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Short communication

Development and validation of a high-performance liquid chromatography-tandem mass spectrometry assay for the quantification of Dexamphetamine in human plasma

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ABSTRACT

Dexamphetamine is registered for the treatment of attention deficit hyperactivity disorder and narcolepsy. Current research has highlighted the possible application of dexamphetamine in the treatment of cocaine addiction. To support clinical pharmacologic trials a new simple, fast, and sensitive assay for the quantification of dexamphetamine in human plasma using liquid chromatography tandem mass spectrometry (LC–MS/MS) was developed. Additionally, it is the first reported LC–MS assay with these advantages to be fully validated according to current US FDA and EMA guidelines.

Human plasma samples were collected on an outpatient basis and stored at nominally −20 °C.

The analyte and the internal standard (stable isotopically labeled dexamphetamine) were extracted using double liquid–liquid extraction (plasma-organic and organic-water) combined with snap-freezing. The aqueous extract was filtered and 2 μL was injected on a C18-column with isocratic elution and analyzed with triple quadrupole mass spectrometry in positive ion mode.

The validated concentration range was from 2.5–250 ng/mL and the calibration model was linear. A weighting factor of 1 over the squared concentration was applied and correlation coefficients of 0.997 or better were obtained. At all concentrations the bias was within ±15% of the nominal concentrations and imprecision was ≤15%. All results were within the acceptance criteria of the latest US FDA guidance and EMA guidelines on method validation.

In conclusion, the developed method to quantify dexamphetamine in human plasma was fit to support a clinical study with slow-release dexamphetamine.

1. Introduction

Dexamphetamine (dextroamphetamine, *S*-amphetamine) (Fig. 1.) is a powerful central nervous system stimulant [1]. It exerts its pharmacological action through the rapid increase of dopamine levels in the striatum and noradrenaline levels in the prefrontal cortex [2].

In Europe, dexamphetamine is used in the therapy of attention-deficit hyperactivity disorder (ADHD) for children between the ages of 6 and 17 years where methylphenidate and atomoxetine were not effective [3,4]. In the United States, in addition to ADHD, dexamphetamine is also licensed to be prescribed to patients that suffer from narcolepsy [5]. Increasing attention is directed towards the possible application of dexamphetamine in the treatment of cocaine dependence, for which no medication is yet approved. Several reviews and studies suggest the promising effectiveness of dexamphetamine in the agonist pharmacotherapy of cocaine dependence [6,7].

A study where the acceptance, safety, and efficacy of dexamphetamine in the therapy of chronic cocaine-dependent patients on heroin-assisted treatment were investigated was recently carried out in the Netherlands [8]. For this trial, a new sustained release formulation of dexamphetamine was developed. To support further pharmacokinetic clinical studies of this formulation, a fast and accurate bioanalytical assay for the quantification of dexamphetamine in human plasma was developed.

Abbreviations: MTBE, methyl *tert*-butyl ether; MF, matrix factor.

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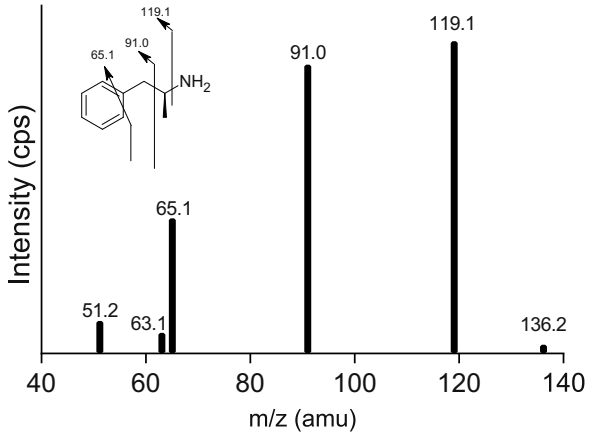

Fig. 1. Molecular structure with suggested fragmentation pathways, product ion mass spectrum of dexamphetamine and parent ion (MH^+) signal at *m/z* 136.2.

Several analytical assays for dexamphetamine have been described in literature [9–23]. The published assays for the quantification of dexamphetamine in plasma cover several analytical methods, such as gas and high-performance liquid chromatography coupled to mass spectrometry (HPLC–MS).

The HPLC–MS methods for human plasma require double liquid–liquid extraction with toxic chloroform, expensive solid-phase extraction, long analysis time or a relatively large injection volume [9,16,18,22]. Additionally, no bioanalytical HPLC–MS method with an isotopically labeled internal standard, which has been fully validated for human plasma according to current bioanalytical guidelines, has been published as far as we know, however. Our goal was to develop a sensitive LC–MS method with a simple, fast and low-cost sample pre-treatment. Previously published methods fall short in one or more of these factors.

This article describes a simple, fast and sensitive method for the quantification of dexamphetamine in human plasma. The assay was fully validated according to the latest US Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines on bioanalytical method validation [24,25]. Additionally, the applicability of the method was tested and demonstrated in clinical study sample analysis with concentrations of dexamphetamine.

2. Experimental

2.1. Chemicals

Dexamphetamine sulfate ($(C_9H_{13}N)_2SO_4$ was purchased from Fagron (Waregem, Belgium). Deuterated amphetamine ($^2H_6$), used as an internal standard (IS) for the assay was manufactured by and obtained from LGC Standards (Teddington, UK). Methanol and water (UPLC–MS grade) were purchased from Biosolve (Amsterdam, The Netherlands). Methyl *tert*-butyl ether (Lichrosolv grade), 25% ammonia (Emsure grade) and 99% formic acid (Emsure grade) were obtained from Merck (Darmstadt, Germany).

2.2. Stock solutions, calibration standards, and quality control samples

Separate stock solutions for calibration standards and quality control samples (QC samples) of 0.1 mg/mL were prepared in water. These stock solutions were further diluted in water to obtain working solutions. Stock solutions of the IS were prepared at a concentration of 0.1 mg/mL in water. The IS working solution contained 250 ng/mL. All stock and working solutions were stored at −20 °C.

Calibration standards were spiked directly after the preparation of the working solutions. A volume of 50 μL of calibration working solution was added to 950 μL control human K_2EDTA plasma to obtain concentrations of 2.5, 5, 10, 25, 50, 100, 200 and 250 ng/mL. The QC samples were prepared by adding 100 μL QC working solution to 1900 μL control human K_2EDTA plasma. Final concentrations at the lower limit of quantification (LLOQ), QC low, QC mid, QC high and QC above upper limit of quantification (> ULOQ) were 2.5, 7.5, 25, 200 and 2000, respectively.

2.3. Sample preparation

A volume of 20 μL internal standard working solution and 30 μL of 1 M NH_4OH were added to a 200 μL plasma aliquot. The mixture was vortexed for 10 s. 1000 μL of methyl *tert*-butyl ether (MTBE) was added, after which the volume was vortexed for 10 s and mixed in an automatic shaker for 10 min at 1250 rpm. Next, the samples were centrifuged at 20,000*g* for 5 min. The aqueous layer was snap frozen on an ethanolic dry ice bath. The organic layer was then transferred to a 1.5 mL Eppendorf tube. 200 μL of 0.01 M formic acid was introduced to the organic layer and the mixture was vortexed for 10 s and mixed on an automatic shaker for 10 min at 1250 rpm. After centrifuging at 20,000 g for 5 min, the aqueous layer was snap frozen and the organic layer discarded. Next, the aqueous fraction was thawed and filtered over a 0.2 μm spin-filter (10,000 g, 5 min), transferred to an autosampler vial and stored at 2–8 °C until analysis.

2.4. Liquid chromatography

The chromatographic separation was carried out using an HPLC-system consisting of an 1100 series binary pump, column oven, on-line degasser and well plate autosampler (Agilent technologies, Palo Alto, CA, USA). Mobile phase A was prepared by adding 0.5 mL 99% formic acid to 1000 mL of water. Mobile phase B was prepared in the same fashion, with methanol instead of water. Mobile phase A and B were isocratically (60% B) pumped through a Kinetex C18 100 Å column (150 × 2.1 mm I.D., 2.6 μm; Phenomenex, Torrance, CA, USA) at a flow rate of 0.15 mL/min. The analytical column was protected by an inline filter (0.2 μm, Upchurch Scientific, Oak Harbor, WA, USA). The column was maintained at 40 °C during analysis. Volumes of 2 μL were injected using the autosampler at a temperature of 4 °C. The total run time was 5 min. The autosampler needle was rinsed before each injection with methanol. During the first minute, the eluate was directed to waste using a divert valve to prevent the introduction of endogenous compounds into the mass spectrometer.

2.5. Mass spectrometer

An API 3000 triple quadrupole mass spectrometer equipped with a Turbo Ion Spray Interface (TIS) source (AB Sciex, Foster City, CA, USA) operating in the positive ion mode was used as a detector. For quantification, multiple reaction monitoring (MRM) chromatograms were acquired and processed using Analyst® software (AB Sciex). The quadruples were operating at unit resolution (0.7 Da). TIS-MS/MS operating parameters and mass transitions are presented in Table 1. Fig. 1 shows the product ion spectrum of dexamphetamine.

2.6. Validation procedures

The assay validation was performed in accordance with the OECD principles of Good Laboratory Practice (GLP) [26]. Calibration model, accuracy, and precision, selectivity, dilution integrity, lower limit of quantitation, matrix effect, carry-over and stability

Table 1
ESI–MS/MS operating parameters.

General settings	
Parameter	Setting
Run duration	5 min
Ion spray voltage	5500 V
Collision gas	2 AU
Curtain gas	8 AU
Nebulizer gas	5 AU
Turbo gas temperature	500 °C
Turbo gas flow	7 L/min

	Dexamphetamine	Amphetamine-D_6
Mass transition	136.2 → 91.0 *m/z*	142.1 → 93.2 *m/z*
Dwell time	150 msec	
Collision energy	19	
Declustering potential	16	
Collision exit potential	4	
Focussing potential	90	
Entrance potential	10	
Retention time	2.3 min	

Table 2
Assay performance data for the analysis of dexamphetamine in human plasma.

Nominal concentration (ng/mL)	Intra-assay		Inter-assay	
	Bias[a] (%)	CV[a] (%)	Bias (%)	CV (%)
2.50	4.4–5.4	4.8–9.7	4.8	–[b]
7.51	−0.5 to 11.6	3.4–7.8	4.4	5.4
25.0	−0.5 to 3.5	4.6–8.8	1.5	–[b]
200	−2.7 to 5.4	1.2–3.9	2.4	4.2

[a] If multiple validation runs were performed, the range of bias' and imprecisions are listed.
[b] The inter-assay precision could not be calculated because there is no significant additional variation due to the performance of the assay in different runs.

under various conditions were determined according to the latest FDA and EMA guidelines covering bioanalytical method validation [24,25].

The inaccuracy is expressed as the bias and the following equations were used:

$$\text{Intra-assay bias}(\%) = 100\% \cdot (\text{mean measured conc. per run} - \textit{nominal conc.})/(\textit{nominal conc.}) \quad (1)$$

$$\text{Inter-assay bias}(\%) = 100\% \cdot (\text{overall mean measured conc.} - \textit{nominal conc.})/(\textit{nominal conc.}) \quad (2)$$

The precision is expressed as the coefficient of variation (CV) and analysis of variance (ANOVA) was used to calculate the precision:

$$\text{Intra-assay CV}(\%) = 100\% \cdot (\text{SD of the measured conc. per run})/(\textit{mean measured conc. per run}) \quad (3)$$

$$\text{Inter} - \text{assayCV}(\%) = \frac{\sqrt{\left(\frac{s^2_{overall}/\left((n_1+\ldots+n_a-1)-\left((n_1-1)s_1^2+\ldots+\left((n_a-1)s_a^2\right)\right)\right)}{a-1}\right)-\left(\frac{(n_1-1)s_1^2+\ldots+(n_a-1)s_a^2}{n_1+\ldots+n_a-a}\right)}}{\left(\frac{n}{a}\right)}}{meanofruns} \quad (4)$$

Where *s* is the standard deviation, *n* is the number of replicates and *a* is the number of runs.

The matrix factor (MF) was determined by using the following equation:

$$\text{MF} = (\text{area of processed blank sample spiked with neat solution}\ (\textit{matrix present}))/(\textit{area of neat solution}(\textit{matrix absent})) \quad (5)$$

3. Results and discussion

3.1. Development

An easy-to-use and fast double liquid–liquid extraction was developed for the plasma sample preparation. It was found that the robustness and reproducibility of the assay were improved by performing an acid-base extraction. In alkaline solution, dexamphetamine is uncharged and readily partitions into the organic MTBE phase. After acidification of the organic phase (charging dexamphetamine), re-extraction into the aqueous phase was carried out, leaving impurities behind.

MTBE was selected as the organic extraction phase because of its low toxicity and low melting point (−109 °C [27]) which is required for snap-freezing.

The mass spectrometric parameters were optimized for dexamphetamine by performing direct infusion (analyte in 70% methanol). The observed molecular ion and the product ions as shown in Fig. 1 were consistent with data found in literature [13,17]. Analyte response and signal-to-noise ratios (S/N) were investigated for three mass transitions (136 → 65.1, 91.0 and 119.1). It was found that although the transition to 119.1 *m/z* yielded the highest signal, it also showed the lowest S/N however. Low S/N values were also found for 65.1 *m/z*, where these were caused by a relatively low analyte response. The S/N values and the signal strength were optimal for 91.0 *m/z*. It was observed that baseline levels and S/N ratios for all mass transition increased and decreased, respectively from day-to-day, when other drug assays were run on the system prior to a dexamphetamine run. This may be due to the ubiquity of the low mass ions during the ionization process originating from analytes, solvents, and contaminants present in the mass spectrometer. Such a situation is likely to occur in a high-throughput LC–MS/MS setting. The baseline was efficiently reduced to <300 cps before each run by injecting a series of blank samples (10) into the system.

3.2. Regression model

Eight non-zero plasma calibrators were prepared freshly and analyzed in duplicate in five separate analytical runs. The linear regression of the ratio of the areas of the analyte and the IS peaks versus the concentration were weighted with weighting factors of $1/x^2$ (where x = concentration). The linearity was evaluated by means of back-calculated concentrations of the calibrators. The assay was linear over the validated concentration range from 2.50 to 250 ng/mL. Calibration curves were accepted if 75% of the non-

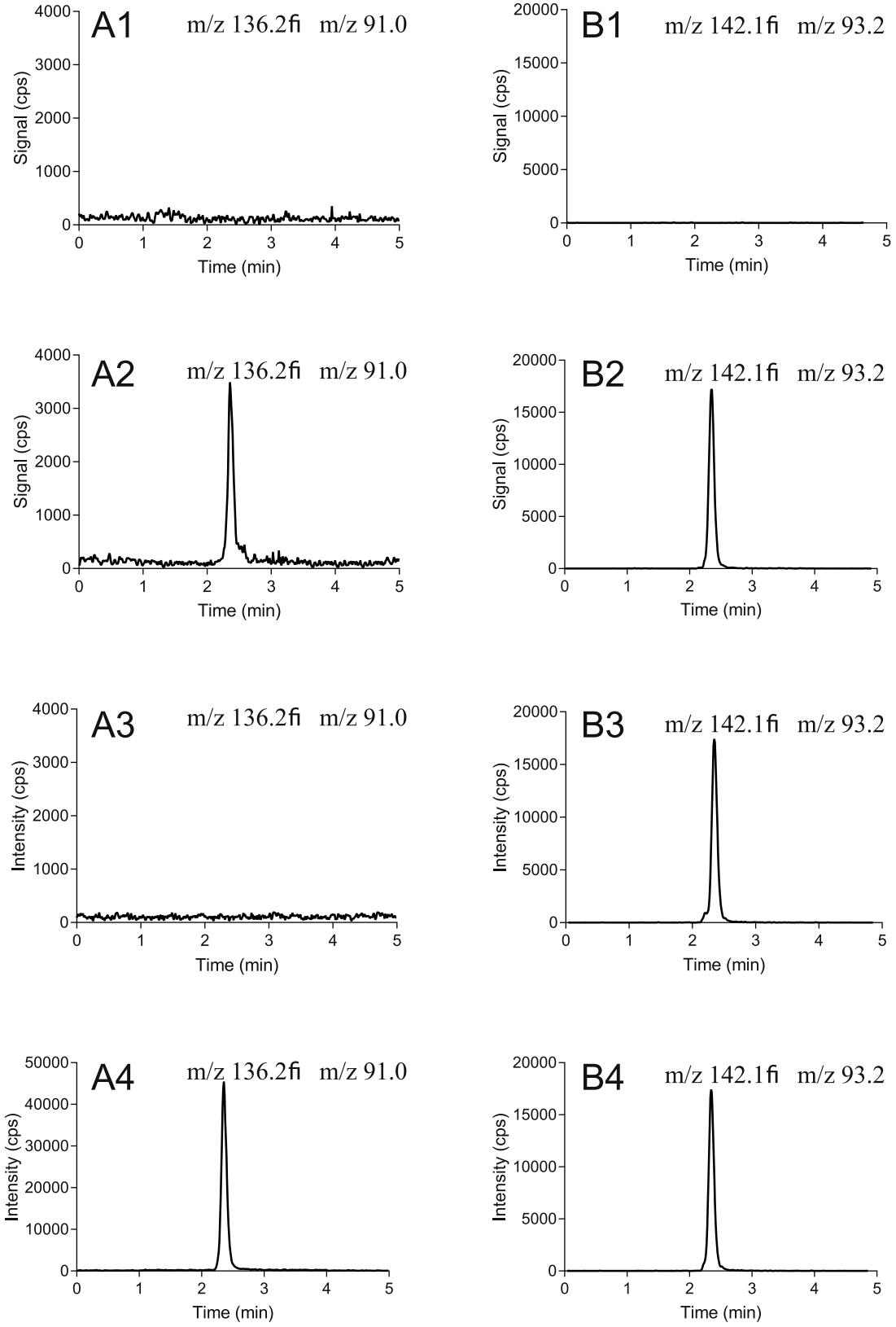

Fig. 2. Representative MRM Chromatograms. Blank human K_2EDTA plasma (A1. Dexamphetamine, B1. D_6-amphetamine); Spiked human K_2EDTA plasma at LLOQ concentration (A2. Dexamphetamine (2.51 ng/mL), B2. D_6-amphetamine (25 ng/mL)); Patient (t = 0 h) sample (A3. Dexamphetamine (0 ng/mL), B3. D_6-amphetamine (25 ng/mL)); Patient (t = 8 h) sample (A4. Dexamphetamine (34 ng/mL), B4. D_6-Amphetamine (25 ng/mL)).

zero calibration standards, including an LLOQ and a ULOQ, had a bias within ±15% of the nominal concentration (±20% for the LLOQ). All calibration curves (n=5) fulfilled these criteria and correlation coefficients were at least 0.997.

Table 3
Stability data for dexamphetamine in stock solution and plasma. All experiments performed in plasma were performed in triplicate in QC low and QC high samples (n=3).

Matrix	Conditions	Nominal conc. (ng/mL)	Measured conc. (ng/mL)	Deviation (%)	C.V. (%)
Water	−20 °C, 17 months	42.9[a]	42.3[a]	−1.3	2.8
Plasma	3 freeze (−20 °C)/thaw cycles	7.51	7.75	3.2	7.8
		200	193	−3.5	0.9
Plasma	Ambient, 24 h	7.51	7.62	1.5	5.6
		200	192	−4.0	2.3
Plasma	−20 °C, 16 months	7.51	8.23	9.6	5.0
		200	207	3.7	3.7
Final extract	2–8 °C, 17 days	7.51	7.42	−1.2	8.0
		200	195	−2.7	1.7
Final extract	2–8 °C, 30 h (reinjection reproducibility)	7.51	7.39	−1.6	1.3
		200	190	−5.2	2.4

[a] Presented are normalized response values, where corrections were made for reference drug weights and solvent volumes.

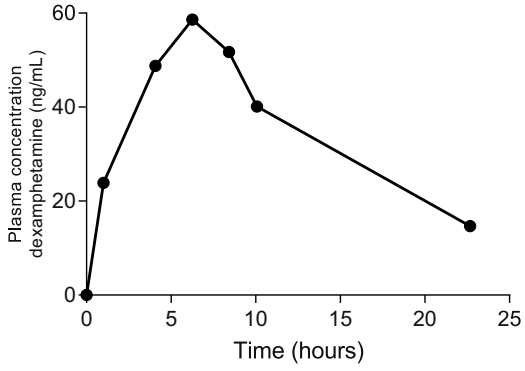

Fig. 3. Typical patients plasma curve after intake of a single dose of sustained-release dexamphetamine (orally, 60 mg).

3.3. Accuracy and precision

Intra- and inter-assay bias and precision (CV%) of the analysis were determined by assaying five replicates of each of the QC samples at LLOQ, low, mid and high concentration level in three separate runs. The concentration of each QC sample was calculated using the calibration standards that were analyzed in duplicate in the same analytical run. Table 2 summarizes the results. The difference between the calculated and the nominal concentrations was used to determine the bias. The bias was within the acceptance criteria of ±15% for low, mid and high concentration levels and within ±20% for the LLOQ level. The imprecision was below the acceptance criteria of 15% of the coefficient of variation (CV), and below 20% for the LLOQ.

3.4. Lower limit of quantification

The analyte response at the LLOQ level was at least 5 times the response compared to a blank response in five validation runs. Fig. 2 shows representative MRM chromatograms of a QC LLOQ sample and a double blank sample.

3.5. Specificity and selectivity

To investigate the specificity and selectivity, six different batches of control plasma were spiked at LLOQ level. Single determinations were performed. The mean deviation from the nominal concentrations was 13.0% with a CV value of 7.9%. There were no peaks observed with areas >20% of the LLOQ in double blank samples of these batches and no interferences were detected at the retention times of the internal standard. Selectivity was therefore considered acceptable.

Cross-analyte and IS interference were tested by spiking control human plasma at ULOQ level with the analyte and IS at nominal concentration separately. The cross-analyte and IS interference at the retention time of the analyte and IS were less than 20% of the peak area of the LLOQ level. For the IS the interference was less than 5%. The cross-analyte and IS interference was considered acceptable.

3.6. Dilution integrity

Five replicate plasma samples at QC > ULOQ level were diluted 10-fold with control human K_2EDTA plasma (50 μL sample and 450 μL control plasma). The intra-assay bias and CV were −1.9% and 1.5%, respectively. The bias and CV were within ±15% and ≤15% which indicates that the study samples can be diluted 10 times in control human matrix with acceptable accuracy and precision. The extended concentration range for dexamphetamine is therefore 2.5–2500 ng/mL.

3.7. Carry-over

Carry-over was determined by analyzing two processed control human plasma samples after a ULOQ sample in three separate runs. Eluting peaks with areas >20% of the LLOQ were not observed in the blank samples injected directly after ULOQ samples, and therefore, the criteria for carry-over were met.

3.8. Matrix factor and recovery

The matrix factor (MF) was determined in six plasma batches (pooled), at QC low and QC high concentration levels. Single determinations were performed. Processed blank samples were spiked with working solutions and compared to matrix free neat solutions.

In addition to the MF, the internal standard-normalized MF was calculated by dividing the MF of the analyte by the MF of the internal standard. The maximal CV of the internal standard-normalized MF calculated from the six plasma batches for the low and high concentrations were 5.1% and 1.5%, respectively. With this, the assay fulfilled the criteria (< 15%). The matrix factor ranged from 0.951 to 1.11. These results (MF around 1) indicate that the use of the stable isotope-labeled standard is effectively minimizing the influence of matrix effects.

3.9. Stability

The stability data are summarized in Table 3. dexamphetamine was stable at −20 °C in plasma and in water for at least 16 and 17 months, respectively. Stability experiments in plasma were performed with QC low and QC high samples. In plasma dexamphetamine was stable for at least 24 h at ambient temperature, indicating that no specific stability precautions were required during sample handling at the clinical site. Final extract stability shows

that the extracts can be injected up to 17 days after sample preparation.

4. Application in a clinical study

During a clinical study with a slow-release formulation of dexamphetamine, patients received doses of 60 or 30 mg once daily. To test the applicability of this assay, dexamphetamine concentrations were measured in 130 patient samples of patients treated with dexamphetamine in this study. A typical patient curve is shown in Fig. 3. All values are within the validated range of 2.5–250 ng/mL. These results demonstrate the applicability of this assay for clinical pharmacokinetic studies.

5. Conclusions

A simple, fast, and sensitive LC–MS/MS assay for the quantification of dexamphetamine in human plasma was developed and validated to support clinical studies. The presented assay was validated according to the current US FDA and latest EMA guidelines. The assay is able to quantify dexamphetamine over a range of 2.5–250 ng/mL in 200 μL samples, with the possibility to dilute the samples containing higher concentrations 10-fold with control plasma prior to analysis. In conclusion, the assay is considered well suited for its purpose and is now used to support clinical studies with dexamphetamine.

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