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Development and validation of LC-HRMS and GC-NICI-MS methods for stereoselective determination of MDMA and its phase I and II metabolites in human urine

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Abstract

3,4-Methylenedioxymethamphetamine (MDMA) is a racemic drug of abuse and its *R*- and *S*-enantiomers are known to differ in their dose-response curve. The *S*-enantiomer was shown to be eliminated at a higher rate than the *R*-enantiomer most likely explained by stereoselective metabolism that was observed in various *in vitro* experiments. The aim of this work was the development and validation of methods for evaluating the stereoselective elimination of phase I and particularly phase II metabolites of MDMA in human urine. Urine samples were divided into three different methods. Method A allowed stereoselective determination of the 4-hydroxy-3-methoxymethamphetamine (HMMA) glucuronides and only achiral determination of the intact sulfate conjugates of HMMA and 3,4-dihydroxymethamphetamine (DHMA) after C18 solid-phase extraction by liquid chromatography–high-resolution mass spectrometry with electrospray ionization. Method B allowed the determination of the enantiomer ratios of DHMA and HMMA sulfate conjugates after selective enzymatic cleavage and chiral analysis of the corresponding deconjugated metabolites after chiral derivatization with *S*-heptafluorobutyrylpropyl chloride using gas chromatography–mass spectrometry with negative-ion chemical ionization. Method C allowed the chiral determination of MDMA and its unconjugated metabolites using method B without sulfate cleavage. The validation process including specificity, recovery, matrix effects, process efficiency, accuracy and precision, stabilities and limits of quantification and detection showed that all methods were selective, sensitive, accurate and precise for all tested analytes.

Keywords

stereoselective method; MDMA; phase I metabolites; phase II metabolites; urine

INTRODUCTION

3,4-Methylenedioxymethamphetamine (MDMA), known as 'Adam' or 'Ecstasy', is a popular rave drug leading to feelings of euphoria and energy and a desire to socialize.^[1, 2] Recent publications of the Substance Abuse and Mental Health Services Administration

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Supporting information

Supporting information may be found in the online version of this article.

report that MDMA consumption is increasing again.^[3] MDMA may lead to severe acute poisonings and also to long-term neurotoxic effects.^[1,2] Direct injection of ecstasy into the brain fails to reproduce the neurotoxic effects seen after systemic administration.^[4] Therefore, the metabolism of MDMA may play a role in its neurotoxicity.^[5–8]

In vivo studies with MDMA showed two main metabolic pathways as shown in Fig. 1. One major pathway includes *O*-demethylenation to 3,4-dihydroxymethamphetamine (DHMA), followed by *O*-methylation mainly to 4-hydroxy-3-methoxymethamphetamine (HMMA). HMMA and DHMA can be further conjugated by uridine-diphospho-glucuronyltransferases or by sulfotransferases. A minor pathway includes demethylation to 3,4-methylenedioxyamphetamine (MDA) followed by demethylenation to 3,4-dihydroxyamphetamine (DHA), *O*-methylation to 4-hydroxy-3-methoxyamphetamine (HMA) and conjugation.^[9–11] In urine samples of recreational MDMA users, HMMA sulfate and glucuronide are the major metabolites.^[12]

MDMA is a racemic drug and *R*- and *S*-MDMA differ in their dose–response curves for neurotoxicity and *in vivo* kinetics.^[1,2,13–16] The *S*-enantiomer is eliminated at a higher rate than the *R*-enantiomer^[1,2,13–16] most likely explained by stereoselective metabolism that was observed in various *in vitro* experiments.^[17–19] For *in vivo* studies, suitable stereoselective methods are needed. GC-MS is the most common instrumental technique for chiral analysis of MDMA and its phase I metabolites.^[17,20–22] Derivatization of the enantiomers with a chiral derivatization reagent is a commonly used approach, resulting in the formation of diastereomers, which are amenable to separation by achiral chromatography methods. Different chiral derivatization reagents were employed such as *S*-trifluoro-acetylpropyl chloride (*S*-TPC),^[23] *R*- α -methoxy- α -trifluoromethyl-phenylacetyl chloride (*R*-MTPCl)^[21,22] and *S*-heptafluoro-butyrylpropyl chloride (*S*-HFBPCl).^[17,20] Sample preparation reached from simple liquid-liquid extraction (LLE) simultaneous to derivatization^[21] to more time-consuming and expensive solid-phase extractions (SPEs).^[16]

However, for stereoselective determination of phase II MDMA metabolites, suitable approaches are missing in the literature. Only for achiral analysis of glucuronides and sulfates of HMMA, but not of DHMA, an high performance liquid chromatography-mass spectrometry (HPLC-MS) assay was recently published after protein precipitation but without method validation.^[12]

As stereoselective analysis of MDMA's main metabolites could not be accomplished by LC-high-resolution mass spectrometry (LC-HRMS), different methods were needed to allow stereoselective analysis of MDMA and its metabolites. Therefore, the aim of this work was to develop and validate an LC-HRMS method for studying the stereoselective elimination of MDMA phase II metabolites, a GC-MS method for stereoselective analysis of the sulfates after cleavage and a GC-MS method for MDMA and its unconjugated metabolites, all for human urine.

EXPERIMENTAL

Chemicals and reagents

Hydrochlorides of racemic MDA, HMA, DHA, MDMA, HMMA and DHMA were obtained from Lipomed (Bad Saeckingen, Germany). Methanolic solutions (1 mg/ml) of MDA-*d*₅ and MDMA-*d*₅ were from LGC Promochem (Wesel, Germany), 4-hydroxymethamphetamine (pholedrine), 3,4-dihydroxy-benzylamine (DHBA) and morphine 6-glucuronide (M6G), hexamethyldisilazane (HMDS) and sulfatases (EC no. 3.1.6.1) from *Aerobacter aerogenes* were from Sigma-Aldrich (Steinheim, Germany). Phenylephrine glucuronide was purchased from Toronto Research Chemicals (Toronto, Canada). *R/S*-

DHMA sulfates, *R/S*-HMMA sulfate and single diastereomers of HMMA glucuronides were synthesized in the authors' laboratory as described in Refs [19,24] as well as the derivatization reagent *S*-HFBPrCl as described in Ref. [25]. *S*-MDMA was obtained in the authors' laboratory through enantioseparation of racemic MDMA as described in Ref. [17.] Isolute Confirm C18 cartridges (500 mg, 3 ml) were obtained from Biotage (Grenzach-Wyhlen, Germany). The AdultaCheck-6 tests were from Mahsan (Hamburg, Germany). Water was purified with a Millipore filtration unit and acetonitrile of HPLC grade was obtained from VWR Prolabo (Darmstadt, Germany). Cyclohexane, ethyl acetate, methanol, sodium ethylenediaminetetraacetate (EDTA-Na), sodium bicarbonate, sodium carbonate and sodium metabisulfite (analytical grade) were from Merck (Darmstadt, Germany).

Urine samples

Human urine samples (blank and fortified) were divided into the following methods: method A, LC-HRMS for glucuronides and sulfates; method B, GC-MS with negative-ion chemical ionization (GC-NICI-MS) for sulfates after cleavage and method C, GC-NICI-MS for MDMA and its unconjugated metabolites.

Method A: LC-HRMS for glucuronides and sulfates

Sample preparation—A 100- μ l aliquot of urine, fortified with 10 μ l internal standard (IS) solution (M6G1 μ M, phenylephrine glucuronide 10 μ M) and diluted with 1 ml water, was submitted to SPE using C18 cartridges, previously conditioned with 1 ml methanol and 1 ml water. Columns were washed with 0.5 ml water and eluted with 1 ml methanol into autosampler vials. Eluates were evaporated to dryness under a stream of nitrogen at 70 °C, reconstituted in 100 μ l water/acetonitrile (1 : 1, v/v), and 10- μ l aliquots injected into the LC-HRMS.

Apparatus and procedure—Analysis was performed with a Thermo Fisher (TF, Dreieich, Germany) Accela LC system consisting of a degasser, a quaternary pump and a TF Pal autosampler coupled to a TF Exactive system equipped with a heated electrospray ionization II (HESI II) source.

The LC conditions were as follows: Phenomenex (Aschaffenburg, Germany) Chirex 3012 (25 $\times$ 4.6 mm, 5 μ m); gradient elution with 10 mM aqueous ammonium formate buffer containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). The flow rate was 1 ml/min with the following gradients: 0–6 min 20% A, at 6.1 min 30% A, hold for 13 min, and at 19.1 min 20% A, hold for 1 min. The MS conditions were as follows: HESI-II, positive mode, sheath gas, nitrogen at a flow rate of 55 arbitrary units (AU), auxiliary gas, nitrogen at a flow rate of 18 AU, vaporizer temperature, 250 °C; spray voltage, 2.2 kV; ion transfer capillary temperature, 250 °C, maximum injection time, 250 ms.

The mass spectrometer was operated in the full scan (m/z 100–2000) and high-energy collision-induced dissociation (HCD) mode (fragmentor voltage 25 eV) with a mass resolution of 25 000. For quantification, the protonated molecules of the target analytes with their accurate masses were used: m/z 262.0744 (DHMA sulfates), m/z 276.0900 (HMMA sulfate), m/z 372.1653 (HMMA glucuronides), m/z 462.1759 (IS M6G) and m/z 344.1340 (IS phenylephrine glucuronide).

Method B: GC-NICI-MS for sulfates after cleavage

Sample preparation—For sulfate cleavage, 100 μ l of urine was adjusted to pH 7.1 by the addition of 200 μ l of potassium phosphate buffer (500 mM, pH 7.1) and incubated with 30 μ l *Aerobacter aerogenes* solution for 2 h at 37 °C in a water bath. After addition of 10- μ l IS

solution (MDMA-*d*₅, MDA-*d*₅, pholedrine, DHBA, 250 µM each) and 400-µl aqueous carbonate buffer (35 g/l sodium bicarbonate and 15 g/l sodium carbonate, pH 9) containing 3% sodium metabisulfite and 3% EDTA-Na, the first derivatization step was performed according to Peters *et al.*^[15] with modifications.

After addition of 50-µl *S*-HFBPCL (0.1 M in dichloromethane) and 500-µl cyclohexane/ethyl acetate (1 : 1, v/v), the reaction vials were sealed and left on a rotary shaker for 30 min. After centrifugation (14 000 g, 2 min), 400 µl of the organic phase was transferred into autosampler vials and evaporated to dryness under a stream of nitrogen at 65 °C. To the dry extracts, 50 µl of HMDS was added and the second derivatization step was performed under microwave irradiation (450 W, 5 min). Aliquots (3 µl) were injected into the GC-MS.

Apparatus and procedure—The samples were analyzed by an Agilent Technologies (AT, Waldbronn, Germany) 6890 Series GC system combined with an AT 5973 network mass-selective detector, an AT 7683 series injector and an AT-enhanced Chem Station G1701CA (version C.00.00, 21 December 1999). The GC conditions were as follows: splitless injection mode; column, 5% phenyl methyl siloxane (HP-5 MS; 30 m × 0.25 mm i.d.; 250 nm film thickness); injection port temperature, 280 °C; carrier gas, helium; flow rate, 1 ml/min and column temperature, 150 °C increased to 245 °C at 15 °C/min, hold for 7 min, increased to 340 °C at 40 °C/min and hold for 4 min. The NICI-MS conditions were as follows: transfer line heater, 280 °C; NICI, methane (2 ml/min); source temperature, 150 °C; solvent delay, 6 min and selected-ion monitoring (SIM) mode with the following ions (quantifiers are underlined): *m/z* 392, 412, 432, (MDA); *m/z*, 397, 417, 437 (MDA-*d*₅); *m/z* 271, 414, 486 (HMA); *m/z* 251, 492, 420 (DHA); *m/z* 420 (DHBA); *m/z* 490 (pholedrine); *m/z* 426, 446, 466 (MDMA); *m/z* 431, 451, 471 (MDMA-*d*₅); *m/z* 448, 500, 520 (HMMA) and *m/z* 265, 506, 434 (DHMA). An NICI mass list with relative abundances of ions is given in Supporting Information. Elution order of *R* and *S* enantiomers were evaluated using incubations of *S*-MDMA with recombinant CYP2D6 and human liver cytosol (HLC) as described in detail in Ref. ^[18].

Method C: GC-NICI-MS for MDMA and its unconjugated metabolites

A 100-µl aliquot of urine was processed as described for method B, with the exception of addition of the enzyme solution and the incubation. The extracts were analyzed as described in the Section on Apparatus and Procedure for method B.

Data analysis

Calibration with internal standardization for methods A–C was accomplished with a 6-point curve for each analyte. Calibration model and weighting factors are given in Tables 1–3. For data evaluation, TF LCQuan 2.6 software determined peak areas. Previously, the files acquired by the GC-MS Chemstation software were converted to Magnum files with Mass Transit 2.6 and further to rawfiles using Xcalibur 2.0.7 software. For method A, the settings were as follows: peak detection algorithm genesis; signal-to-noise threshold 0.5. For methods B and C, the settings were as follows: peak detection algorithm “ICIS”; baseline window 50, area noise factor 5 and peak noise factor 10.

Method validation

Preparation of calibration and quality control (QC) samples—Separate stock solutions (10 mM) of each racemic analyte were prepared in water, stock solutions for *R/S*-DHMA 3-sulfate, *R/S*-DHMA 4-sulfate, *R/S*-HMMA sulfate, *S*-HMMA glucuronide and *R*-HMMA glucuronide in water/acetonitrile, in duplicate. Working solutions (1 and 0.1 mM) were prepared by dilution from each stock solution. Spiking solutions for calibration standards and QC samples were prepared by mixing appropriate amounts of the

corresponding stock or working solution to obtain concentrations ten times higher than the corresponding urine concentration. All solutions were stored in aliquots at -20°C .

Calibration standards were prepared from 100 μl urine and 10 μl of the corresponding fortifying solution. Final calibration concentrations for method A were as follows: 0.5, 5, 10, 25, 50, 75 μM (*R/S*-DHMA 3-sulfate, *R/S*-DHMA 4-sulfate); 0.2, 3, 17, 33, 50, 67 μM (*R/S*-HMMA sulfate) and 0.2, 1.7, 3.3, 8, 17, 25 μM (*S*-HMMA glucuronide, *R*-HMMA glucuronide). Final calibration concentrations of the single enantiomers for methods B and C were as follows: 0.1, 2.5, 5, 10, 15, 25 μM (MDA, HMA, DHA); 0.1, 7.5, 25, 37.5, 75, 125 μM (MDMA, HMMA) and 0.1, 2.5, 7.5, 15, 25, 40 (DHMA). QC sample pools were prepared at three different concentrations (LOW, MED and HIGH; Tables 1–3). Each pool was prepared by transferring a defined volume of the corresponding fortifying solution to volumetric flasks to which blank urine was then added stepwise until the final volume was reached.

Specificity—Ten blank urine samples from different sources were analyzed for peaks interfering with the detection of analytes or IS. Two zero samples (blank sample + IS) were analyzed to check for appropriate IS purity and the presence of native analytes.

Recovery, matrix effects and process efficiency—Recovery, matrix effect (ME) and process efficiencies (PEs) for method A were performed at QC LOW and HIGH concentrations using six different urine sources according to the simplified approach described by Matuszewski *et al.*^[26]

Recovery (RE, percentage) for methods B and C was calculated at QC LOW and HIGH concentrations by comparison of peak areas of fortified urine samples *versus* those in aqueous solution. Coefficients of variation (CVs) (% , $n = 6$) were calculated with peak areas and peak area ratios of analyte to IS.

Creatinine values of the blank urine samples were determined with AdultaCheck and ranged from 10 to 100 mg/dl.

Calibration model—Replicates ($n = 6$) at each concentration level were analyzed as described above. The regression lines were calculated using non-weighted, a weighted $[1/X]$ and a weighted $[1/X^2]$ least-squares regression models. Second-order models with the same weighting factors and a double logarithmic model were also calculated. The final choice of model was made after calculating validation data using these alternatives. Daily calibration curves (single measurement per level) were prepared with each batch of validation samples. The back-calculated concentrations of all calibration samples were compared to their respective nominal values and quantitative accuracy was required within 20% of target.

Accuracy and precision—QC samples (LOW, MED, HIGH) were analyzed according to the procedures described above in duplicate on each of 8 days. For reproducibility of the sulfate cleavage, QC MED of method A was analyzed additionally, according to the sulfate cleavage procedure (method B).

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. Intra-day and inter-day precision were calculated as relative standard deviation (RSD) according to Ref.^[27].

Stability—Processed sample, freeze-thaw and long-term stability were investigated at QC LOW and HIGH concentrations ($n = 6$ each) according to Ref.^[28].

Limits—The lowest point of the calibration curve was defined as the limit of quantification (LOQ) of the method and fulfilled the requirement of LOQ, signal-to-noise ratio of 10 : 1 determined via the peak heights. The limit of detection (LOD) was tested with extracts ($n = 5$ each) fortified at low analyte concentrations. The LOD was determined as the concentration where the signal-to-noise ratio of 3 : 1 was given.

RESULTS AND DISCUSSION

LC-HRMS for glucuronides and sulfates (method A)

For sample preparation, simple protein precipitation as described for achiral LC-MS analysis of HMMA conjugates^[12] was tested, but showed large variations in urine samples from different sources (data not shown). As described in the Experimental section for method A, the developed SPE procedure was finally successful. Thanks to the high selectivity and sensitivity of the high-resolution LC-HRMS apparatus, only a small urine volume was necessary. Separation and analysis of the glucuronides and sulfates were performed on a chiral column with LC-HRMS. Figure 2 shows typical LC-HRMS chromatograms of the accurate masses of an extract of the corresponding QC MED indicating *R/S*-DHMA 3- and 4-sulfates, *R/S*-HMMA sulfate, *R*- and *S*-HMMA glucuronides. Preliminary studies showed that these conjugates were the most abundant phase II metabolites in human urine, while DHMA 3- and 4-glucuronides and conjugates of DHA and HMA were only detected in traces. Stereoselective separation could be achieved for the glucuronides, but unfortunately not for the sulfates, although several chiral columns were tested in preliminary studies. Therefore, the *R/S* sulfate ratios were determined after selective cleavage as described in the Experimental section under method B.

Cleavage of the sulfates (method B)

For selective cleavage of the sulfates, initial experiments using a HIGH concentration (Table 1) of *R/S*-DHMA sulfate, *R/S*-HMMA sulfate and *R*- and *S*-HMMA glucuronides were performed. To assess the final yield of deconjugated sulfates and glucuronides, the enantiomers of DHMA and HMMA were quantified using a calibration curve of the respective free compounds. The percentage of cleavage was calculated via the molar ratio of deconjugated DHMA and HMMA to the intact conjugates. Sulfatase from *Aerobacter aerogenes* was suitable as it cleaved in HIGH concentrations of the sulfates of *R/S*-DHMA and *R/S*-HMMA by 85% (RSD 8) to *R*-DHMA, 82% (RSD 5) to *S*-DHMA, 93% (RSD 11) to *R*-HMMA and 95% (RSD 14) to *S*-HMMA, but less than 1% of the *R*-HMMA and *S*-HMMA glucuronides. These data, measured by method B, showed sufficient selectivity and reproducibility and no stereoselectivity.

GC-NICI-MS for MDMA and its unconjugated metabolites (methods B and C)

For MDMA and metabolites extraction and derivatization, different approaches are published in the literature.^[15,21,29] Owing to its excellent chromatographic properties, its distinct mass spectra and previous successful application,^[25] *S*-HFBPCL was selected, although it was not commercially available. Furthermore, the application of this derivatization reagent allowed ionization in the NICI mode, which leads to increased sensitivity and enabled reduction of sample volume to 100 μ l.

Direct *S*-HFBPCL derivatization of human urine without extraction was tested analogously to a published oral fluid procedure.^[30] However, in different urine samples ($n = 6$), the *S*-HFBPCL derivatization of dihydroxy and hydroxy-methoxy metabolites was not reproducible (data not shown). To overcome this problem, trimethylsilylation of the hydroxy groups after the *S*-HFBPCL derivatization of the amino group was performed as previously described.^[16,21,22] However, derivatization with HMDS was performed under microwave

irradiation for 5 min instead of 20–60-min incubation at 80 °C, reducing total preparation time to about 35 min. Under these conditions, no *S*-HFBPCl derivatization of the hydroxy groups was observed.

Figure 3 shows typical GC-MS chromatograms of the extract masses (method C) of the QC MED, indicating sufficient stereoselective separation of MDMA and its unconjugated metabolites MDA, HMA, DHA, DHMA and HMMA. The elution order of the enantiomers could be determined for MDMA using the available *S*-enantiomer. In contrast to Ref. [21] where the enantiomers of DHA, DHMA, HMA and HMMA were only assigned as enantiomer 1 and 2, in the present study, the elution order could be determined by incubation of *S*-MDMA with CYP2D6 and HLC containing catechol-O-methyltransferase according to Ref. [18] With our derivatization procedure, the *R*-enantiomers of all metabolites were identified to elute before the *S*-enantiomers. Enantiomer differentiation was essential for determining MDMA's stereoselective urinary elimination kinetics.

Method validation

The described procedures were validated according to recommendations on method validation in the context of quality management with forensic-toxicological investigations published by the GTFCh^[27] and internationally accepted recommendations.^[31–35]

Specificity—Blank urine samples from ten different sources were analyzed for chromatographic interference for all three methods. For the LC-HRMS analysis (method A), no interference peaks were detected for any of the analytes in blank samples or after addition of the IS solution.

GC-NICI-MS analysis with (method B) and without sulfate cleavage (method C) showed no interfering matrix peaks. Zero samples (containing the IS mixture) had no relevant amounts of unlabeled analytes of the respective deuterated IS used, but minor peaks were observed for *m/z* 414 for *S*-HMA and *m/z* 420 for *R*-DHA with retention time differences of 0.1 min resulting from the IS pholedrine. However, these peak areas were about 25% of those at the LOQ and were, therefore, negligible.

Recovery, matrix effects and process efficiency—RE, ME and PE data for the LC-HRMS analysis (method A) are listed in Table 1. All analytes could be extracted with REs over 75% with acceptable CVs. No differences in RE between LOW and HIGH concentrations could be observed for the sulfate metabolites, but slight differences were observed for the glucuronides. No notable MEs were observed for any of the analytes.

RE for the GC-MS analysis with (method B) and without sulfate cleavage (method C) was calculated at QC LOW and HIGH concentrations. As the derivatized analytes were not available, RE from urine samples could be determined only relative to aqueous solution with the same concentrations of the respective analytes. The RE data and CVs, calculated from the peak areas only and from ratios of analyte *versus* IS, are given in Tables 2 and 3. Except for the dihydroxy compounds, the acceptance criteria (AC) with CVs of 15% (20% near the LOQ) were fulfilled. Simultaneous extraction and derivatization of DHA and DHMA were not reproducible with CVs between 33 and 80% due to different urine matrices. However, the AC also were fulfilled when looking at the CVs calculated from the ratio of analyte *versus* IS (DHBA for DHA and DHMA), which was generally used for quantification.

Calibration model—Calibration curves using six concentration levels with six replicates each were constructed to evaluate the calibration model. The limits for the LC-HRMS analysis (method A) were assessed after analysis of about 20 positive urine samples and

additionally based on data published by Shima *et al.*^[12] Calibration ranges for GC-MS analysis (methods B and C) were selected based on published urine concentrations for MDA, MDMA, HMA and HMMA determined after controlled administration of MDMA.^[36] Calibration ranges for all analytes are given in Tables 1–3 and should allow quantification without further dilution.

A weighted calibration model was used to account for unequal variances (heteroscedasticity) across the calibration range. A weighted linear and second-order regression model indicated curvature in the data for some analytes. As there were several possibilities with different ISs and calibration models, the final decision was made after evaluation of the accuracy and precision data. The final calibration models, ISs and weighting factors are given in Tables 1–3. Concerning methods B and C, a second-order regression model showed the best results for all analytes. That could be explained by the large calibration range compared to those in other publications, in which calibration ranges were unlikely to cover concentrations after recreational MDMA ingestion.^[21]

Accuracy and precision—QC samples (LOW, MED and HIGH) were analyzed in duplicate on each of 8 days as was proposed by Peters.^[28] QC concentrations were determined from daily calibration curves. Calibrator concentrations were within 20% of target based on the full calibration curve. Accuracy, intra-day and inter-day precision were calculated as described above (Tables 1–3).

For method A, M6G was a suitable IS for the glucuronides and sulfates. For methods B and C, respective deuterated analogs were employed for MDA and MDMA. The dihydroxy compounds showed acceptable accuracy and precision with DHBA as IS. *S*-pholedrine and also *R*-pholedrine proved to be the best ISs for HMA and HMMA; however, MDA-*d*₅ and MDMA-*d*₅ were alternatives for HMA in methods B and C and for HMMA in method C.

All analytes fulfilled the validation parameters. Intra-day and inter-day precision were satisfactory for the conjugated DHMA and HMMA enantiomers determined after sulfate cleavage of a medium conjugate concentration (QC MED sample from method A). Accuracy for unconjugated DHMA after cleavage was slightly below the AC of –15%.

Stability—Phase II metabolites (method A) were stable in processed samples stored on the autosampler for 48 h at 8 °C. No degradation after three freeze/thaw cycles and after storage at –20 °C for 2 and 6 months was observed.

The derivatized analytes from methods B and C were stable on the autosampler for 24 h at ambient temperature. During three freeze/thaw cycles, no analyte degradation was observed. Long-term stability measurements were previously published and no instability could be observed up to after 6 months.^[21,37,38]

Limits—The LOQs and LODs of all analytes are listed in Tables 1–3. The LOQs were consistent with the lowest calibrator with less than 20% bias as compared to the target concentration. Concerning the GC-MS methods (B and C), LOD and LOQ are comparable to previously published methods,^[21,22,37] although the sample volume was markedly lower.

CONCLUSION

Stereoselective analysis of all major MDMA metabolites in human urine was accomplished using three methods: LC-HRMS for glucuronides and sulfates, GC-MS for chiral analysis of sulfates after cleavage and GC-MS for MDMA and its unconjugated metabolites. This work is the first to describe analytical procedures for the stereoselective analysis of all major

MDMA metabolites, particularly the phase II metabolites. The reported methods were fully validated and showed good reproducibility and accuracy, and will, therefore, be suitable for studying the stereoselective urinary elimination of MDMA and its metabolites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Metabolic pathways of MDMA in humans.

Figure 2.
Typical LC-HRMS chromatograms of a processed urine sample (QC MED) indicating the given analytes.

Figure 3. Typical GC-MS chromatograms of a processed urine sample (QC MED) indicating the given analytes.

LC-HRMS method validation data (method A)

Table 1

Analyte	QC sample	Nominal concentration (µM)	Mean calculated concentration (µM)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)	RE (%), (CV, %)	ME (%), (CV, %)	PE (%), (CV, %)	Calibration range (µM)	Calibration model	Internal standard	LOD (µM)	LOQ (µM)
R/S-DHMA 3-sulfate	LOW	1.0	0.85	-15.3	4.6	9.3	96(19)	-2(12)	93(15)	0.5-75	Linear 1/X ²	M6G	0.1	0.5
	MED	20.0	19.2	-4.2	4.8	6.7	-	-	-					
	HIGH	60.0	53.5	-10.9	4.9	6.4	88(13)	-7(12)	83(22)					
R/S-DHMA 4-sulfate	LOW	1.0	0.88	-12.1	6.4	9.7	96(11)	-1(5)	95(11)	0.5-75	Linear 1/X ²	M6G	0.1	0.5
	MED	20.0	19.7	-1.3	4.5	7.4	-	-	-					
	HIGH	60.0	54.4	-9.3	3.8	6.5	89(12)	0(8)	87(15)					
R/S-HMMA sulfate	LOW	0.33	0.31	-6.4	4.7	10.3	96(14)	-1(8)	96(11)	0.2-67	Quadratic 1/X ²	M6G	0.03	0.2
	MED	25.0	24.1	-3.6	5.1	7.3	-	-	-					
	HIGH	58.0	55.5	-4.8	6.1	7.5	90(13)	3(9)	93(7)					
S-HMMAglucuronide	LOW	0.33	0.28	-15.3	4.5	7.8	94(5)	-1(6)	92(7)	0.2-25	Linear 1/X ²	M6G	0.03	0.2
	MED	6.5	6.2	-7.3	3.2	5.4	-	-	-					
	HIGH	20.0	20.0	0.0	3.4	6.1	83(15)	11(6)	92(18)					
R-HMMAglucuronide	LOW	0.33	0.28	-16.0	4.7	6.6	95(5)	-1(8)	93(7)	0.2-25	Linear 1/X ²	M6G	0.03	0.2
	MED	6.5	6.2	-7.6	3.0	5.0	-	-	-					
	HIGH	20.0	19.7	-1.7	3.7	6.0	75(7)	12(7)	84(10)					

Table 2

GC-NCI-MS method validation data after sulfate cleavage (method B)

Analyte	QC sample	Nominal concentration (µM)	Mean calculated concentration (µM)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)	RE (%), CV, %CV, % IS corrected	Calibration range (µM)	Calibration model	Internal standard	LOD (µM)	LOQ (µM)
R-MDA	LOW	0.50	0.49	-1.8	3.1	5.5	121 (8/3)	0.1–25	Quadratic 1/X	R-MDA <i>d</i> ₅	0.005	0.1
	MED	7.5	7.1	-4.7	1.2	2.6	–					
	HIGH	20.0	19.4	-3.1	5.9	8.1	101 (3/2)					
S-MDA	LOW	0.50	0.47	-5.4	7.0	8.2	128 (6/2)	0.1–25	Quadratic 1/X	S-MDA <i>d</i> ₅	0.005	0.1
	MED	7.5	7.2	-4.3	3.5	4.2	–					
	HIGH	20.0	19.1	-4.6	5.6	8.9	101 (3/0.3)					
R-HMA	LOW	0.50	0.50	-0.4	11.0	15.5	109(10/5)	0.1–25	Quadratic log log	S-Pholedrine	0.025	0.1
	MED	7.5	7.7	2.4	7.0	9.5	–					
	HIGH	20.0	20.5	2.6	13.3	14.2	97(3/5)					
S-HMA	LOW	0.50	0.51	1.5	10.0	19.3	106(10/5)	0.1–25	Quadratic log log	S-Pholedrine	0.025	0.1
	MED	7.5	7.6	1.7	5.1	8.7	–					
	HIGH	20.0	20.5	2.4	13.2	13.6	96 (2/6)					
R-DHA	LOW	0.50	0.45	-11.0	9.2	13.6	48 (46/19)	0.25–25	Quadratic log log	DHBA	0.25	0.25
	MED	7.5	7.1	-4.7	7.4	10.9	–					
	HIGH	20.0	18.9	-5.5	8.6	8.8	51 (38/13)					
S-DHA	LOW	0.50	0.42	-15.3	7.4	13.0	48 (53/15)	0.25–25	Quadratic log log	DHBA	0.25	0.25
	MED	7.5	7.3	-2.9	6.0	9.4	–					
	HIGH	20.0	19.2	-3.8	8.7	10.2	56 (34/12)					
R-MDMA	LOW	0.50	0.39	-22.5	7.5	11.1	123 (6/6)	0.1–125	Quadratic log log	R-MDMA <i>d</i> ₅	0.005	0.1
	MED	35.0	35.4	1.1	1.8	5.1	–					
	HIGH	100.0	99.1	-0.9	5.0	6.4	97(2/2)					
S-MDMA	LOW	0.50	0.41	-18.6	6.5	11.3	118 (5/3)	0.1–125	Quadratic log log	S-MDMA <i>d</i> ₅	0.005	0.1
	MED	35.0	35.3	0.8	2.0	4.8	–					
	HIGH	100.0	99.7	-0.3	4.5	6.2	97 (3/3)					
R-HMDMA	LOW	0.50	0.45	-9.1	10.5	18.3	97 (17/11)	0.1–125	Quadratic log log	S-Pholedrine	0.05	0.1
	MED	35.0	38.9	11.2	6.0	8.7	–					
	HIGH	100.0	102.7	2.7	8.8	9.3	95 (4/3)					

Analyte	QC sample	Nominal concentration (μM)	Mean calculated concentration (μM)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)	RE (%), (CV, %/CV, % IS corrected)	Calibration range (μM)	Calibration model	Internal standard	LOD (μM)	LOQ (μM)
S-HMMA	LOW	0.50	0.45	-10.1	8.9	15.5	95(10/7)	0.1–125	Quadratic log log	S-Pholedrine	0.05	0.1
	MED	35.0	39.0	11.3	5.5	8.3	–					
	HIGH	100.0	101.9	1.9	7.8	8.9	94 (4/3)					
R-DHMA	LOW	0.50	0.43	-13.8	11.6	17.7	17 (54 /12)	0.25–37.5	Quadratic 1/X	DHBA	0.25	0.25
	MED	10.0	10.4	4.1	14.0	14.5	–					
	HIGH	35.0	33.6	-4.0	8.7	12.1	19 (59 /14)					
S-DHMA	LOW	0.50	0.42	-16.5	14.5	19.2	16 (74 /10)	0.25–37.5	Quadratic 1/X	DHBA	0.25	0.25
	MED	10.0	10.5	5.3	12.0	14.5	–					
	HIGH	35.0	33.6	-4.0	8.1	10.3	19 (60 /14)					
R-HMMA sulfate	MED	12.5	13.6	8.8	8.3	11.4	–	–	–	–	–	–
S-HMMA sulfate	MED	12.5	13.8	10.1	10.0	11.8	–	–	–	–	–	–
R-DHMA sulfate	MED	20.0	16.7	-16.4	10.2	12.7	–	–	–	–	–	–
S-DHMA sulfate	MED	20.0	16.0	-20.0	11.0	14.3	–	–	–	–	–	–

Bold values indicate values outside the acceptance criteria of ±15% or ±20%

GC-NICI-MS method validation data (method C)

Table 3

Analyte	QC sample	Nominal concentration (µM)	Mean calculated concentration (µM)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)	RE (%), (CV%/CV% IS corrected)	Calibration range (µM)	Calibration model	Internal standard	LOD (µM)	LOQ (µM)
R-MDA	LOW	0.50	0.50	-0.3	4.3	9.5	108(13/1)	0.1–25	Quadratic <i>MX</i>	R-MDA <i>d</i> ₅	0.005	0.1
	MED	7.5	7.2	-4.4	2.3	4.3	–					
	HIGH	20.0	18.6	-7.2	2.7	9.4	109(6/1)					
S-MDA	LOW	0.50	0.48	-3.7	4.9	8.6	116(12/4)	0.1–25	Quadratic <i>MX</i>	S-MDA <i>d</i> ₅	0.005	0.1
	MED	7.5	7.3	-3.2	3.4	3.1	–					
	HIGH	20.0	18.9	-5.5	2.8	8.7	106(5/2)					
R-HMA	LOW	0.50	0.46	-8.6	8.9	12.9	98(17/5)	0.1–25	Quadratic log log	S-Pholedrine	0.025	0.1
	MED	7.5	7.6	1.5	9.4	8.0	–					
	HIGH	20.0	20.0	-0.1	6.6	11.4	97 (8/4)					
S-HMA	LOW	0.50	0.46	-8.5	7.1	11.4	93(17/5)	0.1–25	Quadratic og log	S-Pholedrine	0.025	0.1
	MED	7.5	7.5	-0.4	8.9	7.8	–					
	HIGH	20.0	20.0	-0.1	7.2	10.9	99 (7/4)					
R-DHA	LOW	0.50	0.44	-11.0	4.9	9.9	43 (47/7)	0.25–25	Quadratic log log	DHBA	0.05	0.25
	MED	7.5	7.2	-3.3	4.8	7.7	–					
	HIGH	20.0	18.2	-8.9	5.8	8.2	53 (55/5)					
S-DHA	LOW	0.50	0.43	-14.4	6.4	10.2	49 (45/8)	0.25–25	Quadratic log log	DHBA	0.05	0.25
	MED	7.5	7.3	-2.7	10.6	11.5	–					
	HIGH	20.0	18.0	-10.2	6.2	11.3	54 (52/6)					
R-MDMA	LOW	0.50	0.41	-18.7	8.7	15.0	107(15/8)	0.1–125	Quadratic log log	R-MDMA <i>d</i> ₅	0.005	0.1
	MED	35.0	36.2	3.3	1.8	5.8	–					
	HIGH	100.0	95.6	-4.4	2.2	8.7	105(5/4)					
S-MDMA	LOW	0.50	0.42	-16.2	8.9	15.6	104(15/5)	0.1–125	Quadratic log log	S-MDMA <i>d</i> ₅	0.005	0.1
	MED	35.0	36.2	3.3	1.8	5.6	–					
	HIGH	100.0	96.6	-3.4	2.5	7.9	106(5/4)					
R-HMMA	LOW	0.50	0.43	-14.0	9.5	15.4	83 (20/8)	0.1–125	Quadratic log log	S-Pholedrine	0.05	0.1
	MED	35.0	37.8	8.1	6.7	5.8	–					
	HIGH	100.0	99.1	-0.9	5.8	10.9	99 (8/2)					

Analyte	QC sample	Nominal concentration (µM)	Mean calculated concentration (µM)	Accuracy (%)	Intra-day precision (%)	Inter-day precision (%)	RE (%) _n (CV%/CV% IS corrected)	Calibration range (µM)	Calibration model	Internal standard	LOD (µM)	LOQ (µM)
S-HMMA	LOW	0.50	0.41	-17.1	9.8	13.1	81 (18/7)	0.1–125	Quadratic log log	S-Pholedrine	0.05	0.1
	MED	35.0	38.1	8.7	6.5	5.9	–					
	HIGH	100.0	97.8	-2.2	4.8	10.3	99 (9/2)					
R-DHMA	LOW	0.50	0.43	-14.1	7.1	9.1	20 (56 /9)	0.25–37.5	Quadratic 1/X	DHBA	0.05	0.25
	MED	10.0	10.6	6.1	5.5	9.1	–					
	HIGH	35.0	33.8	-3.5	3.9	7.8	26 (80 /10)					
S-DHMA	LOW	0.50	0.42	-15.7	8.9	12.6	19 (62 /15)	0.25–37.5	Quadratic 1/X	DHBA	0.05	0.25
	MED	10.0	10.7	7.2	5.8	8.1	–					
	HIGH	35.0	33.7	-3.7	3.8	7.4	28 (76 /10)					

Bold values indicate values outside the acceptance criteria of ±15% or ±20%