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Short communication

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 rat brain

Melanie Mueller[a,b], Frank T. Peters[a], George A. Ricaurte[b], Hans H. Maurer[a,*]

[a] Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, Saarland University, D-66421 Homburg (Saar), Germany
[b] Department of Neurology, The Johns Hopkins Bayview Medical Center, Baltimore, MD, USA

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ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA) is a psychoactive drug with abuse liability and neurotoxic potential. Mechanisms by which MDMA produces behavioral and neurotoxic effects have yet to be elucidated. By measuring concentrations of MDMA and its metabolites in relevant brain sites, it may be possible to gain insight into mechanisms underlying MDMA actions. For this purpose, an LC–MS assay with electrospray ionization was developed after homogenization of rat brain and enzymatic conjugate cleavage. The method was successfully validated with respect to selectivity, linearity, accuracy, precision, recovery, and matrix effect and its use should help to delineate the neurotoxic mechanism of action of MDMA.

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA; "Ecstasy") is a psychotropic drug which has gained great popularity over the last two decades [1,2]. MDMA produces a state of consciousness with emotional and sensual overtones [3]. In addition to documented abuse liability [4], MDMA has neurotoxic potential toward brain serotonergic and/or dopaminergic nerve terminals [1,2,5–8]. In rats, as well as in squirrel monkeys, rhesus monkeys and baboons, MDMA produces selective neurotoxic effects on serotonergic nerve terminals. In contrast, in mice, MDMA produces selective toxic effects on dopaminergic nerve endings [9].

Despite considerable research, mechanisms underlying MDMA actions are not fully understood. Some observations, such as dose-dependency, high correlation between MDMA levels and subsequent serotonin neurotoxicity [10] point to (but do not establish) the importance of the parent compound (MDMA), while others suggest a possible role for MDMA metabolites [11]. MDMA metabolism proceeds via two pathways at different rates, depending upon the 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 [12].

To assess the role of the parent compound (MDMA) and/or its metabolites in the biobehavioral effects of MDMA, a method is needed to allow for concomitant measurement of MDMA and its metabolites in specific brain target sites. The rat should be an appropriate animal model for measuring concentrations of MDMA and its metabolites in brain tissue for several reasons: (1) MDMA-induced serotonin neurotoxicity is well documented in the rat [13], (2) the neurotoxic profile of MDMA in rats parallels that in primates [6], (3) the behavioral pharmacology of MDMA in the rat is reasonably well characterized [14], and (4) unlike non-human primates, rats are readily available and ideally suited for studies that require sampling of brain tissue. In the following, a simple LC–electrospray

* Corresponding author. Tel.: +49 68411626050; fax: +49 68411626051.
E-mail address: hans.maurer@uks.eu (H.H. Maurer).

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ionization (ESI)-MS method is described for simultaneous quantification of MDMA, HHMA, HMMA, and MDA in brain tissue of rats.

2. Experimental

2.1. Chemicals and reagents

Chemicals and reagents were the same as described in our previous study [15] and were of analytical grade or highest purity available.

2.2. Rat brain tissue samples

Blank rat brain tissue samples were used for validation of the procedure and taken from male Sprague Dawley rats (Harlan, Indianapolis, IN, USA). Rat brain tissue samples for freeze/thaw and proof of applicability experiments were obtained from two different rats previously treated with MDMA or HHMA. The first rat was treated with an 80 mg/kg oral dose of racemic MDMA and sacrificed 3.5 h after treatment by rapid decapitation. The second rat was treated with a 40 mg/kg oral dose of racemic HHMA and sacrificed 1 h after treatment by decapitation. After quickly isolating the brains, cerebellum, midbrain, and pons were discarded and the remaining tissue was stored in liquid nitrogen at −80 °C until further workup. 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 of brain tissue (approximately 100 mg) were accurately weighed. For each mg of tissue, 10 μl of internal standards (ISs) solution were added. Calibrator and quality control (QC) samples were prepared by adding 10 μl of the corresponding analytical standard solution (containing ISs) to each mg tissue immediately before further sample workup. After homogenizing using a Polytron homogenization unit, model PT 10–35 (Kinematica Inc., Bohemia, NY, USA, 15 s, setting 6), 10 μl of glucuronidase solution were added, the samples were briefly 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, the samples were centrifuged (16 000 × *g* for 10 min), and the supernatant was transferred to autosampler vials. Aliquots (5 μl) were analyzed by LC–MS as previously described [15].

2.4. Quantification procedure

MDMA and its metabolites HHMA, HMMA, and MDA were quantified by comparison of their peak area ratios (analyte vs. IS) to calibration curves in which the peak area ratios of spiked calibrators had been plotted vs. their concentrations (0.1–5 μg/g for HMMA and HHMA, 1–50 μg/g for MDA, and 2–100 μg/g for MDMA) using a weighted ($1/x^2$) first-order calibration model. Table 1 reports which IS was used for which analyte.

2.5. Assay validation for brain tissue analysis

The LC–MS assay was fully validated according to international guidelines. The experimental design was based on that proposed by Peters [16].

2.5.1. Preparation of solutions

The following solutions were prepared in 0.01 M HCl: analytical standard solutions containing MDMA (0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 μg/ml), MDA (0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 μg/ml), HHMA, HMMA (0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 μg/ml each), and the ISs MDMA-d_5, MDA-d_5, and pholedrine (1.0 μg/ml each); solutions for the preparation of quality control samples containing MDMA (0.45, 4.5, and 9.0 μg/ml), MDA (0.225, 2.25, and 4.5 μg/ml), HHMA, HMMA (0.0225, 0.225, and 0.450 ng/ml each), and the ISs MDMA-d_5, MDA-d_5, and pholedrine (1.0 μg/ml each); and a solution of the ISs (MDMA-d_5, MDA-d_5, and pholedrine, 1.0 μg/ml each). All solutions were preserved with 3% of each, SMBS and EDTA (250 mM) and stored at 4 °C.

2.5.2. Preparation of QC samples

QC samples were prepared daily at three different concentrations by exactly weighing aliquots of blank brain tissue and adding 10 μl of the corresponding spiking solution/mg blank brain tissue: 4.5 μg/g (MDMA), 2.25 μg/g (MDA), and 0.225 μg/g (HHMA and HMMA each), low QC sample (LOW); 45 μg/g (MDMA), 22.5 μg/g (MDA), and 2.25 μg/g (HHMA and HMMA each), medium QC sample (MED); 90 μg/g (MDMA), 45 μg/g (MDA), and 4.5 μg/g (HHMA and HMMA each), high QC sample (HIGH).

2.5.3. Selectivity

Blank brain tissue samples from 6 different rats were prepared as described above to check for peaks that might interfere with the detection of the analytes or the ISs.

2.5.4. Calibration model

After aliquots of blank brain tissue were exactly weighed, 10 μl of the corresponding analytical standard solutions was added/mg tissue and the mixture was homogenized to obtain calibration samples at the following concentrations: 2, 5, 10, 20, 50, and 100 μg/g of MDMA, 1, 2.5, 5, 10, 25, and 50 μg/g of MDA and 0.1, 0.25, 0.50, 1, 2.5, and 5 μg/g of each HHMA, and HMMA. Replicates ($n = 6$) at each concentration were analyzed as described above. Daily calibration curves using the same concentrations (single measurement

Table 1
Intercepts, slopes (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 ± S.D.) ($n = 8$)	Slopes (mean ± S.D.) ($n = 8$)	R^2 (range) ($n = 8$)	Level	Nominal concentration, μg/g	Recovery, % mean ± S.D. ($n = 5$)
MDMA	MDMA-d_5	0.001927 ± 0.01266	0.001318 ± 1.4540e−005	0.9984–0.9999	LOW	2	60 ± 5.6
					HIGH	100	63.5 ± 5.7
HHMA	Pholedrine	0.0003666 ± 0.001597	0.0006731 ± 0.0001032	0.9924–0.9996	LOW	0.1	48.5 ± 2.0
					HIGH	5	38.0 ± 3.2
HMMA	Pholedrine	−0.001320 ± 0.0006943	0.001042 ± 2.3910e−005	0.9959–0.9998	LOW	0.1	64.1 ± 5.6
					HIGH	5	67.0 ± 7.7
MDA	MDA-d_5	−0.01266 ± 0.008234	0.001280 ± 1.7670e−005	0.9977–1.000	LOW	1	54.2 ± 5.1
					HIGH	50	61.1 ± 4.8

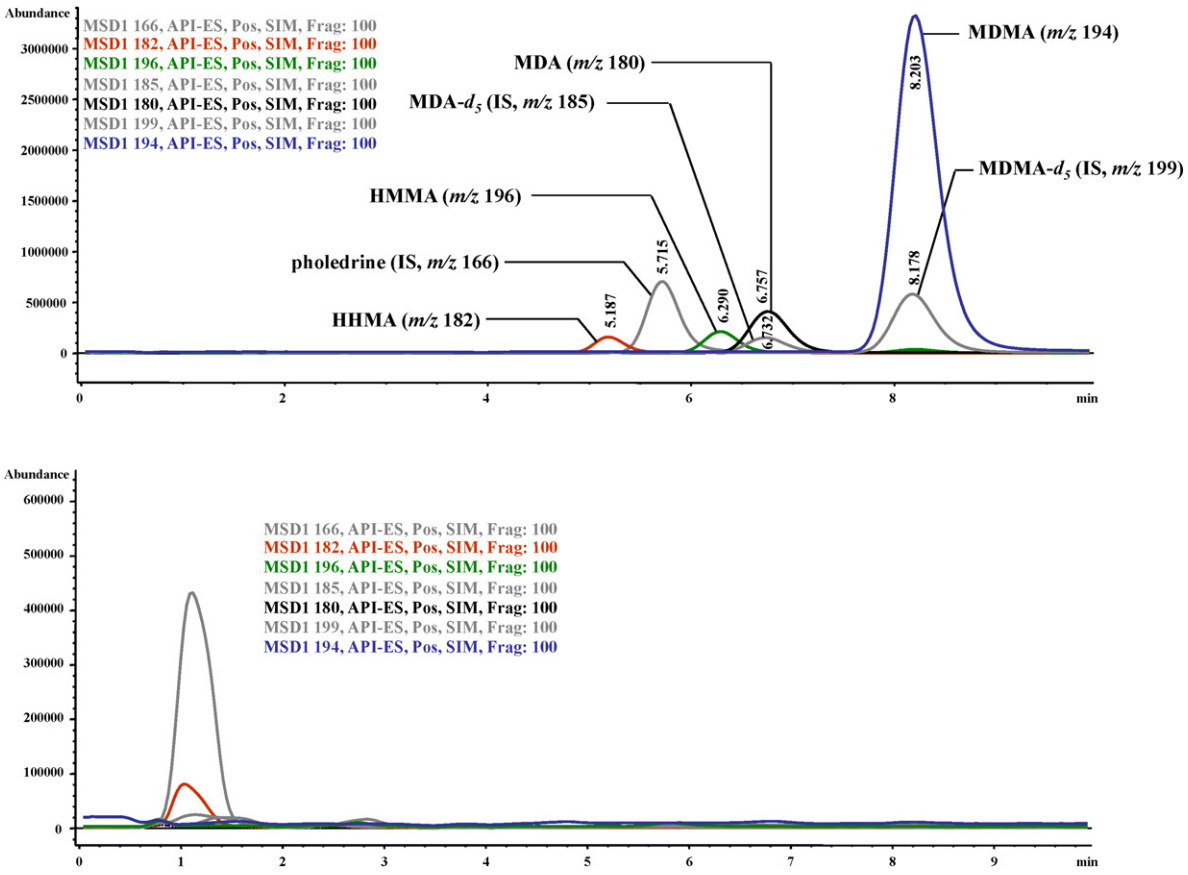

Fig. 1. Mass chromatograms of the given ions of a spiked calibrator containing 50 μg/g MDMA, 25 μg/g MDA, and 2.5 μg/g HMMA, and HHMA, each after enzymatic conjugate cleavage (top part) vs. the mass chromatograms of the given ions of a blank rat brain sample after sample preparation.

per concentration) were prepared with each batch of validation samples.

2.5.5. Accuracy and precision

QC samples (LOW, MED, and HIGH) were analyzed as described above in duplicate on each of eight days using daily calibration curves for calculation of concentrations. 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' [17]. As proposed by Shah et al. [18], accuracy was required to be within ±15% (±20% near LOQ) and precision was required to be ≤15% RSD (≤20% RSD near LOQ).

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 vs. injection time. Instability of processed samples would be

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	QC level	Nominal concentration μg/g	Repeatability RSD (%)	Intermediate precision RSD (%)	Accuracy bias (%)
MDMA	LOW	4.5	1.3	1.5	−1.9
	MED	45	1.5	2.0	−5.2
	HIGH	90	0.5	1.1	−4.5
HHMA	LOW	0.225	1.3	4.6	−1.9
	MED	2.25	2.4	3.3	5.1
	HIGH	4.5	1.8	4.0	6.9
HMMA	LOW	0.225	1.9	4.8	2.8
	MED	2.25	1.1	1.7	3.7
	HIGH	4.5	0.9	1.3	5.0
MDA	LOW	2.25	1.0	1.8	0.6
	MED	22.5	1.9	2.0	−0.9
	HIGH	45	1.1	1.7	0.4

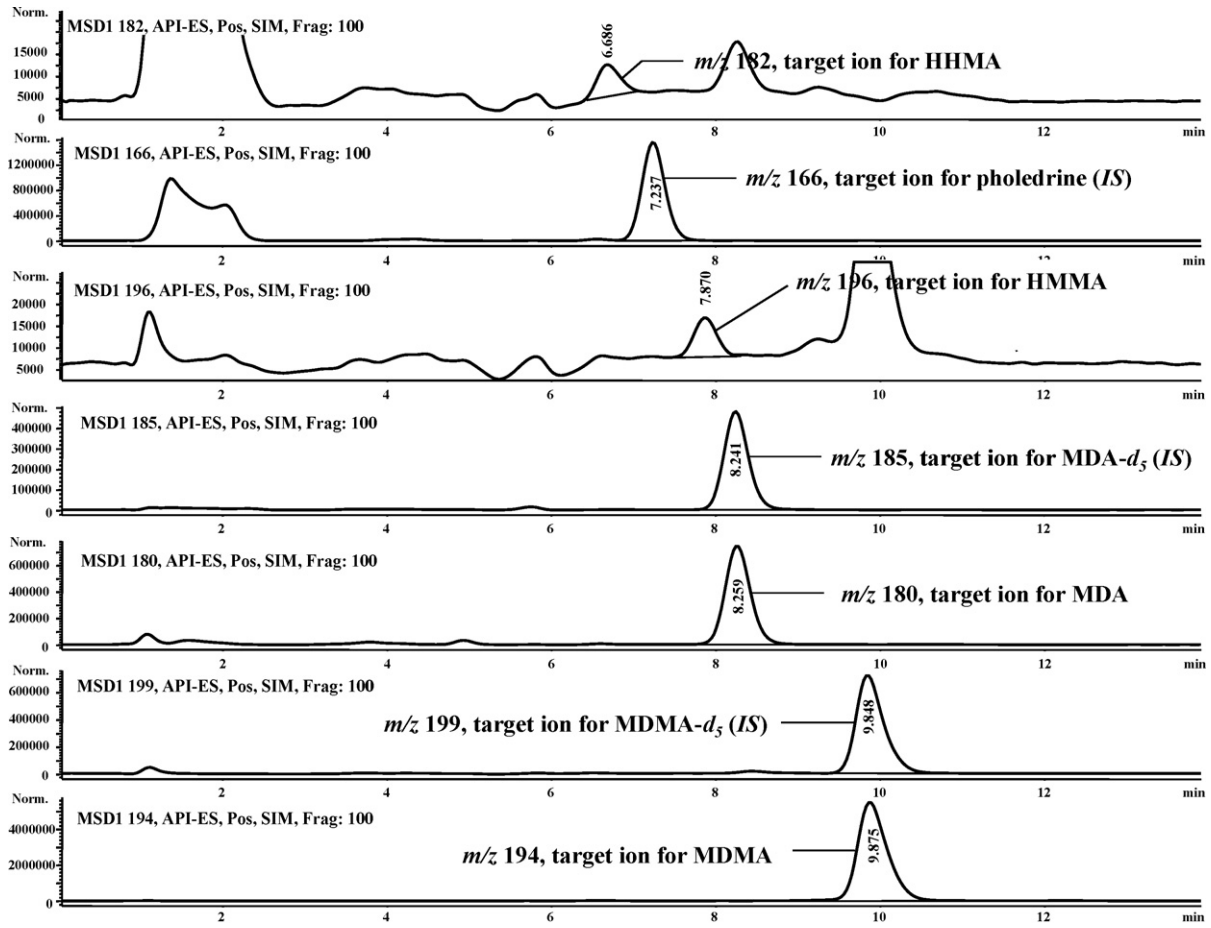

Fig. 2. Mass chromatograms of the given ions of an authentic rat brain tissue sample after sample preparation. The respective brain sample was collected 3.5 h after per oral administration of 80 mg/kg of racemic MDMA. The brain concentrations were determined as 64 μg/g MDMA, 13 μg/g MDA, 0.1 μg/g HHMA, and 0.1 μg/g HMMA. Integration of all peaks was done manually.

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

2.5.7. Freeze-thaw stability

For evaluation of freeze-thaw stability authentic rat brain tissue samples from two different rats, one treated with racemic MDMA, the second treated with racemic HHMA, as specified above, were used. Rat brain tissue samples from both animals were analyzed before (control samples, $n = 3$ for each animal) and after 3 freeze-thaw cycles (stability samples, $n = 3$ for each animal). For each freeze-thaw cycle, the samples were frozen at −80 °C in liquid nitrogen for 21.5 h and thawed. The concentrations of the 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 vs. control samples) and against the presence of the 90% confidence interval (CI) of the stability samples within the 80–120% acceptance interval from the mean of the control samples.

2.5.8. Recovery

To determine the loss of analyte during sample preparation and to evaluate possible matrix effects, extraction samples ($n = 5$, blank brain tissue derived from 5 different rats) at low (2 μg/g of MDMA, 1 μg/g of MDA, and 0.1 μg/g of HHMA and HMMA each) and high (100 μg/g of MDMA, 50 μg/g of MDA, and 5 μg/g of HHMA and HMMA each) concentrations were prepared and analyzed as described above. For control samples ($n = 5$ at each concentration), the respective analytical standard solutions were analyzed immediately without sample preparation. Recovery (mean and S.D.) 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 defined as the limit of quantification (LOQ) of the method (2 μg/g for MDMA, 1 μg/g for MDA, and 0.1 μg/g for HHMA and HMMA each). The LOQs were chosen based on the results of an initial experiment, in which a blood sample was taken 3.5 h after administration of 40 mg/kg of MDMA. The data for the LOW QC (4.5 μg/g for MDMA, 2.25 μg/g for MDA, and 0.225 μg/g for HHMA and HMMA each) were 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 at this concentration [16,19].

2.5.10. Proof of applicability

Rat brain tissues from two different rats, one treated with racemic MDMA, the second treated with racemic HHMA, as specified above, were assayed with the described method.

3. Results and discussion

3.1. Sample preparation

Analysis was performed in brain areas rich in 5-HT terminals (cerebral cortex) in which 5-HT deficits are known to be more severe than in regions containing fibers of passage (hypothalamus)

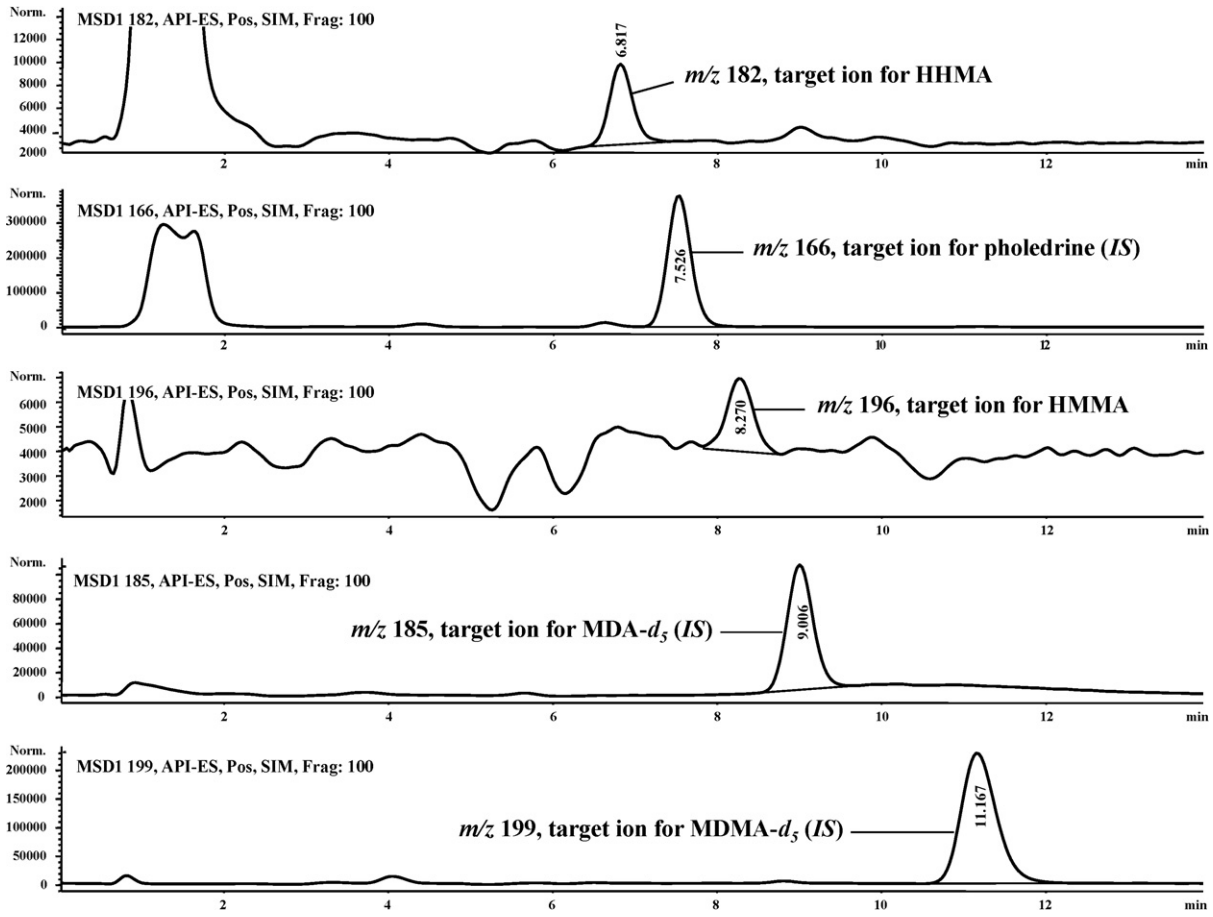

Fig. 3. Mass chromatograms of the given ions of an authentic rat brain tissue sample after sample preparation. The respective brain sample was collected at 1 h after administration of 40 mg/kg of racemic HHMA. The brain concentrations were determined as 0.2 μg/g HHMA and 0.1 μg/g HMMA. Integration of all peaks was done manually.

or cell bodies (brain stem) [6]. A simple sample preparation involving homogenization of small pieces of brain tissue was performed to measure analytes of interest. Addition of preservatives was necessary to prevent oxidation of HHMA during sample preparation and analysis [20]. Because initial experiments showed that HHMA and HMMA cannot be found in their free form in brain, and because quantification of the conjugates was not possible due to the lack of reference standards, conjugate cleavage was necessary prior to the analysis. After homogenization of the brain tissue, the resulting homogenate was at pH 3. Enzymatic conjugate cleavage at this low pH proved to be equally effective as at the optimum of the used enzymes (pH 5) so pH adjustment prior to hydrolysis was not necessary.

3.2. Assay validation for brain tissue analysis

The validation study was carried out in analogy to our previous publication [15]. Therefore, only aspects not already discussed there are specifically addressed here. As shown in Fig. 1 (bottom) for a representative blank rat brain tissue sample, no peaks interfering with the analytes or the ISs were detected in blank brain tissue from six different sources. The individual calibration ranges for each analyte were chosen based on preliminary pharmacokinetic studies in rats after single dose treatment with MDMA (20 mg/kg) (data not shown). Evaluation of a weighted linear regression and a weighted second-order model showed curvature and a better fit of the second-order model for MDMA, HHMA, and HMMA. However, Hartmann et al. [21] have proposed that simpler linear model be accepted if the data for precision and analytical recovery are within acceptable limits. Because this was the case for our data, we decided to accept the linear regression model with a weighting factor or $1/x^2$. The y-intercepts, slopes (mean ± S.D.) and coefficients of determination of all daily calibration curves from the accuracy and precision experiments are shown in Table 1. The results of the accuracy, repeatability (within-day precision) and time-different intermediate precision (combined within-day and between-day effects) experiments are listed in Table 2. They easily met the applied acceptance criteria, even near the LOQ, confirming the LOQ of the described method. Processed samples were found to be stable under the applied conditions, which is in line with our findings for analysis of plasma samples [15] and further shows that matrix components from rat brain had no detrimental effect on analyte stability. All analytes were also stable in authentic rat brain samples over three freeze/thaw cycles (data not shown). In the recovery experiments, peak areas of spiked brain tissue samples were compared to peak areas of the respective neat analytical standard solutions. In this experimental setup, both extraction losses and possible matrix effects may affect the peak areas in the matrix-based samples. However, the recovery data presented in Table 1 indicate that matrix effects, if present at all, were of minor extent and reproducible and hence should not compromise quantification. The somewhat lower recovery of HHMA could be attributable to its catecholamine-like structure which may lead to adsorption onto brain proteins [22].

For proof of applicability, authentic rat brain tissue samples from two different rats, one treated with racemic MDMA, the second treated with racemic HHMA, as specified above, were assayed with the described method. The respective mass chromatograms of the

target ions of these authentic rat brain tissue samples are shown in Figs. 2 and 3, respectively. Brain concentrations 3.5 h after per oral administration of 80 mg/kg of racemic MDMA were determined as 64 μg/g MDMA, 13 μg/g MDA, 0.1 μg/g HHMA, and 0.1 μg/g HMMA (Fig. 2). One hour after per oral treatment with 40 mg/kg of racemic HHMA brain concentrations were determined to be 0.2 μg/g HHMA and 0.1 μg/g HMMA (Fig. 3). In our earlier publication, ratios of MDMA to HMMA and HHMA respectively were found to be much lower in squirrel monkey plasma samples [15]. High polarity of HHMA, HMMA and their phase II conjugates might hamper their passage of the blood brain barrier and therefore explain the low levels of HMMA and HHMA in the authentic brain samples.

4. Conclusions and perspectives

The LC–ESI-MS assay presented here is the first to allow for simultaneous and reliable quantification of MDMA and its metabolites HHMA, HMMA, and MDA in rat brain tissue. This assay should make it possible to explore the relationship between brain concentrations of MDMA and its metabolites and pharmacological and toxic effects of MDMA of interest.

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