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Validated liquid chromatographic–electrospray ionization mass spectrometric assay for simultaneous determination of 3,4-methylenedioxymethamphetamine and its metabolites 3,4-methylenedioxyamphetamine, 3,4-dihydroxymethamphetamine, and 4-hydroxy-3-methoxymethamphetamine in squirrel monkey plasma

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA) is a recreational drug with neurotoxic potential. Pharmacokinetic data of MDMA and its metabolites may shed light on the mechanism of MDMA neurotoxicity. An LC–MS assay with electrospray ionization (ESI) is presented for quantifying MDMA and its metabolites 3,4-methylenedioxyamphetamine (MDA), 3,4-dihydroxymethamphetamine (HHMA), and 4-hydroxy-3-methoxymethamphetamine (HMMA) in squirrel monkey plasma. The method involved enzymatic conjugate cleavage and protein precipitation. Separation was achieved within 14 min. The method was validated according to international guidelines with respect to selectivity, linearity, accuracy, precision, recovery, and matrix effect. The present method should prove useful for acquiring pharmacokinetic and toxicokinetic data in squirrel monkeys.

Keywords: MDMA; Metabolites; LC–ESI-MS; Validation; Neurotoxicity; Plasma

1. Introduction

The drug 3,4-methylenedioxymethamphetamine (MDMA; "Ecstasy") is a psychotropic agent chemically and pharmacologically related to amphetamine and mescaline [1]. Since the early 1980s, MDMA has gained great popularity as a recreational drug [2,3]. Abuse of MDMA is associated with the risk of severe, sometimes fatal intoxication [4–7]. In addition, there is considerable evidence to indicate that MDMA has neurotoxic potential toward brain serotonergic and/or dopaminergic nerve terminals [1–3,8–10]. In rats, squirrel monkeys, rhesus monkeys and baboons, MDMA affects primarily serotonergic nerve terminals. In mice, MDMA produces selective toxic effects on dopaminergic nerve endings. Which animal model best predicts neurotoxic effects in humans is not presently known. However, determination of metabolite formation and calculation of pharmacokinetic and toxicokinetic data of MDMA and its main metabolites in various species may shed light on mechanisms of MDMA neurotoxicity [11].

Systemic metabolism of MDMA may play a role in MDMA neurotoxicity. This is suggested by the observation that direct injection of MDMA into the brain fails to reproduce the neurotoxic effects seen after systemic MDMA administration [12], and the report that alteration of cytochrome P450-mediated MDMA metabolism influences MDMA-induced neurotoxicity [13]. Species differences in neurotoxicity profile (rat/squirrel monkey: serotonin; mouse: dopamine) also suggest that species differences in drug metabolism may influence the neurotoxic

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potential of MDMA. Indeed, by characterizing the formation of various MDMA metabolites in different animal species (rat, mouse, and squirrel monkey), it may be possible to gain insight into mechanisms of MDMA neurotoxicity.

MDMA metabolism proceeds via two pathways, which operate in unison but at different rates, depending on species. The first involves demethylenation to 3,4-dihydroxymethamphetamine (HHMA) followed by *O*-methylation to 4-hydroxy-3-methoxymethamphetamine (HMMA) and *O*-conjugation with sulfate or glucuronic acid. The second entails initial *N*-demethylation to 3,4-methylenedioxyamphetamine (MDA), followed by deamination and oxidation to the corresponding benzoic acid derivatives conjugated with glycine [14]. In primates (humans, squirrel monkeys), the first pathway appears to predominate, whereas in rodents (rats, mice), the second pathway is more active (although both pathways are operant in all species). Metabolites of MDMA such as HHMA and 3,4-dihydroxyamphetamine (HHA) are easily oxidized to their corresponding quinones which, in turn, can form adducts with glutathione and other thiol-containing compounds [15–17]. Recently, such adducts have been implicated in MDMA neurotoxicity [18].

Because of the physicochemical properties of HHMA, extraction from biological samples for simultaneous analysis with MDMA and its less polar metabolites (MDA, HMMA) is difficult. Indeed, to date, only one method for the simultaneous determination of the enantiomers of MDMA and its metabolites HHMA, HMMA, and MDA has been published. Unfortunately, sample preparation for the described GC/MS method is time-consuming and complicated, as it requires a two-step derivatization procedure [19]. We now describe a simple LC–electrospray ionization (ESI)-MS method for simultaneous quantification of MDMA, HHMA, HMMA, and MDA in squirrel monkey plasma.

2. Experimental

2.1. Chemicals and reagents

Methanolic solutions (1000 mg/l) of racemic HMMA and methanolic solutions (100 mg/l) of racemic MDMA-d_5 and MDA-d_5 were obtained from Cerilliant (Round Rock, TX, USA). Methanolic solution of racemic MDMA and MDA were obtained from Lipomed (Cambridge, MA, USA). 4-Hydroxymethamphetamine (pholedrine), 4-methylcatechol, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), and glucuronidase type HP-2 from helix pomatia (glucuronidase activity ≥100.000 units/ml and sulfatase activity ≤7.500 units/ml) were obtained from Sigma–Aldrich (Saint Louis, MO, USA). Racemic MDMA was obtained through the National Institute on Drug Abuse drug supply program (Bethesda, MD, USA), and its identity was confirmed by means of gas chromatography/mass spectrometry. Dosages refer to the free base. Ammonium formate was obtained from Fluka (Steinheim, Germany). Sodium metabisulfite (SMBS) was obtained from E. Merck (Darmstadt, Germany). Formic acid and acetonitrile were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Perchloric acid (PCA) was obtained from J.T. Baker (Phillipsburg, NJ, USA). All chemicals were of analytical grade or highest purity available. HHMA was a kind gift of Arthur Cho, University of California, Los Angeles, Department of Molecular and Medical Pharmacology.

2.2. Squirrel monkey plasma samples

Blank squirrel monkey plasma samples were used for validation of the procedure and taken from squirrel monkeys (*Saimiri sciureus*). For proof of applicability, plasma samples from a squirrel monkey treated with racemic MDMA were used. All animal experiments were carried out according to *The Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health.

2.3. Sample preparation

Aliquots (100 μl) of plasma were preserved with 20 μl of SMBS (250 mM) and 10 μl EDTA (250 mM). One hundred microliters of the corresponding analytical standard solution were added to the calibrator samples. Accordingly, 100 μl of vehicle were added to each non-calibrator sample to adjust the volume. After addition of 100 μl of an aqueous solution of the racemic internal standards (IS) MDMA-d_5, MDA-d_5, and pholedrine (1.0 μg/ml, each) and 10 μl of glucuronidase solution the samples were mixed (15 s) on a rotary shaker and left at 50 °C on a waterbath for 90 min to perform conjugate cleavage. After cooling to room temperature 20 μl of 4-methylcatechol (1 mg/ml) were added to the samples and they were briefly mixed. Then 10 μl of PCA were added and the samples were mixed again on a rotary shaker for 15 s to perform protein precipitation. The samples were centrifuged (16000× *g* for 5 min), and the supernatant was transferred to autosampler vials. Aliquots (5 μl) were injected into the LC–MS system.

2.4. LC–MS analysis

2.4.1. Apparatus

All samples were analyzed using an Agilent Technologies (AT, Waldbronn, Germany) AT Series 1100 LC/MSD, VL version, using ESI in positive ionization mode, including an AT 1100 Series HPLC system which consisted of a degasser, a quaternary pump, a column thermostat, and an autosampler. Isocratic elution was performed on a Zorbax 300-SCX column (Narrow-Bore 2.1 mm × 150 mm, 5 μm) and a Zorbax SCX guard column (4.6 mm × 12.5 mm, 5 μm). The mobile phase consisted of 5 mM aqueous ammonium formate adjusted to pH 3 with formic acid (eluent A) and acetonitrile (eluent B). Until the beginning of the analysis, the HPLC system was flushed with a 30:70 mixture of the two eluents. The flow rate was programmed as follows: 0–4 min 30% B (flow: 0.8 ml/min), 4–11 min 30% B (flow: 1.0 ml/min), 11–14 min 30% B (flow: 0.8 ml/min). These conditions result into an optimized peak shape together with a relatively short run time of a total of 14 min. The following ESI inlet conditions were applied: drying gas, nitrogen (12 l/min, 300 °C) and nebulizer gas, nitrogen (172.5 kPa); capillary voltage, 4000 V; positive selected ion monitoring (SIM) mode with

the following ions: *m/z* 194, 163 (target ion, *t*) for MDMA; *m/z* 199 (*t*), 165 for MDMA-d_5; *m/z* 180, 163 (*t*) for MDA; *m/z* 185, 168 (*t*) for MDA-d_5; *m/z* 182 (*t*), 151 for HHMA; *m/z* 196 (*t*), 165 for HMMA; *m/z* 166 (*t*), 135 for pholedrine; fragmentor voltage, 100 V. Tuning of the mass spectrometer was performed using the autotune feature of the LC–MS ChemStation software (Rev.A.01.01) using the ESI acetonitrile solution tuning mix supplied with the apparatus.

2.4.2. Quantification procedure

MDMA and its metabolites HHMA, HMMA, and MDA were quantified by comparison of their peak area ratios (analyte versus IS) to calibration curves in which the peak area ratios of spiked calibration standards had been plotted versus their concentrations using a weighted ($1/x^2$) second-order calibration model. The spiked calibrators covering a range of 10–500 ng/ml for MDA and of 20–1000 ng/ml for each MDMA, HHMA, and HMMA were prepared as described below using monkey blank plasma. The ISs and analytes used for the calculation of peak area ratios were as follows: MDMA-d_5 for MDMA, MDA-d_5 for MDA and pholedrine for HHMA and HMMA.

2.5. Assay validation for plasma analysis

The LC–MS assay was fully validated according to international guidelines. The experimental design was based on that proposed by Peters [20].

2.5.1. Preparation of solutions

All aqueous solutions were preserved with 3% of each, SMBS and EDTA (250 mM). The following stock solutions were prepared: an aqueous solution (1 mg/ml) of HHMA and a methanolic solution (0.15 mg/ml) of pholedrine. From the aqueous stock solution of HHMA and commercially available methanolic stock solutions (1 mg/ml) of MDMA, HMMA, and MDA the following working solutions were prepared: aqueous solution (5000 ng/ml each) of MDMA, HHMA, and HMMA and an aqueous solution (5000 ng/ml) of MDA. From aqueous working solutions aqueous analytical standard solutions containing MDMA, HHMA, HMMA (20, 50, 100, 200, 500, and 1000 ng/ml each), and MDA (10, 25, 50, 100, 250, and 500 ng/ml) were prepared. Aqueous spiking solutions for the preparation of quality control (QC) samples containing MDMA, HHMA, HMMA (9000, 5000, and 300 ng/ml each), and MDA (4500, 2500, 150 ng/ml) were prepared from the HHMA stock solution (1 mg/ml) and the commercially available stock solutions (1 mg/ml) of MDMA, HMMA, and MDA in methanol. An aqueous solution of the ISs (MDMA-d_5, MDA-d_5, and pholedrine) was prepared from the stock solution of pholedrine and commercially available solutions of MDMA-d_5 and MDA-d_5 in methanol. All solutions were stored at 2 °C.

2.5.2. Preparation of QC samples

QC samples were prepared daily at four different concentrations: 30 ng/ml (MDMA, HHMA, and HMMA each) and 15 ng/ml (MDA), low QC sample (LOW); 500 ng/ml (MDMA, HHMA, and HMMA each) and 250 ng/ml (MDA), medium QC sample (MED); 900 ng/ml (MDMA, HHMA, and HMMA each) and 450 ng/ml (MDA), high QC sample (HIGH); 1800 ng/ml (MDMA, HHMA, and HMMA each) and 900 ng/ml (MDA), above-calibration range sample (ACR). Each QC sample was prepared by spiking monkey blank plasma with a defined volume of the corresponding spiking solution. The samples were thoroughly mixed to obtain homogenous samples. The following volumes of spiking solutions were used at given concentrations and final volumes (volume of spiking solution, final volume): LOW, MED, HIGH (30 μl, 300 μl); ACR (60 μl, 300 μl).

2.5.3. Selectivity

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

2.5.4. Calibration model

Aliquots of blank plasma (100 μl) were spiked with 100 μl of the corresponding analytical standard solutions to obtain calibration samples at the following concentrations: 20, 50, 100, 200, 500, and 1000 ng/ml of each, MDMA, HHMA, and HMMA and 10, 25, 50, 100, 250, and 500 ng/ml of MDA. Replicates ($n = 6$) at each concentration were analyzed as described above. The regression line was calculated using a weighted ($1/x^2$) least-squares linear regression model. A weighted second-order model with the same weighting factors was also calculated. Daily calibration curves using the same concentrations (single measurement per concentration) were prepared with each batch of validation samples.

2.5.5. Accuracy and precision

QC samples (LOW, MED, HIGH, and ACR) were analyzed as described above in duplicate of each of 8 days. The concentrations in the QC samples were calculated based on the daily calibration curves. Accuracy was calculated in terms of bias as the percent deviation of the mean calculated concentration at each concentration level from the corresponding theoretical concentration. Repeatability (within-day precision) and time-different intermediate precision were calculated (as relative standard deviation, RSD) using one-way ANOVA with the grouping variable 'day' [21].

2.5.6. Processed sample stability

For estimation of the stability of processed samples under the conditions of LC–MS analysis, LOW and HIGH QC samples ($n = 8$) were prepared as described above. The supernatants obtained at each concentration were pooled. Aliquots of these pooled extracts at each concentration were transferred to autosampler vials and injected under the conditions of a regular analytical run at 2.3 h intervals over a total run time of 19 h. The stability of the analytes was tested by regression analysis in which the absolute peak areas of each analyte at each concentration were plotted versus injection time. Instability of processed

samples would be indicated by a negative slope significantly different from zero ($p < 0.05$).

2.5.7. Freeze–thaw stability/bench top stability

For evaluation of freeze–thaw stability, QC samples (LOW and HIGH) were analyzed before (control samples; $n = 6$ at each level) and after three freeze–thaw cycles (stability samples; $n = 6$ at each level). For each freeze–thaw cycle, the samples were frozen at −20 °C for 21.5 h, thawed, and kept at ambient temperature for 2.5 h. The concentrations of the QC samples were calculated based on the daily calibration curves. Stability was tested against an acceptance interval of 90–110% for the ratio of the means (stability samples versus control samples) and an acceptance interval of 80–120% from the control samples mean for the 90% confidence interval (CI) of stability samples.

2.5.8. Recovery

To determine the loss of analyte during sample preparation and possible matrix effects extraction samples ($n = 5$) at low (50 ng/ml of MDMA, HHMA, and HMMA and 25 ng/ml of MDA) and high (500 ng/ml of MDMA, HHMA, and HMMA and 250 ng/ml of MDA) concentrations were prepared by spiking blank plasma (100 μl) of five different squirrel monkeys with 100 μl each of the aqueous analytical standard solutions and 100 μl of the IS solution. The extraction samples were prepared and analyzed as described above. For control samples ($n = 5$ at each concentration), 100 μl of purified water was spiked with 100 μl each of the aqueous analytical standard solutions and 100 μl of the IS solution, then diluted with purified water to the same grade as the extraction samples and analyzed immediately without sample preparation. Recovery (mean and SD) was estimated by comparison of the absolute peak areas from extraction samples and control samples for each analyte at each concentration.

2.5.9. Limits

The lowest point of the calibration curve was the limit of quantification (LOQ) of the method (20 ng/ml for MDMA, HHMA and HMMA, each, and 10 ng/ml for MDA). The LOW QC (30 ng/ml for MDMA, HHMA and HMMA, each, and 15 ng/ml for MDA) was used ($n = 16$) to determine whether the criteria established for LOQ based on precision and accuracy (bias) data (20% RSD for precision and ±20% for bias) were met at this concentration [20,22].

2.5.10. Proof of applicability

Plasma samples from a squirrel monkey treated with a dose of racemic MDMA which is equivalent to 1.2 mg/kg body weight of racemic MDMA in human were assayed with the described method. A single dose of 5.08 mg/kg body weight of racemic MDMA was administered orally by orogastric gavage to the animal. Blood samples were collected at 2.5 and 6 h after MDMA administration. At each time point the animal was briefly anesthetized with isoflurane to facilitate blood sampling. Plasma was obtained after the blood samples were centrifuged at 1100 × g for 10 min at 4 °C and SMBS (250 mM) was added at a volume of 30 μl/ml plasma to minimize oxidation of the compounds of interest. Samples were stored at −20 °C until further processed.

3. Results and discussion

3.1. Sample preparation

A simple sample preparation involving protein precipitation with PCA was performed to measure the analytes in a small sample (100 μl) of plasma. Because HHMA and HMMA cannot be found in their free form in plasma (and quantification of the conjugates is not possible due to the lack of reference substances), conjugate cleavage was necessary prior to the protein precipitation. The reaction time during enzymatic cleavage could be reduced from 16 h to 90 min by increasing the reaction temperature from 37 °C to 50 °C. After addition of the preservatives to the plasma samples the pH of the reaction solution was 6. At this point, further pH stabilization was not necessary, because pH fluctuation did not occur in the naturally buffered plasma samples. Furthermore, decreasing the pH of the solution with 0.1 M HCl to 5 did not result in a higher cleavage of the conjugates. Addition of preservatives was necessary to prevent HHMA from oxidation during the procedure [23]. More precisely, EDTA prevents catalytic oxidation by metal ions [24], whereas SMBS acts as a reducing agent. To reduce adsorption of HHMA onto the precipitated proteins, methylcatechol was added to the samples immediately before the protein precipitation, as previously described [23]. Under these conditions, no further purification or derivatization steps were necessary.

3.2. LC–MS analysis

The analytes were separated by LC–MS using a Zorbax 300-SCX column which produced a low background and a good separation of protonated molecules. A representative chromatogram of the second lowest calibrator sample (50 ng/ml MDMA, HMMA and HHMA each, and 25 ng/ml MDA) is shown in Fig. 1. Quantification by ESI-MS was performed in the SIM mode, to enhance sensitivity and precision. The protonated molecular ions of MDMA-d_5, HHMA, HMMA, and pholedrine were used as target ions. Because of their higher abundance, the fragment ions 163 and 168 were used as target ions for MDA and MDA-d_5 instead of m/z 180 and 185.

3.3. Quantification procedure and the choice of suitable ISs

Quantification was carried out by comparison of peak area ratio (analyte versus IS), with calibration curves obtained with spiked calibrators. Unfortunately, deuterated analogues as ISs were not available for HHMA and HMMA. Therefore, the structurally related drug pholedrine was used as IS for both of these metabolites. Earlier studies have shown that if no deuterated analogue is available, the similarity of the side-chain is a good predictor for the suitability of the IS [25].

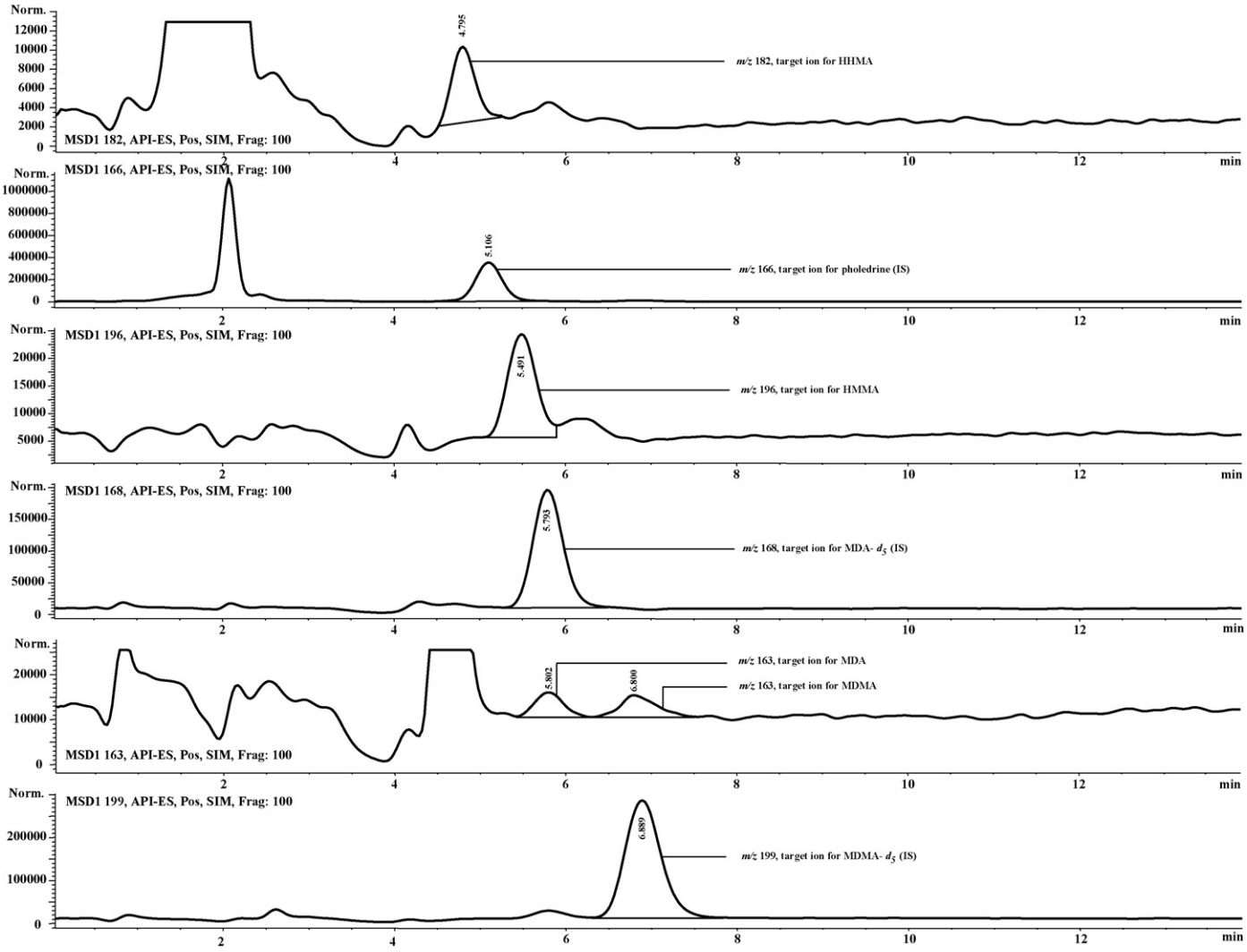

Fig. 1. Mass chromatograms of the given ions of a spiked calibrator containing 50 ng/ml MDMA, HMMA, and HHMA, each and 25 ng/ml MDA after enzymatic conjugate cleavage and protein precipitation. Integration of all peaks was done manually.

3.4. Assay validation for plasma analysis

3.4.1. Selectivity

As exemplified in Fig. 2, no peaks interfering with the analytes or the ISs were detected in blank plasma samples from six different sources. However, in routine application, an interfering peak with the same *m*/*z* as the molecular ion of MDMA, namely 194, occurred in some samples at the retention time of MDMA. In order to avoid difficulties during the quantification procedure, the fragment ion 163 has been used as target ion for MDMA.

3.4.2. Calibration model

Replicates ($n = 6$) of matrix calibrators at six different concentrations from 20 ng/ml to 1000 ng/ml for MDMA, HMMA, and HHMA and from 10 ng/ml to 500 ng/ml for MDA were analyzed. The ratio of the calibration range between the single analytes was chosen based on pharmacokinetic studies in humans after single dose of MDMA (100 mg) where the results showed that MDMA, HHMA, and HMMA plasma concentrations are about the same while MDA plasma concentrations are only about 10% [19]. Evaluation of a weighted linear regression and a weighted second-order model showed curvature and a better fit of the second-order model for all four analytes. The second order model was therefore used for in all further validation experiments and during application to study samples. The inverse of the squared concentration was found to be an appropriate weighting factor to account for unequal variances across the calibration range (heteroscedasticity).

For all following experiments, daily calibration curves were prepared with each batch of validation samples using single measurement per concentration level. In Table 1, intercepts, first and second order terms (mean ± SD) and coefficients of determination of all daily calibration curves from the accuracy and precision experiments and data for recoveries are shown.

3.4.3. Accuracy and precision

QC samples (LOW, MED, HIGH, and ACR) were analyzed in duplicates on each of 8 days as proposed by Hartmann et al. [26]. The concentrations in the QC samples were calculated based on the daily calibration curves. Accuracy, repeatability and time-

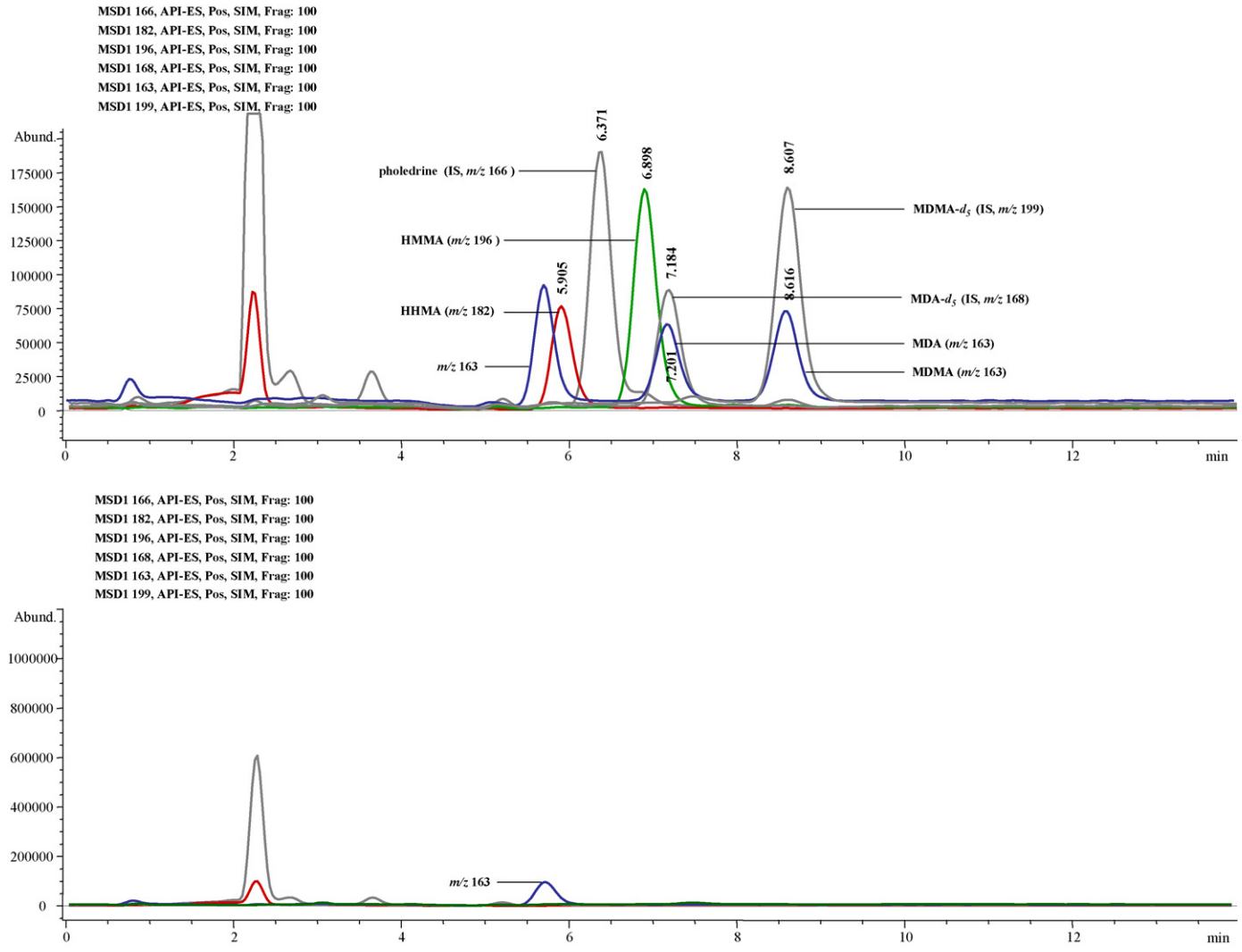

Fig. 2. Merged mass chromatograms of the given ions of a spiked calibrator containing 1000 ng/mL MDMA, HMMA, and HHMA, each and 500 ng/ml MDA (top) and of a squirrel monkey blank plasma sample (bottom), both after enzymatic conjugate cleavage and protein precipitation.

different intermediate precision were calculated as described above. The data for accuracy, in terms of bias, were all within the acceptance limits (±15% of the nominal value) specified by Shah et al. [27], except for value of the ACR QC sample of HHMA. However, at this point it must be mentioned that during the applicability study, where the monkeys were treated with a relatively high dose of MDMA, none of the analyzed plasma samples showed a value for HHMA higher than even the concentration of the MED QC. From this point of view, a dilution of the plasma samples as demanded for evaluation of ACR QC will most probably not be necessary during further studies in monkey plasma. The criteria for repeatability (within-day precision) and

Table 1
Intercepts, first and second order terms (means ± SDs) and coefficients of determination of all daily calibration curves and data for recoveries at low and high concentrations of the LC/MS assay for MDMA and its main metabolites

Analyte	IS	*y*-Intercept (mean ± SD) ($n = 8$)	1st order term (mean ± SD) ($n = 8$)	2nd order term (mean ± SD) ($n = 8$)	R^2 (range) ($n = 8$)	Recovery (mean ± SD) (%)	
						Nominal concentration (ng/ml)	
						50/25 LOW ($n = 5$)	500/250 HIGH ($n = 5$)
MDMA	MDMA-d_5	−0.00225 ± 0.00337	0.00040 ± 0.00003	4.9e-008 ± 2.5e-008	0.9989–0.9999	92.4 ± 5.7	89.2 ± 2.6
HHMA	Pholedrine	0.00391 ± 0.00168	0.00039 ± 0.00004	2.6e-008 ± 2.3e-008	0.9992–1.000	61.9 ± 8.4	59.3 ± 2.1
HMMA	Pholedrine	−0.00075 ± 0.00178	0.00082 ± 0.00003	1.2e-007 ± 5.2e-008	0.9977–1.000	99.1 ± 1.0	97.2 ± 0.6
MDA	MDA-d_5	−0.00421 ± 0.00412	0.00108 ± 0.00029	1.7e-007 ± 1.5e-007	0.9976–0.9999	94.8 ± 13.2	92.9 ± 2.0

Table 2
Repeatability, intermediate precision and accuracy data of the LC/MS assay for MDMA and its main metabolites [$n = 16$ (8 days × 2 replicates) at each level]

Analyte	Repeatability, RSD (%)				Intermediate precision, RSD (%)				Accuracy, bias (%)			
	Nominal concentration (ng/ml)				Nominal concentration (ng/ml)				Nominal concentration (ng/ml)			
	30/15 LOW	500/250 MED	900/450 HIGH	900/450 ACR	30/15 LOW	500/250 MED	900/450 HIGH	900/450 ACR	30/15 LOW	500/250 MED	900/450 HIGH	900/450 ACR
MDMA	5	2.2	1	2	6.4	4.7	2.6	4.7	9.8	5.5	6.8	5.6
HHMA	6.3	2.5	1.2	1.7	10.2	5.4	5.4	7.6	−0.9	−0.7	2.8	18.9
HMMA	5.8	2.3	2	1.8	8.3	3.7	2.5	4	0.7	0.8	3.1	5.5
MDA	7.3	1.9	2	2.1	12.4	2.2	2.8	3.8	3	−1.6	−0.7	−2.0

time-different intermediate precision (combined within-day and between-day effects), namely ≤15% RSD, were fulfilled for all analytes. The results are shown in Table 2.

3.4.4. Processed sample stability

LOW and HIGH QC samples ($n = 10$) were analyzed as described above. Aliquots of the pooled extracts at each concentration were transferred to autosampler vials and injected at time intervals of 2.3 h. Regression analysis, plotting peak area ratio of each analyte at each concentration against injection time, resulted in slopes not significantly different from zero ($p > 0.05$) for all analytes at both concentration levels except for HHMA at the low concentration level. But due to a loss of less than 10% over a 14 h time period, stability of the processed samples

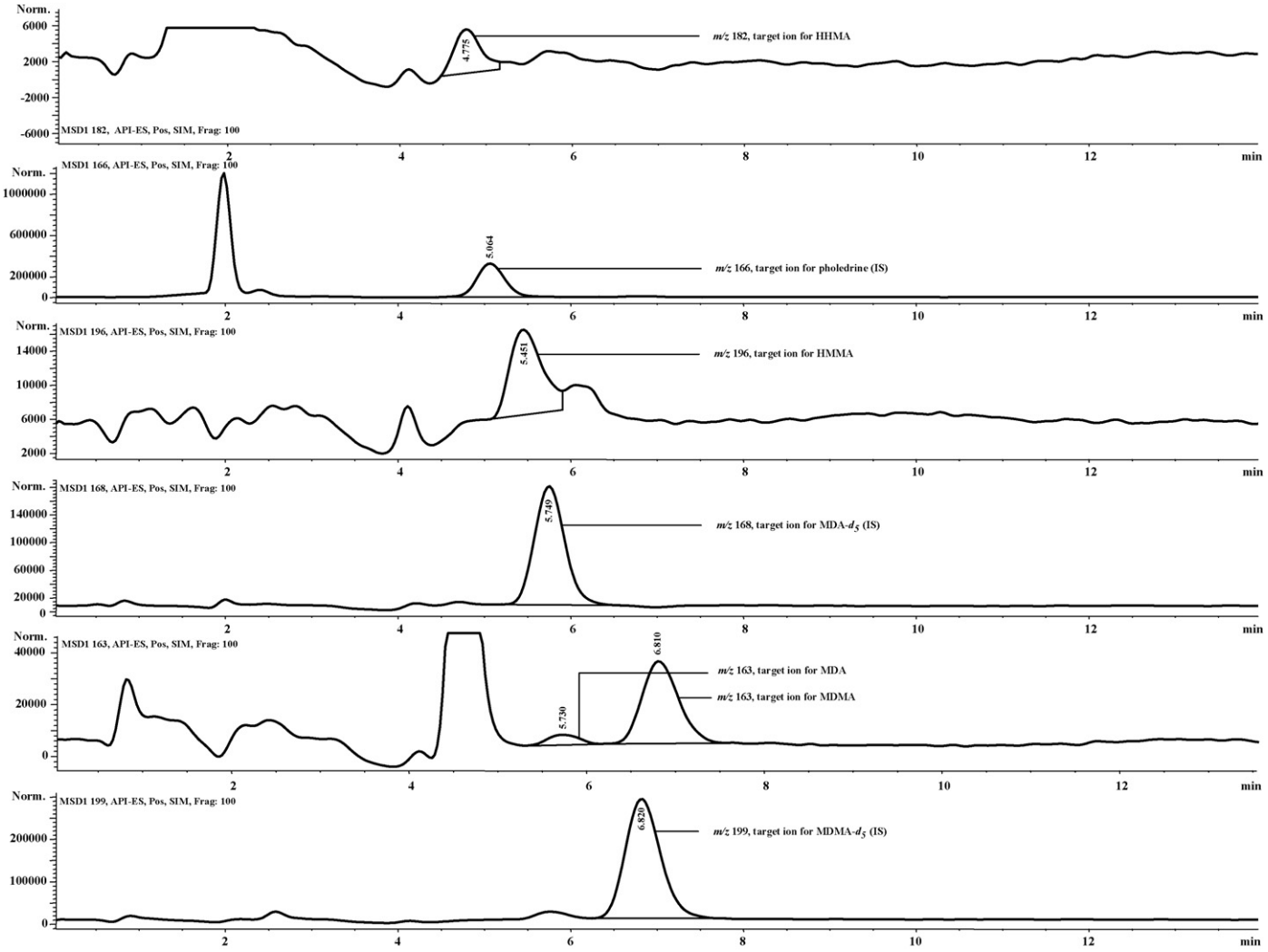

Fig. 3. Mass chromatograms of the given ions of an authentic squirrel monkey plasma sample after enzymatic conjugate cleavage and protein precipitation. The respective blood sample was collected 2.5 h after administration of 1.2 mg/kg of racemic MDMA. The plasma concentrations were determined to be 312 ng/ml for MDMA, 28 ng/ml for MDA, 38 ng/ml for HMMA, and 36 ng/ml for HHMA. Integration of all peaks was done manually.

is within an acceptable range under the conditions of a regular analytical run. However, large runs should be avoided in routine application.

3.4.5. Freeze–thaw stability/bench top stability

For evaluation of freeze–thaw stability, QC samples (LOW and HIGH) were analyzed before (control samples) and after three freeze–thaw cycles (stability samples). For each freeze–thaw cycle, the samples were frozen at −20 °C for 21.5 h, thawed, and kept at ambient temperature for 2.5 h. This procedure allowed the simultaneous evaluation of freeze–thaw stability and bench top stability, i.e. stability of the analytes in the matrix at ambient temperature over the expected maximum period of time needed for preparation of a batch of samples. Both criteria, ratio of means (stability versus control samples) within 90–110% and 90% CI for stability samples within 80–120% of control mean, were fulfilled for all analytes at both concentrations.

3.4.6. Recovery

In LC–MS analysis, the recovery can be influenced by two different effects. First of all, the response in prepared samples in comparison to respective standard solutions can be attributable to the loss of the analytes during the sample preparation. Secondly, possible matrix effects, as ion suppression or ion enhancement, are a well-known phenomenon in LC–MS analytic and can influence the signal intensity [20,22,28–30]. Especially ESI has been reported to be much more susceptible to such effects, caused by co-eluting compounds. Spiking the analytes and IS into blank plasma samples and into purified water samples for the preparation of extraction and control samples allowed the estimation of both, extraction efficiency and possible matrix effects, by comparison of the absolute peak area of extraction sample with those of the control samples. Using protein precipitation, only the combination of possible matrix effects and extraction efficiency can be estimated. The results indicate that all analytes were effectively extracted and

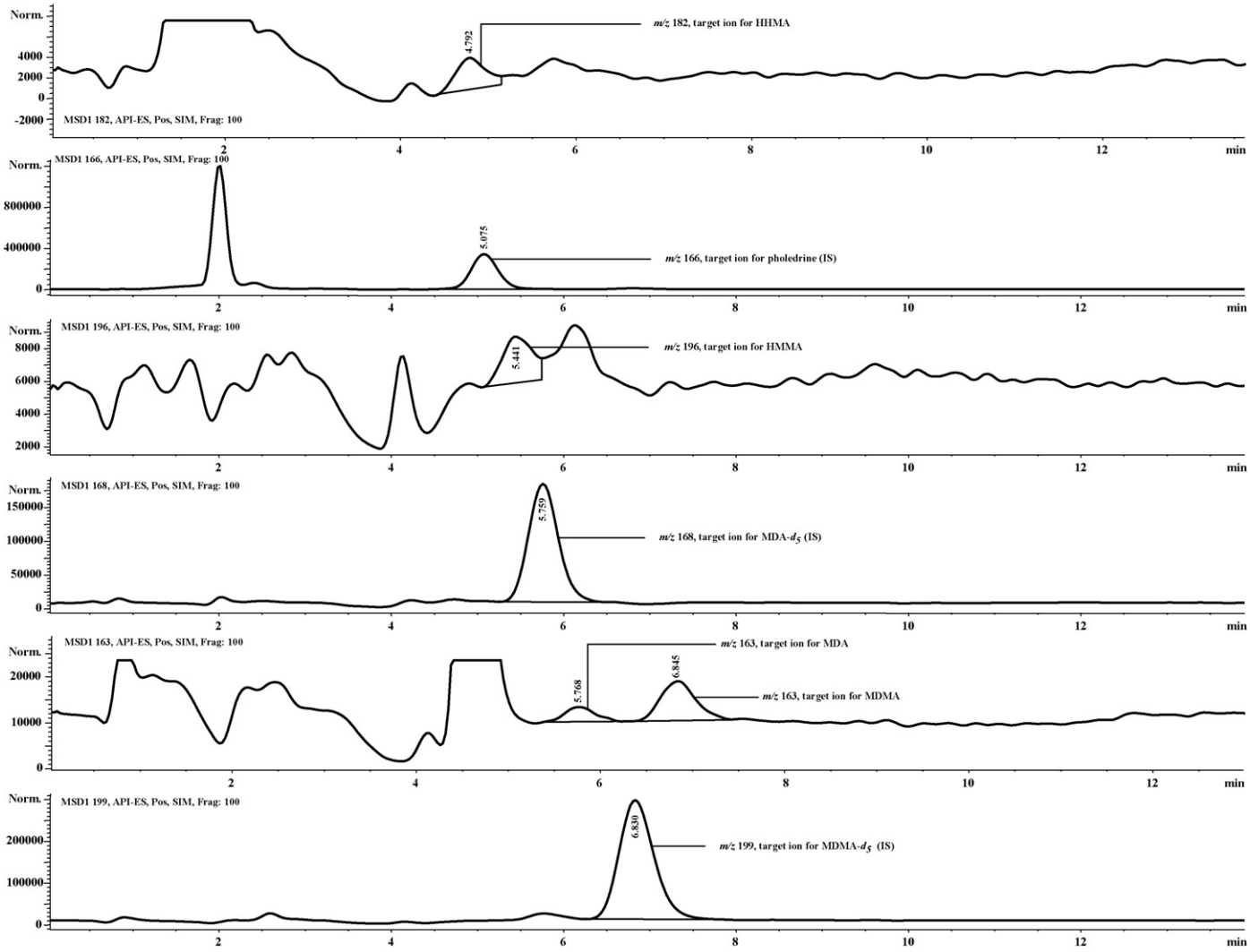

Fig. 4. Mass chromatograms of the given ions of an authentic squirrel monkey plasma sample after enzymatic conjugate cleavage and protein precipitation. The respective blood sample was collected at 6 h after administration of 1.2 mg/kg of racemic MDMA. The plasma concentrations were determined to be 79 ng/ml for MDMA, 15 ng/ml for MDA, and 21 ng/ml for HHMA. HMMA could be detected but was below the LOQ. Integration of all peaks was done manually.

that matrix effects, if present at all, were of minor extent and reproducible and hence should not compromise quantification. However, the lower recovery of HHMA compared to the other compounds could be related to HHMA adsorption onto protein precipitate [23], oxidation of the catecholamine function, or possible matrix effects.

3.4.7. Limits

The lowest point of the calibration curve was the limit of quantification (LOQ) of the method (20 ng/ml for MDMA, HHMA and HMMA, each, and 10 ng/ml for MDA). The LOW QC (30 ng/ml for MDMA, HHMA and HMMA, each, and 15 ng/mg for MDA) was used to determine whether the criteria established for LOQ based on precision and accuracy (bias) data (20% RSD for precision and ±20% for bias) were met on this concentration [20]. The calculated data for precision were for all analytes far below 20% and for bias in a much closer range than ±20%. The results are shown in Table 2.

3.4.8. Proof of applicability

Plasma samples from a squirrel monkey treated with a single dose of racemic MDMA as specified above were assayed with the described method. 2.5 h after treatment with a 1.2 mg/kg human equivalent dose of MDMA the plasma concentrations were determined to be 312 ng/ml for MDMA, 28 ng/ml for MDA, 38 ng/ml for HMMA, and 36 ng/ml for HHMA. Six hours after drug administration the plasma concentrations were 79 ng/ml for MDMA, 15 ng/ml for MDA, and 21 ng/ml for HHMA. HMMA could be detected but was below the LOQ. The results show that the calibration range covers the concentration range in monkey plasma, which is determined at different time points after treatment with a common human equivalent dose of MDMA in monkey. The chromatograms of the authentic monkey plasma sample are shown in Figs. 3 and 4.

4. Conclusions

The LC–ESI-MS assay presented here is the first to allow for simultaneous and reliable quantification of the enantiomers of MDMA and its metabolites HHMA, HMMA, and MDA in squirrel monkey plasma. Using this assay, it should be possible to collect pharmacokinetic and toxicokinectic data in MDMA-treated squirrel monkeys. Such data can be compared to that available in humans. By exploring the relationship between plasma levels of MDMA (and its various metabolites) and serotonin neurotoxicity in squirrel monkeys, it will be possible to assess the relevance of preclinical data to humans and further test the hypothesis that toxic metabolites play a role in MDMA neurotoxicity.

Although the method here described has only been validated for squirrel monkey plasma, it is reasonable to expect that it will also be useful for human plasma. However, this awaits validation. In addition, detection and quantification of the enantiomers of MDMA and its metabolites will require future method development and validation using LC coupled to MS detection.

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