (R^2) in a range within 0.9951–0.9973 ($n=3$).

3.3. Precision, accuracy and robustness

Intra-assay precision and accuracy were determined by testing six replicates of blank plasmas spiked with 2.5, 10 and 50 ng/ml of amphetamine. Inter-assay precision and accuracy were calculated using control samples of 4 and 40 ng/ml (low and high control samples). Both control samples were analysed in 10 analytical batches over a 2-month period. As analytical batches were performed by different scientists with different instruments (same model of GC–MS benchtop instrument), it is possible in addition to have an approach to the method's robustness. Precision was determined by calculating the R.S.D. of the calculated concentrations. Accuracy is expressed as the relative error of the calculated concentrations. The R.S.D. for intra-assay precision was lower than 7.5% for the three concentrations tested. The intra-assay accuracy was lower than 6.2% for the three concentrations tested. The inter-assay precision was 7.3 and 7.2% for the low and high control samples, respectively, and the inter-assay accuracy (mean of the errors absolute values) 7.4% (range 0.25–17.5) and 5.8% (range 0.75–15.6) for the low and high control samples.

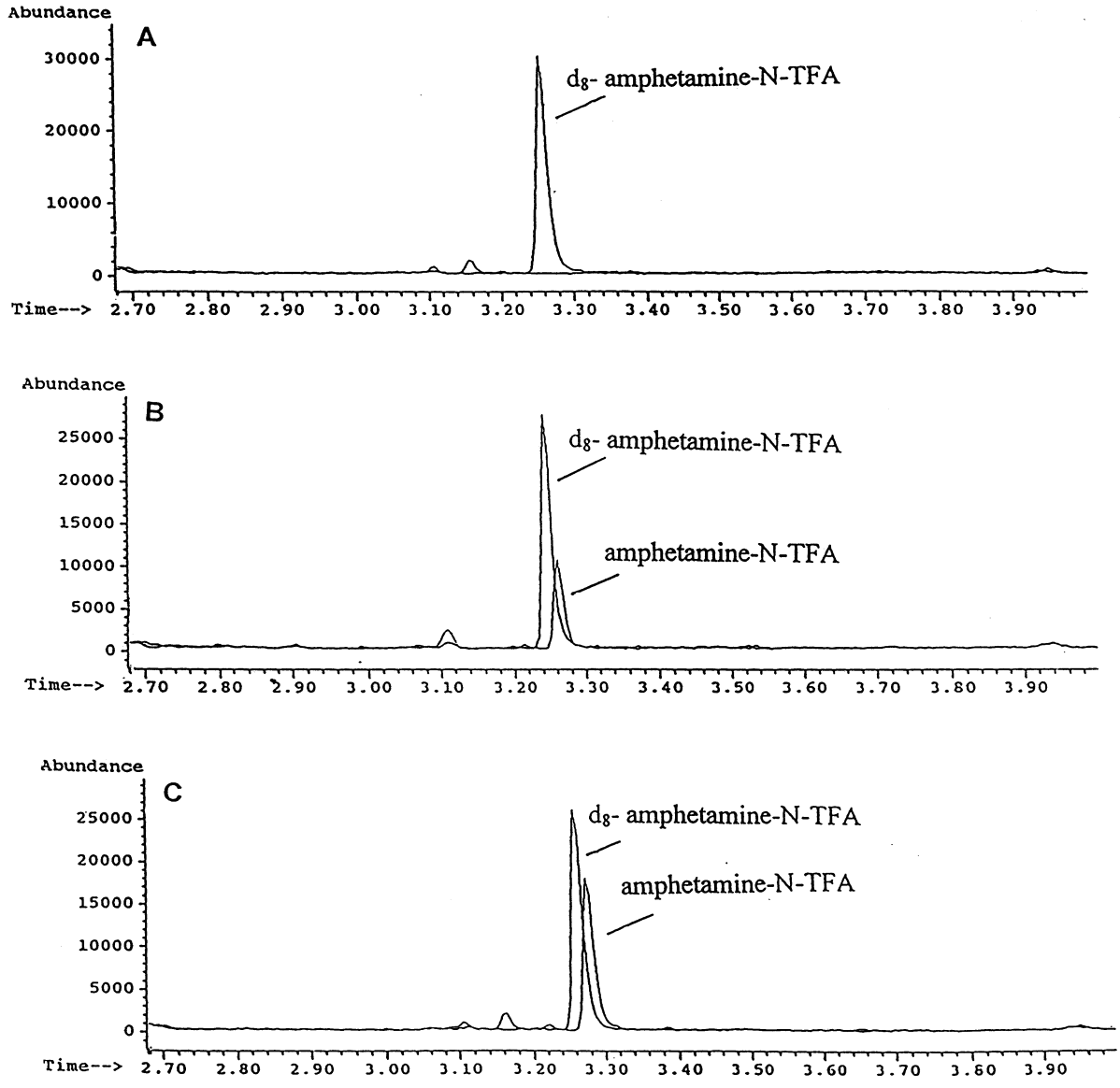

Fig. 2. Representative ion chromatograms for amphetamine (m/z 118) and d_8-amphetamine (m/z 143) of (A) plasma spiked (25 ng/ml) with d_8-amphetamine (ISTD), (B) Plasma spiked with amphetamine (10 ng/ml) and ISTD (25 ng/ml), and (C) plasma from a volunteer 1.5 h after oral administration of 20 mg of amphetamine (calculated concentration of 20.8 ng/ml) and ISTD (25 ng/ml).

3.4. Estimation of limits of detection and quantification

Six replicates of the low concentration value of the calibration curve of amphetamine (2.5 ng/ml) were processed for their calculation. An estimate of the limits of detection and quantification are calculated as three and 10 times respectively the S.D. of the estimated concentration. The detection and quantification limits obtained following this method were 0.43 and 1.42 ng/ml, respectively.

3.5. Clinical studies

All plasma samples from clinical studies were processed following the method described. Table 1 shows some pharmacokinetic parameters after the administration of amphetamine to participants. Fig. 3 shows the plasma concentrations of amphetamine obtained after the administration of the different doses.

4. Discussion

The analysis of amphetamine in biological fluids and, particularly, in blood samples should take into consideration the following aspects: sample preparation, evaporation losses during evaporation and how to avoid them, type of derivatisation, if any, of the final extract before chromatographic analysis, and the most suitable detection system.

In relation to preparation of the sample, the options available are either liquid–liquid or solid phase extraction procedures [11,12,16,19]. Both approaches have been used. Resins combining cation exchange and hydrophobic properties as interaction mechanisms with amphetamine seem the more adequate for solid-phase extraction [19]. When considering costs, liquid–liquid extraction procedures and especially the method reported here with a single extraction are very good alternatives. Independently of the sample preparation procedure selected, one of the main problems to be faced when analysing amphetamine is that the final organic extract has to be pre-treated in some way before its evaporation to avoid analyte losses. Several authors use mixtures of hydrochloric acid with methanol for the conversion in the organic phase of bases to their corresponding less volatile chlorhydrates [12,16,20–22]. Other approaches include the derivatisation of the free base directly in the organic phase or its concentration to a small volume under a gentle nitrogen stream and moderate temperatures that is either analysed directly or with a previous derivatisation step [11,23,24]. All these approaches add some intermediate steps to the method which are sometimes difficult to control and that substantially increase sample

Table 1
Pharmacokinetic parameters of amphetamine doses[a]

Dose (mg)	N	C_{max} (μg/l)	t_{max} (h)	$AUC_{0\text{-}24\ h}$
20	1	36.6	3	482.5
20	1	38.8	2	431.6
30	1	57.3	3	790.2
30	1	57.8	2	753.1
35	1	63.5	2	822.9
35	1	57.5	2	758.6
40	11	69.1 ± 5.75	2.23 ± 0.98	945.4 $\pm$ 71.83

[a] N, number of subjects; data for 40 mg are means $\pm$ S.D.; $AUC_{tn-tn-1} = (C_{n-1}+C_n)/2 \times (t_n - t_{n-1})$, area under curve.

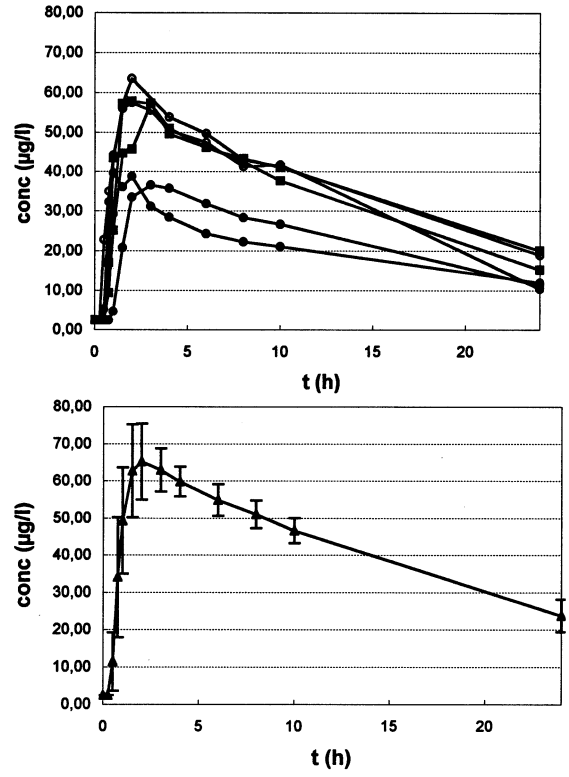

Fig. 3. Time course of (A) amphetamine plasma concentrations (0–24 h) from each volunteer administered orally with 20 mg (filled circle), 30 mg (filled square), 35 mg (open circle), and (B) mean amphetamine plasma concentration with their S.D. from 11 volunteers administered orally with 40 mg (filled triangle).

preparation time. An important point that makes the present method easy to use is the addition of 20 μl of MBTFA to the organic phase before its evaporation, that allows to take samples to dryness without losses of amphetamine [23] with no increases in preparation time. This approach is very useful if the trifluoroacetyl derivative of amphetamine is the compound to be analysed in the chromatographic system. It has been used in the past in qualitative screening procedures for the detection of amphetamine and other stimulants misuse in athletic competitions [23]. This is the first time that this reaction has been used for quantitative work with very good results. Once a final residue is available, amphetamine can be analysed directly in the gas-chromatographic system [20,22] or after being derivatised [11,16,21,24].

While normal amphetamine concentrations in urine allow their direct analysis, in plasma and for pharmacokinetic purposes derivatisation is needed to attain the required sensitivity of about 1 ng/ml. In recent reports [18,25], derivatization reactions for gas chromatographic analysis of drugs and in particular amphetamine and related compounds have been reviewed. Alkylation [12] and mainly acylation [11,16,21,24] of the primary amine are the most common derivatization reactions proposed for amphetamine. Extractive acylation has also been described to form TFA derivatives of primary and secondary amines and also phenols [26,27]. Acylation of amphetamine with MBTFA give many improvements to the method regarding the use of anhydrides [28]. First of all, MBTFA is a potent acylating agent that reacts also at room temperature. In the present method, the derivatisation reaction is made at 70°C during 20 min to ensure complete acylation. In addition, MBTFA is an excellent solvent and the excess of MBTFA does not have to be removed, allowing its direct injection into the chromatographic system. Its low boiling point explains an early elution in the gas chromatographic system that does not interfere with analytes. Finally, it is a reagent that does not damage the chromatographic column as compared with anhydrides [11,21,22,29]. This is the first report where MBTFA has been used in the quantification of amphetamine work.

There are two main options when selecting the best-suited detection system: gas chromatography equipped with a nitrogen phosphorus detector and GC–MS. Both chromatographic systems give similar sensitivities, while specificity will always be better for mass spectrometry. The main advantage of GC–MS when performing quantitative analysis of amphetamine is the commercial availability of deuterated analogues of amphetamine. In a report comparing different internal standards for the analysis of amphetamine and methamphetamine, it was shown that better precision was observed when using deuterated analogues of amphetamine as compared with alkyl derivatives of amphetamine also in use [19]. The use of stable isotope-labelled analogue to amphetamine as internal standard, such as amphetamine-d_8, eliminates problems related with differences in solubility, boiling point (evaporation losses) and chromatographic behaviour as compared with other amphetamine analogues. Amphetamine-d_8 complies with this requirement. In fact precision and accuracy observed during the methods' validation and its further application in a pharmacokinetic study are better than those reported in previous reports probably because of the use of amphetamine-d_8 as ISTD [11].

This analytical procedure has been used in clinical studies of amphetamine pharmacokinetics. The sensitivity of the method is adequate to monitor amphetamine plasma levels during 24 h in the range of doses tested.

The pharmacokinetics of amphetamine was evaluated in the 1960s [29]. As stated previously, today amphetamine has little interest in therapeutics but can be an interesting tool in clinical pharmacology. In fact amphetamine is a prototype of stimulant drug and can be used as control group when trying to characterise the pharmacological profile of amphetamine related compounds like designer drugs (MDMA, MDEA, MDA, etc.). Most work done in the past was performed using dextroamphetamine, nevertheless in many countries this substance is not available and amphetamine racemate is used. Pharmacologically both compounds should be equivalent, taking into account that the racemate contains 50% of dextroamphetamine. Nevertheless studies have to be

performed to ascertain this statement. Results summarised in the present work correspond to dose finding studies with amphetamine racemate in order to have a dose of amphetamine with an activity (psychomotor activity and subjective effects) in healthy volunteers high enough that results obtained can be used as a reference when evaluating the pharmacological profile of other stimulants. Three doses were tested (25, 30 and 35 mg) and their effects were considered too low and finally a dose of 40 mg was selected and administered to 11 healthy volunteers. When comparing AUC and C_{max} in the range of doses studied, variations behave in a linear mode and hence plasma levels can be predicted easily.

5. Conclusions

The major contributions of present study to the analysis of amphetamine in plasma are: (1) the use of MBTFA both to avoid amphetamine loses in the evaporation step of the organic extract before its GC analysis and to obtain TFA derivatives that give the method an easy and fast residue treatment which allows a quantitative analysis; and (2) the relevance of choosing a suitable deuterated internal standard (amphetamine-d_8) in GC–MS chromatography, especially in pharmacokinetics studies.

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