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High-performance liquid chromatography with electrochemical detection applied to the analysis of 3,4-dihydroxymethamphetamine in human plasma and urine

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Abstract

Metabolic activation in the disposition of 3,4-methylenedioxymethamphetamine (MDMA, ''ecstasy'') has been implicated in some of its pharmacological and toxicological effects, with the major metabolite 3,4-dihydroxymethamphetamine (HHMA) as a putative toxicant through the formation of thioether adducts. We describe the first validated method for HHMA determination based on acid hydrolysis of plasma and urine samples, further extraction by a solid-phase strong cation-exchange resin (SCX, benzenesulfonic acid), and analysis of extracts by high-performance liquid chromatography with electrochemical detection. The chromatographic separation was performed in an *n*-butyl-silane (C_4) column and the mobile phase was a mixture of 0.1 *M* sodium acetate containing 0.1 *M* 1-octanesulphonic acid and 4 m*M* EDTA (pH 3.1) and acetonitrile (82:18, v/v). Compounds were monitored with an electrochemical cell (working potentials 1 and 2, +0.05 and +0.35 V, respectively, gain 60 μA). A mobile phase conditioning cell with a potential set at +0.40 V was connected between the pumping system and the injector. Calibration curves were linear within the working concentration ranges of 50–1000 μg/L for urine and plasma. Limits of detection and quantification were 10.5 and 31.8 μg/L for urine and 9.2 and 28.2 μg/L for plasma. Recoveries for HHMA and DHBA (3,4-dihydroxybenzylamine, internal standard) were close to 50% for both biological matrices. Intermediate precision and inter-day accuracy were within 3.9–6.5% and 7.4–15.3% for urine and 5.0–10.8% and 9.2–13.4% for plasma.

Keywords: 3,4-Dihydroxymethamphetamine

1. Introduction

There is widespread misuse of 3,4-methylenedioxymethamphetamine (MDMA, commonly called ''ecstasy'') among young people, particularly in Europe and the United States. Neurodegeneration of serotonergic pathways of the central nervous

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system has been postulated as the major neurotoxicological effect after its continued consumption [1]. It seems that some of the pharmacological and toxicological effects of MDMA in humans are related to metabolic activation in the disposition process of the drug [2–4]. A metabolite of MDMA, 3,4-dihydroxymethamphetamine (HHMA), has been hypothesized as a putative toxicant through the formation of thioether adducts [5,6]. The presence of HHMA has been demonstrated in in vitro studies and appears as a major metabolite of MDMA in human microsomal preparations [7]. HHMA would result from the *O*-demethylenation of MDMA, whereas *O*-methylation of HHMA would result in 4-hydroxy-3-methoxymethamphetamine (HMMA), which appears to be a major metabolite of MDMA in vivo. On the other hand, 3,4-methylenedioxyamphetamine (MDA), an *N*-demethylated metabolite of MDMA, and 4-hydroxy-3-methoxyamphetamine (HMA), an *O*-methylated metabolite of 3,4-dihydroxyamphetamine (HHA), have been identified as minor metabolites [8–10].

Fig. 1 shows a summary of the main metabolic pathways described for MDMA in humans. The analysis of *O*-methylenedioxy derivatives of amphetamine-related substances in different biological samples has been the subject of a number of publications [9,11,12] and there are several excellent reviews on this analytical topic [13,14]. HHMA, however, has catecholamine-like physicochemical properties and, probably for this reason, the analysis of this compound has consistently been omitted from the ana-

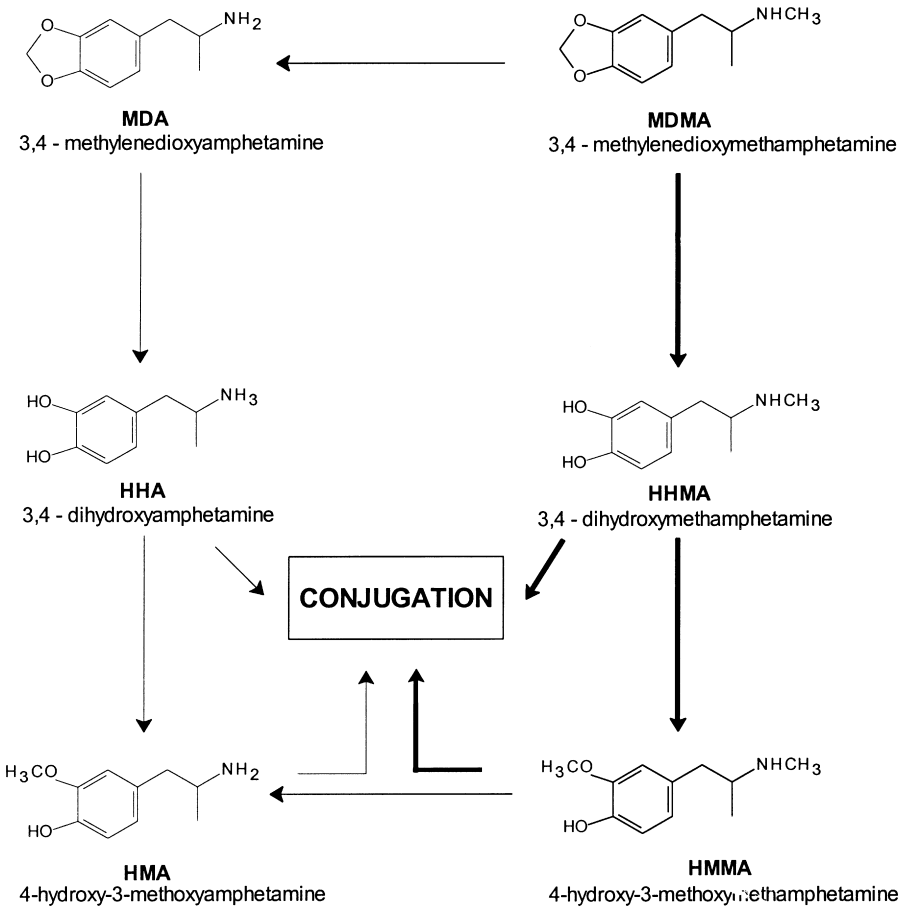

Fig. 1. Metabolic pathways described for MDMA in humans.

lytical methods described for the detection of less-polar MDMA metabolites (MDA, HMMA, and HMA) [9,15]. HHMA is an unstable substance, light- and temperature-sensitive, that auto-oxidises easily to its corresponding quinone. When, instead of following more ordinary analytical approaches in the analysis of amphetamine-like substances, one concentrates on the catecholamine structure of HHMA, high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) (HPLC–ECD) seems a suitable technique. In fact, HPLC–ECD is considered one of the techniques of choice for the analysis of catecholamines in biological fluids [16–20]. Several methods for the extraction of catecholamines from plasma and urine have been developed. These include alumina (Al_2O_3), the formation of cyclic boronates, with further extraction by liquid–liquid extraction procedures and solid-phase extraction methods using phenylboronic acid (PBA) or strong cation-exchange resins (SCX, benzenesulfonic acid) [16–20].

We describe here the first validated method for HHMA determination in human plasma and urine samples by strong cation-exchange solid-phase extraction and HPLC–ECD analysis.

2. Experimental

2.1. Materials and reagents

3,4-Dihydroxymethamphetamine (HHMA) racemate was synthesized following procedures described elsewhere [21]. 3,4-Dihydroxybenzylamine (DHBA), used as internal standard (ISTD), and methylcatechol were purchased from Aldrich (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA), respectively. Ultra-pure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France). HPLC-grade acetonitrile, methanol, hydrochloric acid, perchloric acid, ortho-phosphoric acid 85%, sodium acetate, potassium hydrogen phosphate, potassium dihydrogen phosphate, and sodium hydroxide were obtained from Merck (Darmstadt, Germany). EDTA and 1-octanesulfonic acid were supplied by Fluka (Buchs, Switzerland). Sodium bisulphite was from Sigma. Bond Elut SCX (strong cation-exchange) columns were purchased from Varian (Harbor City, CA, USA) and mounted on a Vac Elut vacuum manifold (Supelco, Bellefonte, PA, USA). Drug-free (blank) urine was purchased from Bio-Rad (CA, USA). Blank plasma was obtained from the blood bank of the Hospital del Mar (Barcelona, Spain).

2.2. Working standards

Standard solutions (1 g/L) of HHMA and DHBA were prepared in methanol. Working solutions at concentrations of 0.1 and 0.01 g/L were prepared by dilution of the stock standard solutions with methanol. All solutions were checked chromatographically for purity by standard techniques. Standard solutions were stored at −20 °C until analysis.

2.3. Calibrations and quality control samples

Calibration standards containing 50, 100, 250, 500, and 1000 μg/L of HHMA for plasma and urine samples were prepared in duplicate daily for each analytical batch by adding suitable amounts of methanol working solutions to 1 mL of pre-checked drug-free plasma and 1 mL of 1:15 diluted urine with water. Samples were processed as described below (Sections 2.7 and 2.8). At the beginning of the study, quality control samples of 60 μg/L (low control), 400 μg/L (medium control), and 850 μg/L (high control) were prepared (using a new set of freshly prepared drug standard solutions) once from bulk drug-free plasma and urine samples, aliquoted, and stored at −20 °C. They were included in each analytical batch to control the daily quality of the analytical process and to check the stability of samples under storage conditions.

2.4. Instrumentation

Chromatographic analysis was carried out using a 1050 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a Model 5100A Coulochem electrochemical detector (ESA, Bedford, MA, USA). Compounds were monitored through a Model 5011 dual porous graphite electrode cell with potential electrodes 1 and 2 set at +0.05 and +0.35 V, respectively. A guard cell set at +0.40 V was positioned between the solvent delivery pump and

injector to condition the mobile phase (Model 5021). The gain of the detector was set at 60 μA and the response time was 4 s. The signal from detector 2 was sent to a Hewlett-Packard Chemstation for liquid chromatography (Rev. A.06.03[509]).

2.5. Chromatographic procedure

Separation was carried out using a 4.6 mm×250 mm Kromasil 100 *n*-butyl-silane (C_4) column (Teknokroma, Barcelona, Spain). The mobile phase was a mixture of 0.1 *M* sodium acetate containing 0.1 *M* 1-octanesulphonic acid and 4 m*M* EDTA (pH 3.1) and acetonitrile (82:18, v/v). It was filtered through a 0.45 μm Hewlett-Packard nylon filter before use. The flow-rate of the mobile phase was maintained at 1 mL/min. Peak areas were measured and the ratios with the internal standard were used for all calculations.

2.6. Redox potential curves

The potential of electrode 2 was optimised to obtain the best signal and the highest sensitivity of HHMA. Additionally, HMMA, HMA, and DHBA were studied.

The redox potentials tested ranged from 0.05 to 0.5 V. Five replicates of 500 μg/L were prepared from methanolic standards, taken to dryness, and reconstituted with mobile phase. Peak areas obtained for the different substances were determined at each redox potential considered.

2.7. Urine sample preparation

One millilitre (diluted urine 1:15 with water) of control and test urine samples was pipetted into 15 mL screw-capped glass tubes. Then, 25 μL of the ISTD solution (DHBA 0.01 g/L in methanol), 100 μL of 250 m*M* sodium bisulphite, and 50 μL of 250 m*M* EDTA were added. Acidic hydrolysis was performed by adding 200 μL of 6 *M* hydrochloric acid. Samples were incubated for 30 min at 100 °C and then cooled to room temperature. The pH was adjusted to 5.5–6 with 1 mL of 1 *M* potassium phosphate buffer (pH 6) and approximately 200 μL of 5 *M* NaOH. After hydrolysis, samples were processed by applying a solid-phase extraction procedure using strong cation-exchange (SCX) columns. The columns were previously activated and conditioned with 2 mL of methanol and 2 mL of 1 *M* potassium phosphate buffer (pH 6) to avoid columns running dry. Samples were forced to pass through columns at no more than 15 mmHg vacuum pressure. After application of the samples, columns were washed with 1 mL of water and 4 mL of methanol. Columns were dried by applying a vacuum (maximum 15 mmHg) for 2 min. Analytes were then eluted with 2 mL of methanol–HCl (99:1) containing 3% (v/v) 250 m*M* EDTA and 3% (v/v) 250 m*M* sodium bisulphite. Eluates were evaporated to dryness under a stream of nitrogen in a water bath at 40 °C. The dried extracts were reconstituted in 200 μL of mobile phase by vigorous vortex mixing and transferred into 200 μL injection vials. Volumes of 30 μL were injected into the chromatographic system.

2.8. Plasma sample preparation

One millilitre of plasma was pipetted into 15 mL screw-capped glass tubes. Then, 25 μL of the ISTD solution (DHBA 0.01 g/L in methanol), 50 μL of methylcatechol 1 g/L, 200 μL of 250 m*M* sodium bisulphite, and 50 μL of 250 m*M* EDTA were added. Acidic hydrolysis was performed by adding 1 mL of 0.5 *M* hydrochloric acid. Samples were incubated for 30 min at 100 °C and then cooled to room temperature. After hydrolysis, proteins in plasma samples were precipitated with 100 μL of perchloric acid and centrifuged at 3000 *g* for 10 min. The supernatant was adjusted to pH 5.5–6 with 1 mL of 1 *M* potassium phosphate buffer (pH 6) and approximately 130 μL of 10 *M* NaOH. A solid-phase extraction procedure using strong cation-exchange (SCX) columns was carried out according to the procedure described above for urine samples. The dried extracts were reconstituted in 200 μL of mobile phase by vigorous vortex mixing and transferred into 200 μL injection vials. Volumes of 30 μL were injected into the chromatographic system.

2.9. Validation protocol

Prior to the application of the method to real samples, an intra- and inter-assay validation protocol

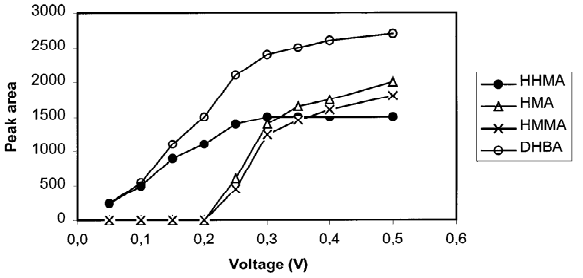

Fig. 2. Redox potential curves for HHMA, HMMA, HMA and the internal standard (DHBA).

was carried out. Selectivity, recovery, linearity, precision, accuracy, limits of detection and quantification were assayed.

3. Results

3.1. Selectivity

Fig. 2 shows redox potential curves obtained for HHMA and DHBA. In addition to HHMA, which was further validated for its detection and quantification, Fig. 2 illustrates two other MDMA metabolites (HMMA and HMA) tested in the development of the method and which can be included in the analytical procedure if needed. For HHMA and DHBA, 0.35 V was the lowest potential where the maximal response was observed. As shown in Fig. 3, a good separation and good response were obtained for a standard mixture of the four compounds studied. DHBA was chosen as internal standard because of its similar physico-chemical properties, eluting at a retention time very close to HHMA.

Changes in the retention times between urine and plasma can be explained by the small variations in the recirculating mobile phases when using a C_4 column, mainly caused by organic solvent evaporation. The changes in retention times can also be explained by the 2-month period between the analysis of plasma and urine, during which the C_4 column can change slightly. However, the small variations do not affect the selectivity and sensitivity of the method.

The selectivity of the method was studied by analyzing several plasma and urine samples ($n=10$) and checking for the presence of interfering substances at the retention times of the compounds of interest with the conditions described. The extraction of HHMA from urine and plasma using SCX columns followed by HPLC–ECD using a C_4 column allows elution of analytes in less than 6 min with a good separation from plasma and urine interference (Fig. 4).

3.2. Recovery

The recoveries of HHMA and the ISTD were calculated by comparing the peak areas obtained when analyzed with the reference substances added

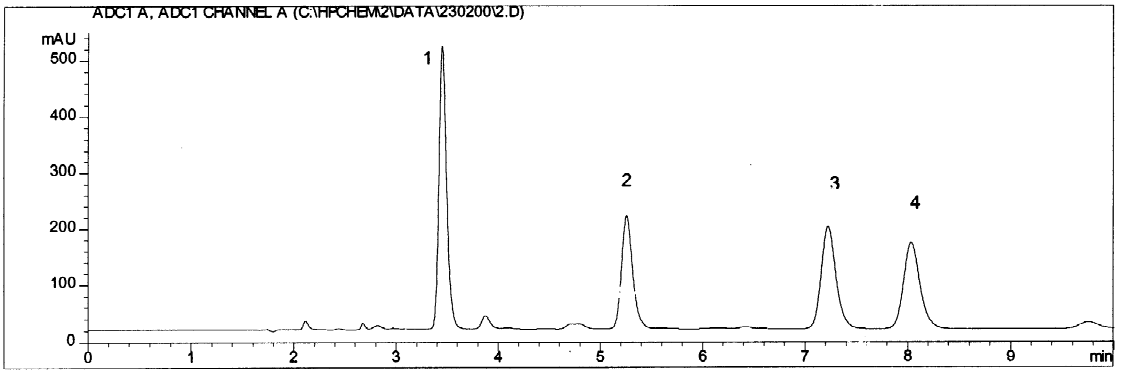

Fig. 3. Chromatogram of a standard mixture of HHMA (2), HMMA (3), HMA (4) and the internal standard (1) using a redox potential of +0.35 V. The standard mixture contained 500 μg/L of each. Separation was carried out using a 4.6 mm×250 mm Kromasil 100 *n*-butyl-silane (C_4) column. The mobile phase was a mixture of 0.1 *M* sodium acetate containing 0.1 *M* 1-octanesulphonic acid and 4 m*M* EDTA (pH 3.1) and acetonitrile (82:18, v/v).

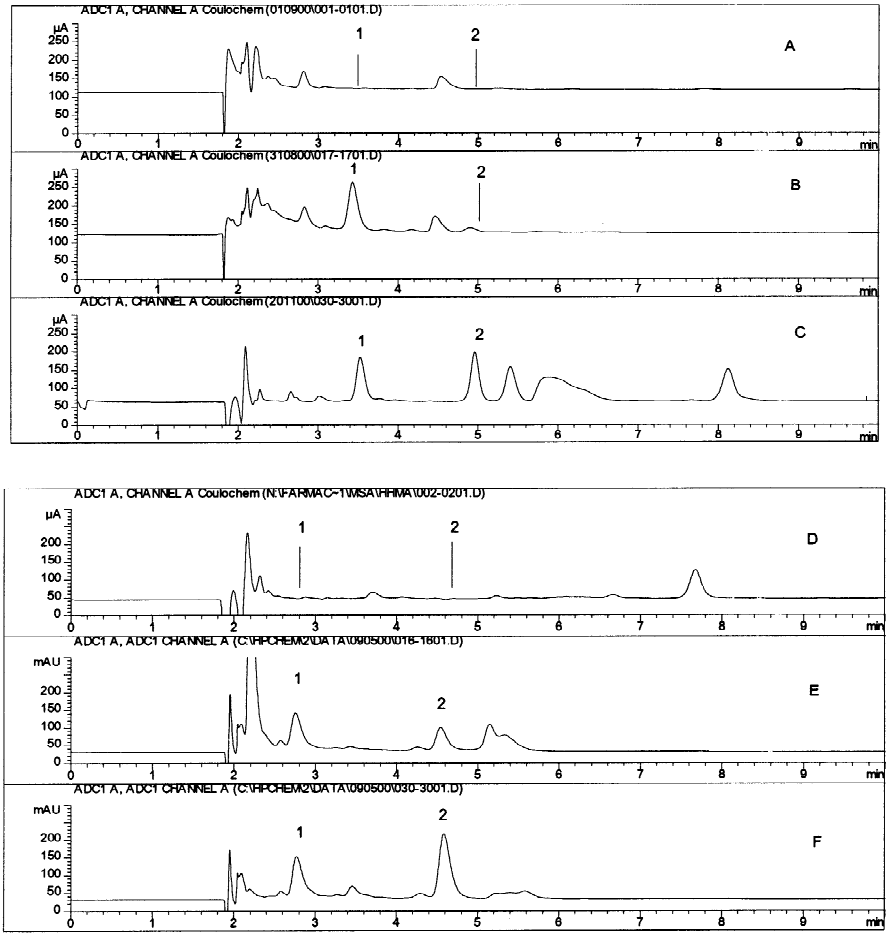

Fig. 4. HPLC chromatograms of an extract of blank plasma (A), an extract of a real plasma sample estimated at 300 μg/L HHMA [with enzymatic hydrolysis (B), with acidic hydrolysis (C)], an extract of blank urine (D), and an extract of a real urine sample estimated at 600 μg/L HHMA [with enzymatic hydrolysis (E), with acidic hydrolysis (F)]. Peak 1 corresponds to DHBA and peak 2 to HHMA. Separation was carried out using a 4.6 mm×250 mm Kromasil 100 *n*-butyl-silane (C_4) column. The mobile phase was a mixture of 0.1 *M* sodium acetate containing 0.1 *M* 1-octanesulphonic acid and 4 m*M* EDTA (pH 3.1) and acetonitrile (82:18, v/v).

prior to and after extraction. For the ISTD, 12 replicates of the same blank plasma or urine were extracted. After the extraction, 250 ng of ISTD was added to the organic phase and taken through the analytical procedure. In parallel, 12 aliquots of the same blank plasma or urine were spiked with the same amount of ISTD and extracted following the complete analytical procedure. The experiment was carried out for HHMA at three different concentrations (four replicates for each concentration). Recoveries obtained were 53% (C.V. 12.7%) and 47% (C.V. 14.1%) in plasma and 58% (C.V. 12.1%) and 49% (C.V. 13.8%) in urine for DHBA and HHMA, respectively

3.3. Linearity

For the study of linearity, four replicates were analyzed at the following concentrations: 50, 100, 250, 500, 750, and 1000 μg/L. Results obtained clearly showed a non-constant variance (SPSS9.0) of the data (heteroscedasticity). To overcome this problem, regression analysis using the weighted least-squares method (with $1/x$ as weighting factor) of the

theoretic concentrations versus peak-areas ratios (HHMA and DHBA) resulted in a slope of 0.00291±0.00006 ($n=4$) and an intercept of −0.06834±0.0109 ($n=4$) for plasma samples, and in a slope of 0.00211±0.00017 ($n=4$) and an intercept of 0.01636±0.0586 ($n=4$) for urine samples. In both cases, the coefficients of determination (r^2) were greater than 0.9900.

3.4. Precision and accuracy

Three replicates at three different concentrations of HHMA (60, 400, and 850 μg/L) were spiked in blank plasma and urine for the determination of intra-assay precision and accuracy. The inter-day precision and accuracy were determined during three different assays. Intermediate precision, expressed as relative standard deviation (RSD) of the calculated concentrations, and inter-day accuracy, expressed as the relative error (ERR%) of the estimated concentrations, varied from 3.9 to 6.5% and 7.4 to 15.3%, respectively, over the whole concentration range for urine. Intermediate precision and inter-day accuracy varied from 5.0 to 10.8% and 9.2 to 13.4%, respectively, over the whole concentration range for plasma. Results are shown in Tables 1 and 2.

3.5. Estimation of limits of detection and quantification

Analyses of four replicates were performed with a spiked plasma and urine containing 50 μg/L of HHMA. The standard deviation (SD) obtained from the values was used as a measure of the noise for the estimation of the limits of detection (3SD) and quantification (10SD). Estimated limits of detection and quantification were 10.5 and 31.8 μg/L for urine and 9.2 and 28.2 μg/L for plasma.

Table 1
Intra-assay and intermediate precision and accuracy obtained for the determination of HHMA in urine

Conc. (μg/L)	No. obs.	Estimated conc. ±SD (μg/L)	Precision (RSD)	Accuracy (error %)
Intra-assay				
60	3	66±4.2	6.4	9.5
400	3	397±51.7	13.0	9.2
850	3	774±29.2	3.8	8.9
Intermediate				
60	9	68±4.2	6.2	13.3
400	9	381±15.0	3.9	7.4
850	9	720±46.6	6.5	15.3

Table 2
Intra-assay and intermediate precision and accuracy obtained for the determination of HHMA in plasma

Conc. (μg/L)	No. obs.	Estimated conc. ±SD (μg/L)	Precision (RSD)	Accuracy (error %)
Intra-assay				
60	3	66±6.1	9.3	10.5
400	3	359±19.6	5.5	10.2
850	3	773±56.5	7.3	9.0
Intermediate				
60	9	68±3.4	5.0	13.4
400	9	387±24.4	6.3	11.6
850	9	876±94.1	10.8	9.2

3.6. Clinical studies

All plasma and urine samples were processed following the method described previously for HHMA quantification. Fig. 5 shows a plasma profile of HHMA and the urinary HHMA excretion profile from a healthy volunteer after administration of 100 mg MDMA.

4. Discussion

The analytical method developed for the extraction of HHMA from plasma and urine has shown suffi-

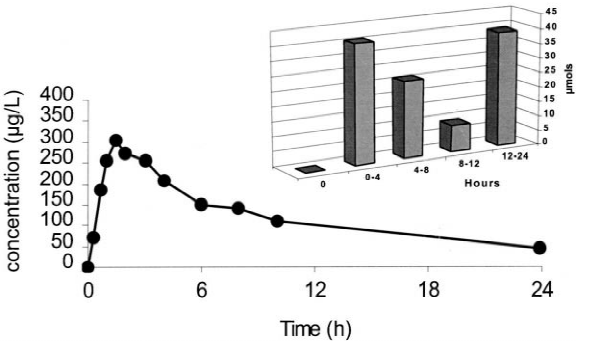

Fig. 5. HHMA plasma concentration versus time course and urinary excretion from a volunteer administered 100 mg of MDMA.

cient selectivity to remove the interfering and co-eluting compounds. The selection of strong cation-exchange (SCX) columns for sample extraction has proven to be adequate in comparison with other alternatives described for the analysis of catecholamines, such as alumina or the formation of boronates assayed during method development (data not shown) [16–20]. A mixed stationary phase with hydrophobic and cation-exchange interactions (Bond Elut Certify) was also checked. The recoveries obtained were very low and variable. Under conditions of pH 5.5–6, HHMA and DHBA were bonded to the surface of the SCX phase and columns could be washed with 1 mL of water and 4 mL of methanol, so that the polar and non-polar components could be washed off without affecting the recovery. Different elution phases were assayed in order to obtain the highest drug recovery. Several elution mixtures at different pH compatible with SCX columns were studied. Buffers with high ionic strength gave good recoveries, however the total volume needed for the elution was very high (2 mL) and it complicated the pre-concentration of the analyte before the chromatographic analysis. Methanol–HCl (99:1) gave the best recovery of HHMA from the cartridges when compared with those observed for an ethyl acetate–ammonia (98:2) mixture. The presence of sodium bisulphite and EDTA during the whole analytical procedure is crucial for preventing HHMA oxidation and degradation. The use of DHBA, a typical internal standard for the analysis of catecholamines, was demonstrated to be the substance of choice for the analysis of HHMA. The extraction procedure when coupled to an analytical method making use of HPLC–ECD facilitates the establishment of a highly sensitive and selective assay procedure for the compound studied. From Fig. 2 it is predicted that the best signal for HHMA is when the redox potential is set at $+0.35$ V, which is still in the rising part of the redox curve for the other two MDMA metabolites, HMA and HMMA.

In liver preparations, Maurer et al. [22] reported the detection of unstable nascent catechol-type metabolites, as their *O*-methylated derivatives, in a reaction catalyzed by catechol-*O*-methyltransferase. In this study, the authors consider this approach interesting for the indirect determination of HHMA in biological fluids as HMMA. Taking into account that HMMA is almost 100% conjugated in biological fluids, the analysis of free HMMA could be an indirect measurement of free HHMA. Nevertheless, HHMA does not appear to be available in its free form, but is completely conjugated to moieties other than glucuronic acid in biological fluids. The preliminary release of HHMA in its free form is then necessary before its stabilization through in vitro enzymatic *O*-methylation. If a hydrolysis step is needed for the analysis of HHMA, then the direct detection of HHMA by HPLC–ECD as proposed in the present study appears to be more suitable.

HHMA has not been detected in its free form either in plasma or in urine under the analytical conditions of the present method. A first attempt to hydrolyse samples with a standard enzymatic procedure [9,10] using β-glucuronidase/aryl sulphatase from *Helix pomatia* was partially successful for the recovery of HHMA from biological fluids (Fig. 4). Higher recoveries of HHMA from its conjugated forms were observed after an acidic hydrolysis procedure both in plasma and urine (Fig. 4). The hydrolysis conditions applied to plasma needed to be weaker than for urine because of the presence of proteins. After acid hydrolysis of the sample, plasma proteins were not fully precipitated and most of them remained soluble. Moreover, it was necessary to perform an additional step with perchloric acid for the precipitation of hydrolysated proteins in order to avoid their presence during the solid-phase extraction. Because of the catechol-type structure of both HHMA and DHBA, once the precipitate formed, a high adsorption of both compounds to this matrix was observed. As a way of circumventing this phenomenon, it was decided to add a blanketing agent. Among other substances investigated, 3-methylcatechol was selected. A relatively large amount of the blanketing agent is needed to prevent HHMA and DHBA adsorption onto the protein precipitate. Because of the lack of functional groups in the methylcatechol structure that can be ionised and interact with the resin under the present analytical conditions, it was easily eliminated during the solid-phase extraction procedure.

This is the first method designed and validated for the direct quantification of HHMA in human plasma and urine samples. The hydrolysis and extraction procedures described, combined with HPLC–ECD

analysis, provide adequate selectivity, accuracy and precision. Taking into account the HHMA plasma and urine concentrations described in Fig. 5 and Ref. [23], the sensitivity achieved seems adequate for its use in clinical studies.

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