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Development and validation of a high-throughput method for the quantitative analysis of D-amphetamine in rat blood using liquid chromatography/MS^3 on a hybrid triple quadrupole-linear ion trap mass spectrometer and its application to a pharmacokinetic study

Nicola Cesari*, Stefano Fontana, Dino Montanari, Simone Braggio

Department of Neurosciences CEDD DMPK, GlaxoSmithKline Medicines Research Centre, Via Fleming 4, 37135 Verona, Italy

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ABSTRACT

Amphetamines are a group of sympathomimetic drugs that exhibit strong central nervous system stimulant effects. D-Amphetamine ((+)-alpha-methylphenetylamine) is the parent drug in this class to which all others are structurally related. In drug discovery, D-amphetamine is extensively used either for the exploration of novel mechanisms involving the catecholaminergic system, or for the validation of new behavioural animal models. Due to this extensive use of D-amphetamine in drug research and its interest in toxicologic–forensic investigation, a specific and high-throughput method, with minimal sample preparation, is necessary for routine analysis of D-amphetamine in biological samples. We propose here a sensitive, specific and high-throughput bioanalytical method for the quantitative determination of D-amphetamine in rat blood using MS^3 scan mode on a hybrid triple quadrupole-linear ion trap mass spectrometer (LC–MS/MS/MS). Blood samples, following dilution with water, were prepared by fully automated protein precipitation with acetonitrile containing an internal standard. The chromatographic separation was achieved on a Waters XTerra C18 column (2.1 mm × 30 mm, 3.5 μm) using gradient elution at a flow rate of 1.0 mL/min over a 2 min run time. An Applied Biosystems API4000 QTRAP™ mass spectrometer equipped with turbo ion-spray ionization source was operated simultaneously in MS^3 scan mode for the D-amphetamine and in multiple reaction monitoring (MRM) for the internal standard. The MS/MS/MS ion transition monitored was m/z 136.1 → 119.1 → 91.1 for the quantitation of D-amphetamine and for the internal standard (rolipram) the MS/MS ion transition monitored was m/z 276.1 → 208.2. The linear dynamic range was established over the concentration range 0.5–1000 ng/mL (r^2 = 0.9991). The method was rugged and sensitive with a lower limit of quantification (LLOQ) of 0.5 ng/mL. All the validation data, such as accuracy, precision, and inter-day repeatability, were within the required limits. This method was successfully applied to evaluate the pharmacokinetics of D-amphetamine in rat. On a more general extent, this work demonstrated that the selectivity of the fragmentation pathway (MS^3) can be used as alternative approach to significantly improve detection capability in complex situation (e.g., small molecules in complex matrices) rather than increasing time for sample preparation and chromatographic separation.

1. Introduction

D-amphetamine ((+)-alpha-methylphenetylamine, Fig. 1A) is a drug that acts as stimulant of the central nervous system, by increasing levels of norepinephrine, serotonin, and dopamine in the brain. Amphetamine was first synthesized in 1887 by Lazăr Edeleano in Berlin, Germany [1]. It was one of a series of compounds related to the plant derivative ephedrine, which had been isolated from Ma-Huang that same year by Nagayoshi Nagai [2]. No pharmacological use was found for amphetamine until 1929, when psychopharmacologist Gordon Alles resynthesized and tested it on himself, in search of an artificial replacement for ephedrine [3]. From 1933 Smith, Kline and French began selling the racemic volatile base form of the drug under the name Benzedrine Inhaler as a decongestant.

In 1935 appeared the first report of amphetamine use as a clinical treatment for narcolepsy [4].

In the past years the pharmaceutical industry has promoted many uses for amphetamine, including treatment of schizophrenia, opiate addiction, infantile cerebral palsy, seasickness, radia-

* Corresponding author. Tel.: +39 0458219673; fax: +39 0458218153.
E-mail address: nicola.2.cesari@gsk.com (N. Cesari).

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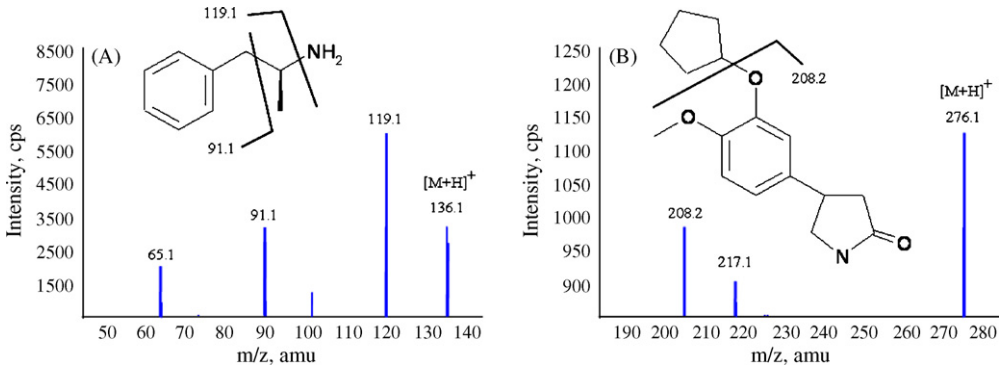

Fig. 1. Chemical structures and product ion spectra of $[M+H]^+$ of D-amphetamine (A) and rolipram (B).

tion sickness, and persistent hiccups. During World War II amphetamine was extensively used to combat fatigue and increase alertness in soldiers.

To date, when used within the recommended doses, amphetamine is an oral prescribed treatment, in both children and adults, for anti-depressant therapy in treatment-resistant depression and for attention-deficit hyperactivity disorder (ADHD). Beneficial effects for ADHD include improved impulse control, improved concentration, decreased sensory over-stimulation, decreased irritability and decreased anxiety.

In drug discovery, when the research is focused in looking for new drugs acting at central nervous system, D-amphetamine is extensively used either for the exploration of novel mechanisms involving catecholaminergic system, or for the validation of new behavioural animal models.

Due to an extensive use of D-amphetamine in drug research and to its interest in toxicologic–forensic investigation, a specific and high-throughput method, with minimal sample preparation, is necessary for routine analysis of D-amphetamine in biological samples.

In the past, numerous methods have been developed for the determination of D-amphetamine in biological samples including those using capillary electrophoresis with UV detection (CE/UV) [5], and those using a liquid chromatography (LC) with a UV detector [6–10], or a fluorescence detector [11]. One of the major limitations of these methods is a poor selectivity, which *de facto* limits the sensitivity and requires complicated sample preparations to remove matrix interferences and long separation times up to 30 min to resolve these from the analyte.

Amphetamine has been successfully analyzed in biological matrices by using gas chromatography (GC) with either a nitrogen phosphorus detector [12] or mass spectrometric detection (MS) (see [13,14] for review). This technique has the advantage of great peak separation but it requires time consuming sample preparations methodologies such as liquid–liquid extraction and solid phase extraction. In addition, the derivatization of amine and hydroxyl moieties may be necessary to prevent peak tailing and improve the sensitivity.

In the recent years liquid chromatography (LC) coupled to mass spectrometry (MS) has been successfully applied for the analysis of a wide variety of small molecules in biological matrices, including D-amphetamine, due to the high sensitivity and selectivity of LC–MS/MS methodology. The selectivity of MS/MS in bioanalysis has enabled users to decrease significantly the analysis time. Even with unit resolution on triple quadrupole systems, in the majority of cases, single chromatographic peaks will be observed. Hence, several LC–MS/MS applications for analysis of D-amphetamines have been developed recently for the determination of D-amphetamine in urine, plasma and in serum samples [15–17]. Despite the highest sensitivity achieved with this technique, however, in some cases, the presence of endogenous species in biological extracts can still lead to interferences, even in MS/MS mode. In order to improve selectivity, several scenarios can be used to eliminate the interferences, but most of them lead to an increase in the analysis time and/or a decrease in sensitivity. Wang et al. [15] reported a failure in their first approach of developing a LC–MS/MS assay for D-amphetamine and diphenhydramine in dog plasma using simply a protein precipitation for the cleaning up of the samples. They reported the presence of strong interferences from the sample matrix and low recoveries, not eliminable simply using protein precipitation and LC–MS/MS.

In the following work, we propose another alternative to improve selectivity in quantitative applications, using MS^3 on a hybrid quadrupole-linear ion trap (QqLIT). The concept relies on the selectivity of the fragmentation pathway as opposed to the fragment mass. In a hybrid quadrupole-linear the final quadrupole can be operated as either a standard radiofrequency (RF/DC) resolving quadrupole mass filter or as a linear ion trap with axial ion ejection [18]. This combination of mass analysers can be used to perform quadrupole or trap scans while switching from one to the other in a few milliseconds. The performance of either mode is not compromised by this switching [19]. Thus it is possible to perform one scan in quadrupole mode (i.e., selected reaction monitoring) and the next scan performs a trap experiment. Thus a large number of combinations of different scan types can be performed. Of particular interest for quantification methodologies is the use of the scan modes MRM and MS^3 in combination. Since MS^3 is simply adding another stage of fragmentation, it is not unreasonable to assume that the probability of having compounds that will have common precursor ion, 1st fragment ion and 2nd fragment ion would be very low. Additionally a concomitant MRM scan can be performed to monitor other transitions such as an internal standard.

Very few authors reported the use of MS^3 on a hybrid quadrupole-linear ion trap mass spectrometer for the quantitative analysis of drugs in biological fluids [20,21] and, to the best of our knowledge, this appears to be the first complete validation of a quantitative method using LC–MS^3. Herein we described, for the first time the validation of a quantitative method using fully automated protein precipitation, fast liquid chromatography and MS^3 as scan function by using a hybrid quadrupole-linear ion trap mass spectrometer. Using this approach we obtained a specific and sensitive method for the quantitative analysis of D-amphetamine in rat blood that was successfully applied to pharmacokinetic studies.

2. Experimental

2.1. Chemicals and reagents

D-Amphetamine sulphate salt (>99.7%) was purchased from Sigma–Aldrich (Steinheim, Germany). Rolipram was purchased

from Sigma–Aldrich (Steinheim, Germany). DMSO was purchased from Riedel De-Haen (Sigma–Aldrich Steinheim, Germany). Acetonitrile (HPLC grade) was purchased from Mallinkrodt Baker (Deventer, Germany). Formic acid (mass spectrometry grade) was purchased from Fluka (Sigma–Aldrich, Steinheim, Germany). Ammonia solution 32% extra pure was purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate (99.5%) was purchased from Sigma–Aldrich (Steinheim, Germany). Deionised water (18.2 MΩ/cm) was generated in-house using a Milli-Q System from Millipore (Bedford, MA, USA).

2.2. LC–MS/MS/MS instrumentation

The LC–MS/MS system used in this work consisted of an Agilent 1100 series vacuum degasser, binary pump and thermostated column compartment, a CTC PAL autosampler (Zwinger, Switzerland) and an Applied Biosystems/MDS SCIEX API 4000QTRAP (Concord, ON, Canada) equipped with turbo ion-spray (TIS) source. System control and data analysis were provided by the Applied Biosystems Analyst 1.4.2 software.

2.3. Liquid chromatographic conditions

The chromatographic separation was achieved with a Waters XTerra C18 column (2.1 mm × 30 mm, 3.5 μm particle size) at 25 °C. The mobile phases, delivered at a flow rate of 1 mL/min, were pH 9 ammonium bicarbonate buffer (phase A) and acetonitrile, containing 0.1% (v/v) formic acid (phase B), programmed as follows: 5% acetonitrile during 0.3 min, linearly increased to 95% in 0.7 min, kept that percentage for 0.5 min, decreased to 5% in 0.1 min (original conditions), and equilibrated for 0.4 min, which resulted in a total run time of 2 min (detailed LC conditions are shown in Table 1). Autosampler temperature was kept at 5 °C and the injection volume was set at 5 μL.

2.4. Mass spectrometer conditions

Mass spectrometer was operated in positive ion mode; MS^3 and selected reaction monitoring (SRM) were used for the data acquisition of amphetamine and rolipram (internal standard), respectively. The MS parameters for the analysis were as follows: ion source temperature, 650 °C; ion-spray voltage, 4000 V. In the MS^3 experiment the first precursor ion isolated was the protonated D-amphetamine (m/z 136.1) which was fragmented using collision gas flow (CAD) at the instrument setting of 6 and collision energy (CE) of 10 eV giving the second precursor ion at m/z 119.1. The second precursor ion was trapped and accumulated in the linear ion trap (LIT) using a fixed LIT fill time of 100 ms and excitation time of 40 ms. The resulting MS^3 spectrum showed an intense peak at m/z 91.1; therefore, for the quantitation of D-amphetamine, the MS/MS/MS ion transition monitored was m/z 136.1 → 119.1 → 91.1 with the LIT set to perform a mass scan centered at m/z 91.1 and a mass window of 20 amu. The time required to perform the MS^3 was 277 ms. For the internal standard (rolipram) the MS/MS ion transition monitored was m/z 276.1 → 208.2 using a dwell time of 10 ms. The final MS cycle time, including both MS^3 and SRM experiments, was 290 ms. Product ion mass spectra for D-amphetamine and rolipram are shown in Fig. 1.

Table 1
Gradient elution timetable.

Time (min)	%A	%B
0.00	95	5
0.30	95	5
1.00	5	95
1.50	5	95
1.60	95	5
2.00	95	5

A, ammonium bicarbonate 10 mM buffered at pH of 9 with ammonia; *B*, acetonitrile containing 0.1% (v/v) formic acid.

2.5. Preparation of the standard and quality control (QC) samples

Stock solutions of D-amphetamine were all 1 mg/mL and dissolved in appropriate volume of water:acetonitrile (1:1, v/v). Standard working solutions were serially diluted from stock solutions with water:acetonitrile (1:1, v/v). Stock solution concentration of rolipram, as the internal standard (IS), was 1 mg/mL and dissolved in dimethyl sulfoxide (DMSO). Drug free rat blood samples were collected from not treated animals into K3EDTA tubes and then diluted 1:1.71 (v/v) with water. 190 μL of rat blank blood:water (1:1.71) were added with 10 μL working standard solution to give calibration standards. Blood calibration concentrations of D-amphetamine were 0.5, 1, 2, 4, 10, 20, 40, 100, 200, 400, 1000 ng/mL. Quality control (QC) samples were prepared with blank blood at LLOQ, low, medium and high concentrations, which were set at 0.5, 1.2, 120, 600 ng/mL.

2.6. Sample preparation

Blood samples, including calibration standard and QC samples, were prepared by protein precipitation. Rat blood samples were stored as 200 μL aliquots at −20 °C; the aliquots were thawed at room temperature before analysis and were deprotenized adding 400 μL of acetonitrile containing rolipram (IS) at a concentration of 20 ng/mL. Samples were vortexed and centrifuged at 3000 rpm for 3 min at 5 °C. The supernatants (100 μL) were transferred to another 96 well plate and added with 80 μL of water. After vortexing for 10 s at 300 × *g*, 5 μL were used for injection in the chromatographic system.

2.7. Method validation

The method validation assays were carried out by following the currently accepted US Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance [22]. The following parameters were determined for the validation of the analytical method of D-amphetamine in rat blood: selectivity, linearity, lower limit of quantification (LLOQ), precision, accuracy, extraction recovery, matrix effect and stability.

2.8. Selectivity

Selectivity was evaluated by comparing chromatograms of six blank blood samples from six different sources to make sure there were no significant interfering peaks at retention time at LLOQ of the analytes.

2.9. Linearity and lower limit of quantification

A line ($y = ax + b$) was fitted through the standard curve ranged by a weighted linear regression (weight = $1/x^2$) of peak area ratio of D-amphetamine to IS (y) versus actual concentration of the analyte (x). LLOQ, defined in the presented study is the lowest blood concentration in the calibration curve that can be measured by precision and accuracy. The precision and accuracy were evaluated by the relative standard deviation (RSD) and relative error (RE), respectively. The acceptable value of RSD was below 20% and RE was within ±20%.

2.10. Accuracy and precision

The within-run precision and accuracy were determined by analyzing QC samples ($n = 6$) as described above. The between-run precision and accuracy were also carried out by analyzing QC samples in 6 batches on different days. The precision was presented as RSD and accuracy was as RE.

2.11. Extraction recovery and matrix effect

The used concentrations of D-amphetamine for the evaluation of recovery and matrix effect were set at 1.2, 120 and 600 ng/mL. 20 ng/mL IS were used with each experimented concentration.

The extraction recoveries were determined by comparing the response ratio of extracted blood standards with those of extracted blank blood spiked with corresponding concentrations. The response was defined as the peak area of analyte divided by the peak area of IS. Six different sources of blank blood were used to assess the matrix effect. The absolute and relative matrix effect was previously defined by Matuszewski et al. [23]. The absolute matrix effect was evaluated by comparing the peak areas of analytes added to extracted blank blood with those of extracted water and the RSD of the mean peak areas of analytes in the extracted blank blood indicated the relative matrix effect.

2.12. Stability

The stability of D-amphetamine in rat blood was assessed using QC samples, prepared with blank blood at LLOQ, low, medium and high concentrations, which were set at 0.5, 1.2, 120, 600 ng/mL.

QC samples were kept at different storage conditions and were expressed as relative error (RE%) with respect to nominal concentration. Bench-top stability was evaluated by keeping D-amphetamine blood QC samples at ambient temperature (25 °C) for 6 h. Short-term and long-term stability were determined following storage of six QC samples at −20 °C for 1-week and 3 months,

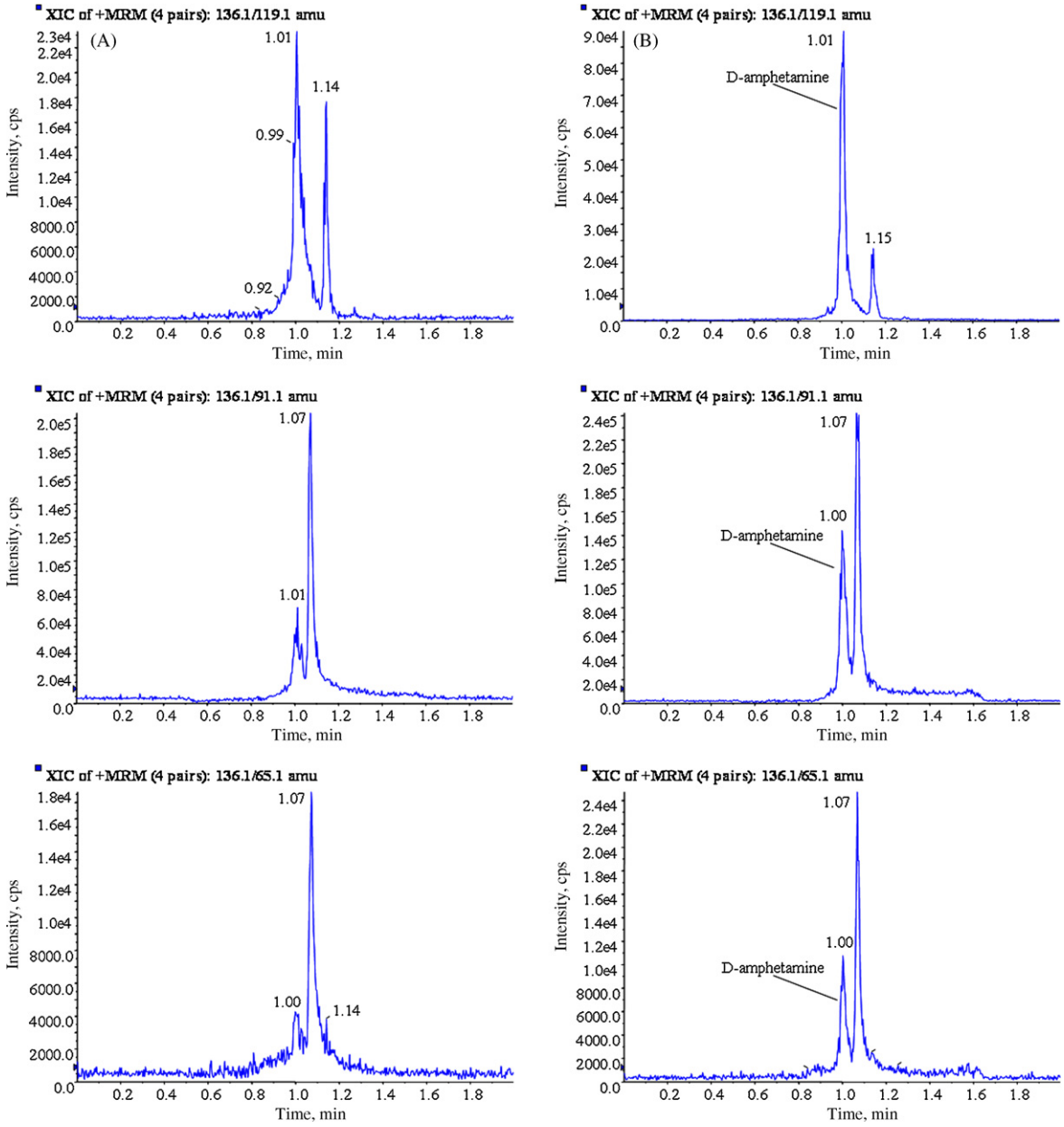

Fig. 2. Representative MRM chromatograms of D-amphetamine. (A) Double blank rat blood. (B) Blank rat blood spiked with D-amphetamine at 50 ng/mL.

respectively. Freeze–thaw stability was carried out by analyzing D-amphetamine samples processing three freeze–thaw cycles at −20 °C.

2.13. Pharmacokinetic study in rat

The present method was applied to a pharmacokinetic study of D-amphetamine during the D-amphetamine-induced sensitization test in rat.

In this test, three male Sprague–Dawley rats (Charles River Laboratories) received a chronic intraperitoneal administration of D-amphetamine at 1 mg/kg (1 mL/kg) during 8 days. At the same time, three other male Sprague–Dawley rats received a chronic intraperitoneal administration of D-amphetamine at 1 mg/kg (1 mL/kg), co-administered with a GSK test compound. D-Amphetamine levels were determined to exclude PK interaction in the co-administration.

All blood samples were collected into K_3EDTA tubes at 0.33, 1, 2, 4 h after administration and, immediately, aliquoted to 200 μL, by mixing 70 μL of whole blood with 130 μL of water. Diluted blood samples were stored at −20 °C prior to the analysis.

3. Results and discussion

3.1. Method development

The most widely used mass spectrometers in the bioanalytical laboratory are triple quadrupole and ion trap mass analysers. For a full scan MS–MS experiment, triple quadrupole analysers have a relatively poor duty cycle as only one precursor to fragment pair is stable throughout the instrument at one time. Thus most of the ions from the source are wasted. But for quantification, using multiple reactant monitoring (MRM), the high duty cycle provides excellent sensitivity. For conventional ion traps, once the ions are introduced into the trap, all of the different steps of ion manipulation occur within the same volume, but at different times. The advantage of this process, compared to triple quadrupole analysers, is that a complete mass spectrum can be obtained for each pulse of ions introduced into the ion trap. Thus increased scanning sensitivity is obtained. The development of a hybrid triple quadrupole-linear ion trap mass analyser combines the functionality of both without compromising the performance of either. The final quadrupole can be operated as either a standard radiofrequency (RF/DC) resolving quadrupole mass filter or as a linear ion trap with axial ion ejection.

Fig. 1(A and B) shows MSMS spectra of D-amphetamine and rolipram. At least three major fragments (*m*/*z* 119, 91 and 65) can be used by MRM for the quantitative analysis of D-amphetamine in biological fluids such as plasma. Unfortunately, each one of these MRM transitions demonstrated varying degrees of interference, especially at the low level, thus effectively increasing the lower limit of quantification (LOQ), as can be seen in Fig. 2(A and B), where chromatograms of blank blood samples (Fig. 2A) are compared with D-amphetamine spiked blood samples (Fig. 2B) for all the MRM transitions used for the quantitation of the D-amphetamine. Since D-amphetamine has a specific fragmentation pathway, MS^3 was investigated to improve the LOQ and the selectivity of the method.

During the second stage of fragmentation in a 4000 QTRAP, the fragmentation is normally performed in the Q3 mass analyser at low pressure (4.2×10^{-5} Torr). In trap, fragmentation is carried out using the technique of dipole excitation [19]. Under these conditions, if an ion fragments easily (low excitation energy and short time), it is possible to observe fragmentation at efficiencies >90% and with a low mass cut-off (LMCO) of 0.26× precursor *m*/*z*. The ability to induce more efficient fragmentation with shorter excitation time has benefits for quantitative LC–MS^3 analysis. Performing the fragmentation on a shorter time scale enables collection of more points across the LC peak and generally translates into better precision on the measurement. Furthermore, as it was previously demonstrated, MS^3 enables an increase in selectivity by almost an order of magnitude over LC–MS/MS (MRM). This is demonstrated with the analysis of D-amphetamine in plasma. Fig. 1(A) shows the MS/MS spectrum of D-amphetamine. The most intense fragment during in-cell fragmentation is *m*/*z* 119, which is 2–3× more intense than the next best candidates (*m*/*z* 91 and 65), but might not necessarily be the most selective. Fig. 2(A and B) shows that interferences are observed in all three MRM traces that could be used for D-amphetamine. Although the extent of interference varies with the LC and MRM conditions used, sudden changes in the level of the interference can have a serious impact on the accuracy and precision of the method.

Using the same sample set, we performed MS^3 of 136 > 119 and used extracted ion chromatogram (XIC) of 91 fragment ion to generate calibration curves. Since the first fragmentation step occurs in the collision cell, the time required to perform the MS^3 is on the order of 277 ms (including isolation, fragmentation and scanning) which is still reasonable for quantitation of single analyte. Fig. 3(A) and (B) shows the XIC for fragment 91 when blank rat blood (A) and rat blood spiked with D-amphetamine at 50 ng/mL (B) were injected. It is clear from these chromatograms that the interferences are completely eliminated, as a result of the increased selectivity. Using this approach, the quantitation limit for D-amphetamine was 2.5 pg on column (see Section 3.2.2.) and, when compared to MRM for the corresponding fragment 91, MS^3 represents a 200× improvement in detection limit.

As said before, in a linear ion trap the final quadrupole can be operated as standard radiofrequency (RF/DC) resolving quadrupole mass filter or as a linear ion trap with axial ion ejection. This combination of mass analysers can be used to perform quadrupole or trap scans while switching from one to the other in a few milliseconds.

In this study, D-amphetamine was sampled using MS^3 scan function (ion trap mode) with a scan time of 277 ms, while the internal standard (rolipram) was sampled by using SRM, setting a second experiment with a scan time of 10 ms. The final cycle time (290 ms) enabled the collection of at least 13 data across the LC peak.

In this study, by using the unique selectivity of MS^3, we demonstrated that automated protein precipitation (see Section 2.6.), even though it is not considered a highly efficient sample preparation methodology such as SPE and LLE, may be employed for the analysis of D-amphetamine in rat blood.

3.2. Method validation

3.2.1. Selectivity

The selectivity of the method was tested by comparing the chromatograms of different lots of blank blood and spiked blood. All blank rat blood lots were found to be free of interferences with respect to rolipram transition 276.1 → 208.2 in the SRM scan mode. On the other hand, the MRM analysis of blank blood using the three transitions of D-amphetamine 136.1 → 119.1, 136.1 → 91.1 and 136.1 → 65.1 showed the presence of strong interferences at the same retention time of the analyte. The intensity of the interferences was so high as to increase the LLOQ up to 100 ng/mL in whole blood. MRM representative chromatogram of double blank rat blood is shown in Fig. 2(A), while an example of spiked rat blood chromatogram with D-amphetamine and rolipram at whole blood concentration of 50 ng/mL is shown in Fig. 2(B). Interferences were avoided on the D-amphetamine chromatogram only using the transitions 136.1 → 119.1 → 91.1 with MS^3 scan mode. D-Amphetamine MS^3 representative chromatogram of blank rat blood is shown in Fig. 3(A), while an example of spiked rat blood chromatogram with D-amphetamine at whole blood concentration of 50 ng/mL is shown

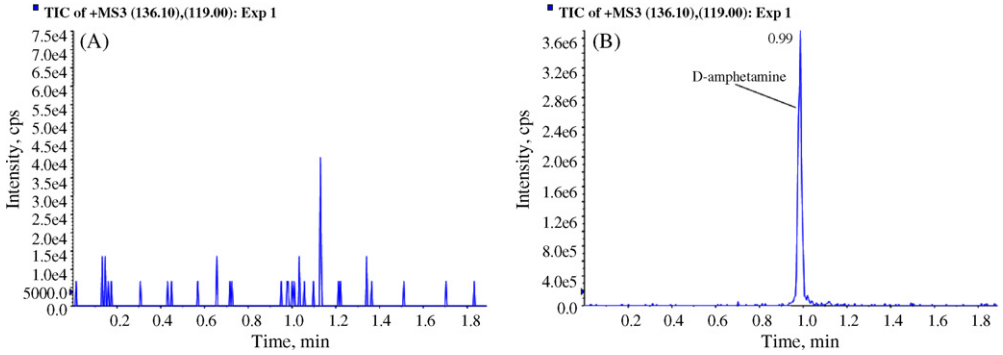

Fig. 3. Representative MS^3 chromatograms of D-amphetamine. (A) Double blank rat blood. (B) Blank rat blood spiked with D-amphetamine at 50 ng/mL.

in Fig. 3(B). The method was set up using SRM scan mode for the IS and MS^3 scan mode for D-amphetamine.

3.2.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were constructed from working standard solutions at eleven concentrations of D-amphetamine ranging 0.5–1000 ng/mL in whole rat blood by plotting peak area ratio (y) of D-amphetamine to the internal standard, rolipram, versus D-amphetamine concentrations (x). Chromatographic peaks were integrated using Analyst 1.4.1 and the regression parameters of slope, intercept, correlation coefficient and quantification of samples calculated using the software Statsoft Statistica (v. 8). The standard calibration curves for D-amphetamine were linear over the concentration range 0.5–1000 ng/mL with $r^2 = 0.9991$ when evaluated by weighted $1/x$. A typical equation for the calibration curves was: $y = 2.035x + 0.1869$. The lower limit of quantification, defined as the lowest concentration on the calibration curve, was validated using an LLOQ sample (LLOQ QC) for which an acceptable accuracy, expressed as relative error (RE%), within ±20% and a precision, expressed as relative standard deviation (RSD), below 20% were obtained. The lower limit of quantification was 0.5 ng/mL, with precision and accuracy reported in Table 2 with RE% within ±20% and RSD lower than 20%.

3.2.3. Precision and accuracy

Intra-day precision and accuracy were determined performing eight replicates analysis of QC blood samples on the same day at four QC concentration levels: LLOQ QC 0.5 ng/mL, Low QC 1.2 ng/mL, Mid QC 120 ng/mL and High QC 600 ng/mL. Precision was expressed as the RSD and accuracy as RE% determined by comparing the calculated concentration using calibration curves to known concentration. The same procedure was performed once a day for five consecutive days to determine inter-day precision and accuracy. Table 2 summarizes the intra- and inter-day precision and accuracy for D-amphetamine in rat blood. The intra-day precision for the LLOQ QC, Low QC, Mid QC and High QC was 7.96%, 11.12%, 8.93% and 6.14%, respectively and the accuracy ranged from −6.87% to 0.43% throughout the four concentrations. The inter-day precision for the LLOQ QC, Low QC, Mid QC and High QC was 7.19%, 8.27%, 2.65% and 5.65%, respectively and the accuracy ranged from −1.97% to 2.74% throughout the four concentrations.

3.2.4. Recovery and matrix effect

The extraction recoveries of D-amphetamine using protein precipitation were calculated by comparing the peak areas of six replicates of extracted blood QC samples (Low QC, Mid QC and High QC) to those of post-extraction blood blanks spiked with the corresponding concentrations. Extraction recoveries are expressed as the percentage of the ratio of the mean peak area of the analyte spiked into blood pre-extraction to the mean peak area of the analyte into blood post-extracted. Mean D-amphetamine recoveries in rat blood were 86%, 101% and 98% at concentration of 1.2, 120 and 600 ng/mL.

The matrix effect on D-amphetamine in rat blood was evaluated by comparing the peak areas of six replicates of extracted blood QC samples (Low QC, Mid QC and High QC) to those obtained extracting

Table 2
Summary of precision and accuracy of D-amphetamine in rat blood.

QC nominal concentration (ng/mL)	Intra-day ($n = 8$)			Inter-day ($n = 8$)		
	Measured concentration (ng/mL) (mean ± SD)	RSD (%)	RE[a] (%)	Measured concentration (ng/mL) (mean ± SD)	RSD (%)	RE[a] (%)
0.5 (LLOQ QC)	0.50 ± 0.04	7.96	11.10	0.49 ± 0.03	7.19	−1.97
1.2	1.12 ± 0.12	0.43	−6.87	1.19 ± 0.01	8.27	−0.44
120	116.55 ± 10.41	8.93	−2.87	118.94 ± 3.15	2.65	−0.88
600	567.55 ± 34.84	6.14	−5.41	616.45 ± 34.85	5.65	2.74

[a] RE is expressed as [(mean measured concentration)/(nominal concentration) − 1] × 100.

Table 3
Matrix effect of D-amphetamine in rat blood.

QC nominal concentration (ng/mL)	($n = 6$)		
	Spiked blood peak area ($\times 10^5$) (mean ± SD)	Spiked water:acetonitrile peak area ($\times 10^5$) (mean ± SD)	ME[a] (%)
1.2	1.99 ± 0.17	1.73 ± 0.21	−15.44
120	184.42 ± 17.10	158.09 ± 21.43	−16.65
600	1003.22 ± 118.56	882.55 ± 118.98	−13.67

[a] ME is expressed as [100 − (mean peak area in blood)/(mean peak area in water:acetonitrile 1:1)] × 100.

Table 4
Stability of D-amphetamine in rat blood.

Sample condition	QC nominal concentration (ng/mL)	Measured concentration (ng/mL) (mean ± SD)	RE[a] (%)
Bench-top[b] ($n = 5$)			
	1.2	1.11 ± 0.14	7.69
	120	112.34 ± 8.02	6.38
	600	595.81 ± 29.04	0.70
Short-term[c] ($n = 5$)			
	1.2	1.29 ± 0.19	−8.08
	120	119.97 ± 4.70	0.03
	600	658.62 ± 19.21	−9.77
Long-term[d] ($n = 5$)			
	1.2	1.32 ± 0.25	−10.11
	120	105.50 ± 3.17	12.08
	600	523.40 ± 22.90	12.77
Freeze–thaw[e] ($n = 5$)			
	1.2	1.13 ± 0.22	5.83
	120	103.33 ± 5.21	13.89
	600	509.67 ± 39.15	15.06

[a] RE is expressed as [(mean measured concentration)/(nominal concentration) − 1] × 100.
[b] Exposed at room temperature (25 °C) for 6 h.
[c] Stored at −20 °C for 1-week.
[d] Stored at −20 °C for 3 months.
[e] After three freeze–thaw cycles.

the pure compound spiked in water:acetonitrile 1:1 at the same QC concentrations. The percentage of matrix effect for D-amphetamine at three level QC concentrations (Low QC, Mid QC and High QC) is reported in Table 3 and was evaluated as the percentage of the ratio of the mean peak area of the analyte spiked into blood to the mean peak area of the analyte spiked into water:acetonitrile 1:1. No significant matrix effect was observed for D-amphetamine in rat blood.

3.2.5. Stability

The stability of D-amphetamine in rat blood was assessed using Low QC, Mid QC and High QC under different storage conditions and is expressed as the relative error (RE%) with respect to nominal concentration. Bench-top stability was evaluated by exposing D-amphetamine spiked blood QC samples at room temperature (25 °C) for 6 h. Short-term and long-term stability were determined using spiked blood QC samples after a period of storage at −20 °C for 1-week and 3 months, respectively. Freeze–thaw stability was determined by thawing at room temperature and refreezing at −20 °C spiked blood QC samples for three cycles. Results, as shown in Table 4, demonstrated a good stability of D-amphetamine during all tested storage conditions and confirmed the applicability of the method for routine analysis.

3.3. Application to pharmacokinetic study

We applied this analytical method to the quantification D-amphetamine in rat blood in support to a D-amphetamine-induced sensitization test, an animal model able to evaluate the active involvement of a test compound with the catecholaminergic system. In this experiment three male Sprague–Dawley rats were intraperitoneally administered with a saline solution of D-amphetamine at 1 mg/kg (1 mL/kg) during 8 days. At the same time three different male Sprague–Dawley rats received intraperitoneal administration of D-amphetamine at 1 mg/kg (1 mL/kg) during 8 days but co-administered with a GSK test compound able to reverse the central side effects induced by the administration of the D-amphetamine. Blood samples were collected from all treated rats at 0.33, 1, 2, 4 h after administration and analyzed with described LC–MS/MS/MS method. Pharmacokinetic parameters were obtained from the blood concentration-time profiles with standard non-compartmental method using WinNonlin version 4.0 (Pharsight Corp., Mountain View, CA, USA). The mean blood concentration-time profiles of D-amphetamine when administered alone and during the co-administration are shown in Fig. 4. The pharmacokinetic parameters used to investigate potential drug–drug interaction were the time characterized by

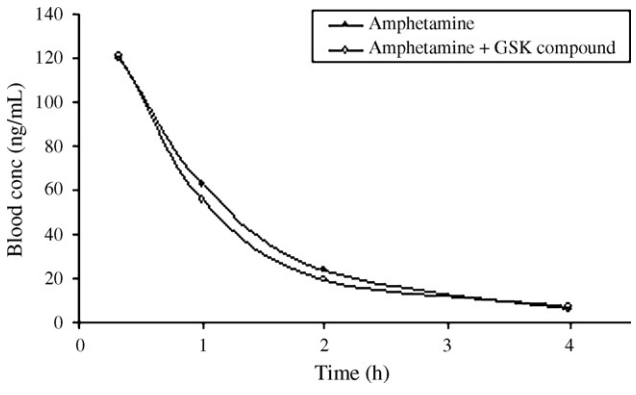

Fig. 4. Mean blood concentration-time profile D-amphetamine after intraperitoneal administration with and without co-administration of GSK test compound.

Table 5
Pharmacokinetic parameters of D-amphetamine in male rat after intraperitoneal administration with and without co-administration of GSK test compound.

Parameter	D-Amphetamine (mean ± SD)	D-Amphetamine with GSK test compound (mean ± SD)	End-point fold change
C_{max} (ng/mL)	120.3 ± 5.9	121.1 ± 42.6	1.0
T_{max} (h)	0.33	0.33	1.0
AUC (0 → 4 h) (h ng/mL)	144.9 ± 30.3	132.1 ± 11.6	0.9

the maximum concentration (T_{max}), the maximum concentration observed (C_{max}) and the area under the blood concentration-time curve (AUClast). All described PK parameters obtained for D-amphetamine in rat blood when administered alone and during the co-administration with a GSK test compound are shown in Table 5. D-amphetamine showed a good PK profile when intraperitoneally administered at 1 mg/kg with a C_{max} around 100 ng/mL reached at 20 min after administration. The results obtained indicated that PK profile of D-amphetamine was not modified by the co-administration with a GSK test compound as demonstrated by the end-point fold change around 1.0 for all determined PK parameters.

4. Conclusion

The validation of a LC–MS/MS/MS method for the quantitative analysis of D-amphetamine in rat blood is described. The method is very sensitive with a LLOQ of 0.5 ng/mL in whole blood and good linearity, precision and accuracy were achieved. It was demonstrated that the selectivity of the fragmentation pathway (MS^3) can be used to significantly improve detection limits in the presence of interferences. Using MS^3 for quantitation can provide a high degree of selectivity that can be applied in a much more generic fashion than increasing time for sample preparation for chromatographic separation.

Therefore, the selectivity achieved by MS^3 offers the advantage of analyzing D-amphetamine directly in rat blood, using automated protein precipitation as sample preparation and fast gradient liquid chromatography.

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