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Simultaneous liquid chromatographic-electrospray ionization mass spectrometric quantification of 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy) and its metabolites 3,4-dihydroxymethamphetamine, 4-hydroxy-3-methoxymethamphetamine and 3,4-methylenedioxyamphetamine in squirrel monkey and human plasma after acidic conjugate cleavage

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA, Ecstasy) is a psychoactive drug with abuse liability and neurotoxic potential. Specimen preparation of a recently presented LC–MS assay with electrospray ionization for quantifying MDMA and its main metabolites in squirrel monkey plasma was modified to include acidic hydrolysis to obtain total 3,4-dihydroxymethamphetamine and 4-hydroxy-3-methoxymethamphetamine. Method re-validation for squirrel monkey plasma and full validation for human plasma showed selectivity for all analytes. Recoveries were $\geq 71.0\%$. Changed specimen preparation or matrix did not affect accuracy or precision. No instability was observed after repeated freezing or in processed samples. Plasma MDMA and metabolites quantification, derived pharmacokinetic and toxicokinetic data and neurotoxicity research will benefit from this validated method.

Keywords

MDMA; Metabolites; LC–ESI–MS; Human plasma; Validation; Acidic hydrolysis

1. Introduction

Since the early 1980s, 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy) has gained great popularity as a recreational drug [1,2]. Abuse of MDMA is associated with the risk of severe, sometimes fatal intoxication [3–6]. In addition, there is considerable evidence to

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indicate that MDMA has neurotoxic potential for brain serotonergic and/or dopaminergic nerve terminals, depending on species [1,2,7–10].

MDMA metabolites may be involved in some of the effects caused by MDMA use (e.g., hyperthermia, increase in blood pressure). Some studies implicate systemic metabolism in MDMA neurotoxicity [11,12]. By characterizing the formation of various MDMA metabolites in different species (human, rat, mouse, and squirrel monkey), insight may be gained into processes underlying MDMA neurotoxicity. Also helpful for that matter might be measuring concentrations of MDMA and its metabolites in relevant brain sites [13].

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 [14,15]. 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. Metabolites of MDMA such as HHMA and 3,4-dihydroxyamphetamine (HHA) are oxidized to the corresponding quinones that can form adducts with glutathione and other thiol-containing compounds [16–18]. Such adducts have been implicated in MDMA neurotoxicity [19].

Recently, we presented a simple validated LC–electrospray ionization (ESI)-MS method for simultaneous quantification of MDMA, HHMA, HMMA, and MDA in squirrel monkey plasma after enzymatic cleavage of conjugates and protein precipitation [20]. During preliminary experiments designed to adapt this method to analysis of human plasma, LC–MS results were compared to GC–MS results for the same specimens obtained with a recently published assay for determination of MDMA, MDA, HMMA, HMA, and MDEA in human plasma [21]. Surprisingly, HMMA concentrations determined with the GC–MS method were considerably higher than those determined with the LC–MS method. Similar differences were noted with squirrel monkey specimens. Discrepancies were traced to different methods for conjugate cleavage during specimen preparation, namely enzymatic hydrolysis in the LC–MS and acidic hydrolysis in the GC–MS method [21]. The optimum conditions for different hydrolysis procedures were thoroughly investigated during method development. A full account of these experiments is beyond the scope of the present paper and will be reported elsewhere (Mueller et al., *Anal. Bioanal. Chem.*, 2009, in revision). Based on these findings, we have modified our recently described method [20] by replacing enzymatic with acidic conjugate cleavage. Here, we describe re-validation of the method with respect to analysis of squirrel monkey plasma, and full validation of the method for analysis of human plasma specimens in order to be able to correlate monkey and human plasma concentrations relevant in the neurotoxic risk assessment. Finally, this new method may be useful also in clinical and forensic toxicology.

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). HHMA and methanolic solutions of racemic MDMA and MDA were obtained from Lipomed (Cambridge, MA, USA). 4-Hydroxymethamphetamine (pholedrine), 4-methylcatechol, and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) were purchased from Sigma–Aldrich (Saint Louis, MO, USA). The National Institute on Drug Abuse (Bethesda, MD, USA) supplied racemic MDMA; identity and purity were confirmed by GC–MS. Ammonium formate was obtained from Fluka (Steinheim, Germany), sodium metabisulfite (SMBS) from E. Merck (Darmstadt, Germany), and formic acid and acetonitrile from Fischer Scientific (Fair Lawn,

NJ, USA). Perchloric acid (PCA) was purchased from J.T. Baker (Phillipsburg, NJ, USA) and hydrochloric acid (HCl) from Merck (Darmstadt, Germany). All chemicals were of analytical grade or highest purity available.

2.2. Squirrel monkey plasma specimens

Blank plasma for assay validation was collected from squirrel monkeys (*Saimiri sciureus*). 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. Human plasma specimens

Blank human plasma specimens from 8 healthy adult volunteers were used for method validation. Two different batches of pooled authentic human plasma specimens following controlled administration of MDMA were evaluated in freeze–thaw experiments. Participants provided informed consent to participate in this Institutional Review Board-approved protocol and adhered to Federal guidelines for the protection of human subjects.

2.4. Specimen preparation and LC–MS analysis

Aliquots (100 μ l) of human or squirrel monkey plasma were preserved with 20 μ l of SMBS (250 mM) and 10 μ l of EDTA (250 mM). After addition of 100 μ l of an aqueous solution of the racemic internal standards (IS) MDMA-*d*₅, MDA-*d*₅, and pholedrine (1.0 μ g/ml, each), and 300 μ l 0.5 M HCl, samples were mixed (15 s) on a rotary shaker and left at 100 °C for 80 min for conjugate cleavage. After cooling to room temperature, 20 μ l of 4-methylcatechol (1 mg/ml) was added and briefly mixed. 10 μ l PCA (69–70%) was added and samples immediately mixed again on a rotary shaker for 15 s for protein precipitation. Samples were centrifuged (16 000 $\times g$ for 5 min), and supernatants transferred to autosampler vials. Aliquots (5 μ l) were injected into the LC–MS system. Samples analysis was performed on an Agilent Technologies (AT) Series 1100 LC–MSD, using electrospray ionization (ESI) in positive ionization mode with the following ions: *m/z* 194 (target ion), 163 for MDMA; *m/z* 199 (t), 165 for MDMA-*d*₅; *m/z* 180 (t), 163 for MDA; *m/z* 185 (t), 168 for MDA-*d*₅; *m/z* 182 (t), 151 for HHMA; *m/z* 196 (t), 165 for HHMA; *m/z* 166 (t), 135 for pholedrine (for selectivity reasons, ions for MDMA and MDA different from those used in ref. [20]). The isocratic mobile phase consisted of 5 mM aqueous ammonium formate adjusted to pH 3 with formic acid: acetonitrile (70:30, v/v). The stationary phase was a Zorbax 300-SCX column (2.1 mm $\times$ 150 mm, 5 μ m). For details see [20].

2.5. Assay validation

The LC–MS assay with the modified specimen preparation was re-validated for squirrel monkey plasma analysis and validated for human plasma analysis. The following validation parameters were evaluated: selectivity, accuracy and precision, processed sample stability, freeze/thaw stability, and recovery/matrix effect. The experimental design was based on that proposed by Peters [22].

2.5.1. Preparation of QC samples

QC samples were prepared daily at three concentrations: 30 ng/ml (MDMA, HHMA, and HHMA) and 15 ng/ml (MDA), low QC sample (LOW); 500 ng/ml (MDMA, HHMA, and HHMA each) and 250 ng/ml (MDA), medium QC sample (MED); 900 ng/ml (MDMA, HHMA, and HHMA each) and 450 ng/ml (MDA), high QC sample (HIGH). QC samples were prepared by fortifying monkey and human blank plasma with 30 μ l of aqueous drug solutions (prepared and stored as previously described [20]), and mixing thoroughly to obtain homogenous samples at a final volume of 300 μ l at each concentration level.

2.5.2. Selectivity

Selectivity was evaluated in blank plasma specimens from 6 squirrel monkeys and 8 human individuals as well as in two zero samples for each species as described in the previous validation study [20].

For human plasma specimens, a further experiment was performed to check for interferences from exogenous compounds that might be present in samples from drug users. QC LOW ($n = 2$ for each interference) were fortified at 1000 ng/ml cocaine, benzoylecgonine, norcocaine, norbenzoylecgonine, ecgonine ethyl ester, ecgonine methyl ester, anhydroecgonine ethyl ester, ecgonine, Δ^9 -tetrahydrocannabinol, 11-hydroxy- Δ^9 -tetrahydrocannabinol, 11-nor-9-carboxy- Δ^9 -tetrahydrocannabinol, amphetamine, methamphetamine, ephedrine, pseudoephedrine, morphine, normorphine, morphine-3- β -D-glucuronide, morphine-6- β -D-glucuronide, codeine, norcodeine, 6-monoacetyl-morphine, acetylcodeine, hydrocodone, hydromorphone, oxycodone, diazepam, lorazepam, oxazepam, alprazolam, nitrazepam, flunitrazepam, temazepam, nordiazepam, and phencyclidine that might interfere with MDMA and metabolites' quantification. Target analyte concentrations were calculated from daily calibration curves; low QC concentrations were required to be within $\pm 20\%$ of target to rule out interference.

2.5.3. Accuracy and precision, limit of quantification

QC LOW, MED, and HIGH ($n = 6$ at each concentration) were prepared from squirrel monkey plasma and analyzed by acidic and enzymatic specimen preparation techniques [20]. Means and variances of peak area ratios of both hydrolysis methods for each analyte at each concentration were compared by two-tailed t -tests and F -tests, respectively.

QC LOW, MED, and HIGH ($n = 6$ at each concentration for each species) were prepared using human and squirrel monkey blank plasma according to the modified procedure described above. Means and variances of human and monkey samples for each analyte at each concentration were compared by two-tailed t -tests and F -tests, respectively.

In the recently published method [20], the lowest point of the calibration curve was the limit of quantification (LOQ) of the method. The LOW QC was used to determine whether the criteria established for LOQ based on precision and accuracy (bias) data [20% RSD for precision and $\pm 20\%$ for accuracy] were met at this concentration [22,23]. Limits of quantification (LOQ) could be considered the same as in the original method [20], if LOW QC results obtained with the modified method and/or alternate matrix were not significantly different.

2.5.4. Processed sample stability

Processed sample stability under LC–MS conditions was evaluated as described in the previous validation study [20] using LOW and HIGH squirrel monkey and human QC samples ($n = 8$). Stability of analytes was tested by regression analysis in which absolute peak areas of each analyte at each concentration were plotted against injection time. Instability of processed samples would be indicated by a negative slope significantly different from zero ($p < 0.05$).

2.5.5. Freeze–thaw stability/applicability

For evaluation of freeze–thaw stability, authentic human plasma specimens from two different plasma pools were analyzed before (fresh, $n = 3$ for each pool) and after 3 freeze–thaw cycles (frozen, $n = 3$ for each pool). For each freeze–thaw cycle, samples were frozen at -20°C for 21.5 h and thawed to room temperature. Analytes were considered stable, if the ratio of the means was within an acceptance interval of 90–110% and if the mean values of fresh samples were within 80–120% of the 90% confidence interval (CI) of frozen samples.

2.5.6. Recovery/matrix effect

Loss of analyte during specimen preparation and possible matrix effects were determined as described in the previous validation study [20] using LOW and HIGH QC ($n = 5$ at each level) prepared in blank plasma from five different sources in both species. Recoveries (mean and SD) were estimated by comparing absolute peak areas from extracted and control samples for each analyte at each concentration in each species.

3. Results and discussion

Since modification of specimen preparation might affect all validation parameters with exception of freeze/thaw stability, the original, extensively validated method [20] had to be re-evaluated. Re-evaluation of calibration model, accuracy, precision, and LOQ, was considered sufficient if the quantitative response, i.e. the peak area ratio (analyte vs internal standard), was unaffected by acidic cleavage, in which case further experimental re-evaluation would be not necessary.

Because changing sample matrix from squirrel monkey to human plasma might affect all validation parameters, a second validation was necessary. Concerning the quantitative calibration model, accuracy, precision, and LOQ, peak area ratios of squirrel monkey QC and human QC samples were compared after both were processed according to the modified procedure. If comparisons were not significantly different, calibration model, accuracy, precision, and LOQ for human plasma samples could be considered the same as for squirrel monkey plasma samples.

Results in detail: re-validation/validation could prove method selectivity in plasma of both species. Fig. 1 depicts mass chromatograms of a human blank specimen after acid hydrolysis and protein precipitation, representative of the 8 human blank specimens evaluated. None of the tested drugs that might be present in authentic plasma specimens from drug users interfered with the quantification of either analyte in LOW QC samples. Differences between nominal and target concentrations for all analytes were $\leq 15\%$. Spiking potentially interfering compounds to QC LOW rather than to blank plasma samples does not only allow the detection of possible changes in analyte peak retention but also changes in chromatographic behavior which could influence the quantification of the compounds of interest.

Comparison of mean peak area ratios from both methods for each analyte at three different concentration levels in squirrel monkey plasma samples showed that acidic hydrolysis did not affect quantitative accuracy. No significant differences were observed (t -test, $p > 0.05$) with exception of the HIGH QC samples of HHMA and the LOW and HIGH QC samples of HHMA. However, these observed differences were not relevant for quantification with mean values within -9.7% and 2.8% of those obtained with the original method. Precision was not negatively affected by acidic hydrolysis either, as shown by comparison of variances. A significant difference was only found for MDMA in the LOW QC samples (F -test, $p = 0.015$), but the variance for specimens processed with acid hydrolysis was even lower than that obtained with the original method. Comparison of mean peak area ratios in squirrel monkey and human QC samples processed in parallel yielded statistically significant, but not quantitatively relevant differences (-5.7 and 1.7%), only between QC samples of HHMA, HIGH QC samples of MDA, and MED QC samples of MDMA. These results indicate that the described method also can be used for quantification of MDMA, MDA, HHMA, and HHMA in human plasma specimens.

Analyte stability in processed samples was not affected by changed specimen preparation. Over a total run time of 17.5 h at an average ambient temperature of $22\text{ }^{\circ}\text{C}$ no analyte instability in either species was found.

In the freeze/thaw experiments, both criteria were fulfilled for all analytes at all tested concentrations in authentic human plasma. Mean ratios were within 97.4–103.4% and mean values of fresh samples ranged from 86.4 to 116.9% of the 90% CI of frozen samples. Mass chromatograms of an authentic human plasma specimen after acid hydrolysis and protein precipitation are shown in Fig. 2.

In LC–MS analysis, recovery can be influenced by loss of analytes during specimen preparation and by matrix effects, i.e., ion suppression or ion enhancement, a well known phenomenon in LC–MS analysis, influencing signal intensity [22–27] with ESI being especially susceptible. Comparison of peak areas of extraction and control samples indicated that all analytes were effectively recovered and that matrix effect, if present, was of minor importance and reproducible. Hence matrix effect should not compromise quantification. Data for recoveries at low and high analyte concentrations in squirrel monkey and human plasma are presented in Table 1.

4. Conclusions

Acidic hydrolysis should be employed if MDMA metabolites are to be analyzed in human or squirrel monkey plasma to maximize the recovery of MDMA metabolites. After modifying specimen preparation from enzymatic to acid hydrolysis, this LC–ESI-MS assay achieves simultaneous, reliable quantification of MDMA and metabolites HHMA, HMMA, and MDA in squirrel monkey and human plasma. This new method improves pharmacokinetic and toxicokinetic data derived from controlled administration of MDMA to squirrel monkeys and humans, allowing direct comparison between the two species. By exploring these relationships, it will be possible to assess the relevance of preclinical data to understanding human MDMA toxicity, and further, to test the hypothesis that toxic metabolites play a role in MDMA neurotoxicity. In addition, this method should prove useful for determination of MDMA and its major metabolites in clinical and forensic toxicology.

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Fig. 1. Merged mass ion chromatograms of a fortified calibrator containing 1000 ng/ml MDMA, HMMA, and HHMA, and 500 ng/ml MDA (top) and of a human blank plasma specimen (bottom), both after acidic conjugate cleavage and protein precipitation. Integration of all peaks was done manually.

Fig. 2. Mass ion chromatograms of an authentic human plasma specimen after acidic conjugate cleavage and protein precipitation. Plasma concentrations were 145 ng/ml MDMA, 105 ng/ml HHMA and 181 ng/ml HMMA. MDA could not be detected at the method's limit of quantification of 10 ng/ml. Integration of all peaks was done manually.

Table 1

Recovery data at low (50 ng/ml for MDMA, HMMA, and HHMA, each and 25 ng/ml for MDA) and high concentrations (500 ng/ml for MDMA, HMMA, and HHMA, each and 250 ng/ml for MDA,) for the LC-MS assay of MDMA and its main metabolites in human and squirrel monkey plasma.

Analyte	Recovery (mean $\pm$ SD) (%) in squirrel monkey plasma		Recovery (mean $\pm$ SD) (%) in human plasma	
	Nominal concentration (ng/ml)		Nominal concentration (ng/ml)	
	50/25 LOW (<i>n</i> = 5)	500/250 HIGH (<i>n</i> = 5)	50/25 LOW (<i>n</i> = 5)	500/250 HIGH (<i>n</i> = 5)
MDMA	83.9 $\pm$ 5.1	71.0 $\pm$ 1.6	88.7 $\pm$ 4.5	77.4 $\pm$ 0.7
HHMA	78.2 $\pm$ 2.8	74.4 $\pm$ 1.1	76.6 $\pm$ 2.0	80.9 $\pm$ 0.4
HMMA	96.6 $\pm$ 2.8	98.0 $\pm$ 1.2	100.2 $\pm$ 2.5	101.9 $\pm$ 0.8
MDA	98.6 $\pm$ 2.1	98.3 $\pm$ 1.6	98.6 $\pm$ 1.2	101.9 $\pm$ 1.9