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Short communication

Optimization of enantioselective high-performance liquid chromatography-tandem mass spectrometry method for the quantitative determination of 3,4-methylenedioxy-methamphetamine (MDMA) and its phase-1 metabolites in human biological fluids

Giorgia Sprega [a], Giorgi Kobidze [a], Alfredo Fabrizio Lo Faro [a,*], Simona Pichini [b], Tivadar Farkas [c], Anastasio Tini [a], Antonina Mskhiladze [d], Francesco Paolo Busardò [a], Bezhan Chankvetadze [e]

[a] Department of Excellence-Biomedical Sciences and Public Health, Università Politecnica delle Marche, Ancona 60121, Italy
[b] National Centre on Addiction and Doping, Istituto Superiore di Sanità, Rome, Italy
[c] Phenomenex Inc., 411 Madrid Ave, Torrance, CA 90501, USA
[d] Faculty of Natural Sciences, Mathematics, Technologies and Pharmacy, Sokhumi State University, Polytkovskaya 61, Tbilisi 0186, Georgia
[e] Institute of Physical and Analytical Chemistry, School of Exact and Natural Sciences, Tbilisi State University, I. Chavchavadze Ave 1, Tbilisi 0179, Georgia

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ABSTRACT

Recently we published in this journal an enantioselective high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of 3,4-methylenedioxymethamphetamine (MDMA) and its major phase-1 metabolites, 4-hydroxy-3-methoxyamphetamine (HMA), 4-hydroxy-3-methoxy-methamphetamine (HMMA) and 3,4-methylenedioxyamphetamine (MDA) in human plasma, sweat, oral fluid and urine. Since we did not achieve simultaneous enantioseparation of all 4 compounds with a single chiral column, two amylose-based chiral columns were used alternatively. Further optimization of the mobile phase in the present study enabled baseline separation of all four pairs of enantiomers on a single Lux AMP column. In addition, by optimization of the column dimension and applied flow-rate it became possible to complete the separation within 6 minutes. These new methods were applied to the analysis of human plasma, oral fluid and urine. While results on the concentration of MDMA and its metabolites in various biological fluids were reported in our recent publication, in the present study an attempt was made to hydrolyze glucuronides in urine samples by using alternatively, hydrochloric acid or glucuronidase and to evaluate the effect of hydrolysis on the concentration and enantiomeric distribution of hydroxy metabolites of MDMA such as HMA and HMMA.

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA) is one of the widely used recreational synthetic drugs in recent decades. It is also known under the names Ecstasy or Molly on the illegal market. Perhaps the popularity of MDMA among drug abusers is caused by the fact that it combines both, hallucinogenic and stimulant effects. It is estimated that about 20 million people worldwide consumed this drug for recreational purpose in 2021[1]. According to a 2021 survey by the U.S. National Institute on Drug Abuse, 0.8% of the U.S. population over the age of 12 reported using Ecstasy in the previous 12 months [1]. MDMA alters mood and perception, producing feelings of increased energy, pleasure, emotional warmth, and distorted sensory and time perception. Although MDMA is considered as one of the safest recreational drugs, its use may come with side effects such as nausea, muscle cramping, involuntary teeth clenching, blurred vision, chills and sweating. Despite its undesirable effects mentioned above, MDMA has become associated with a therapy and healing in recent years. Since July 2023 Australia is the first country who registered MDMA as prescription medication [2] and its approval for the treatment of post-traumatic stress disorder is expected by U.S. Drug and Food Administration (FDA) in coming years. It is also expected that MDMA approval as a prescription medication will

* Corresponding author.
E-mail address: a.lofaro@staff.univpm.it (A.F. Lo Faro).

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facilitate its recreational use and that the number of its abusers will increase significantly. Thus, development of a fast bioanalytical method for the quantitative determination of MDMA and its metabolites in various biological matrixes is in high demand in clinical toxicology and forensic laboratories.

MDMA (Fig. 1) undergoes demethylenation leading to the formation of 4-hydroxy-3-methoxymethamphetamine (HMMA) and further N-demethylation by formation of 4-hydroxy-3-methoxyamphetamine (HMA), as well as N-demethylation by formation of MDA (Fig. 1). 3,4-Dihydroxyamphetamine (HHA) and 3,4-dihydroxymethamphetamine (HHMA) are metabolic intermediates [3].

MDMA is a chiral molecule with one asymmetric center and is available as a 1:1 (racemic) mixture of *S*-(+)- and *R*-(-)- enantiomers. Significant differences in the pharmacodynamic, pharmacokinetic and toxicokinetic properties of MDMA enantiomers are well known [4–10]. Therefore, for a proper description of pharmacodynamics, metabolism, pharmacokinetics and toxicokinetics of MDMA enantioselective analytical methods should be used. Recently we have proposed HPLC-MS/MS methods for the simultaneous separation of enantiomers of MDMA and its major phase-1 metabolites, MDA, HMA and HMMA and applied it to human biological samples such as plasma, oral fluid, urine and sweat [11]. The disadvantage of these methods was that two chiral columns were required to be used alternatively. In the present study we describe our attempts to simplify the previously described methods by using a single chiral column and to shorten analysis time. It is also worth mentioning that hydroxy metabolites of MDMA, such as HMA and HMMA undergo glucuronidation and are present mostly in the form of their glucuronides in urine. Therefore, additional emphases were made in the present study on the hydrolysis of glucuronides and evaluation of the change in concentration and enantiomeric distribution of HMA and HMMA following the hydrolysis of glucuronides.

2. Experimental part

2.1. Reagents and standards

MDMA, MDMA-d_5, MDA and MDA-d_5 standards were purchased from Cerilliant (Round Rock, TX, USA). HMA and HMMA standards were acquired from Lipomed (Basel, Switzerland) and Cayman Chemical (Ann Arbor, MI, USA), respectively. Standards were stored at −20° C until analysis. LC-MS grade water, methanol, acetonitrile, formic acid, chloroform and ethyl acetate were obtained from Carlo Erba (Cornaredo, Italy). Ammonium hydroxide (25% w/w aqueous solution) and hydrochloric acid (37% w/w aqueous solution) were purchased from Honeywell Fluka™ (Morristown, NJ, USA). Lux AMP chiral columns with proprietary chiral selector were supplied from Phenomenex Inc. (Torrance, CA, USA). The columns had the dimensions 250 ×4.6 mm and 150 ×4.6 mm and both of them were packed with a chiral stationary phase made of a polysaccharide-based chiral selector coated onto a high-pH stable silica support with 3 micrometers nominal particle size and 100 nm nominal pore size.

2.2. Instrumentation

Separations of MDMA and its metabolites were performed with a HPLC 1290 Infinity II (Agilent Technologies Italia S.p.a. Milan, Italy) system coupled to a mass spectrometer (6470 A Triple Quadrupole LC-MS) equipped with an electrospray ionization source (ESI) operated in positive ion mode. Data were acquired with MassHunter® Workstation Quantitative Analysis 10.0 Software (Agilent). The optimization process was conducted automatically with the "Mass Hunter Optimizer" tool provided by Agilent and manually confirmed. Autosampler and column oven temperatures were maintained at 10° C and 20° C, respectively.

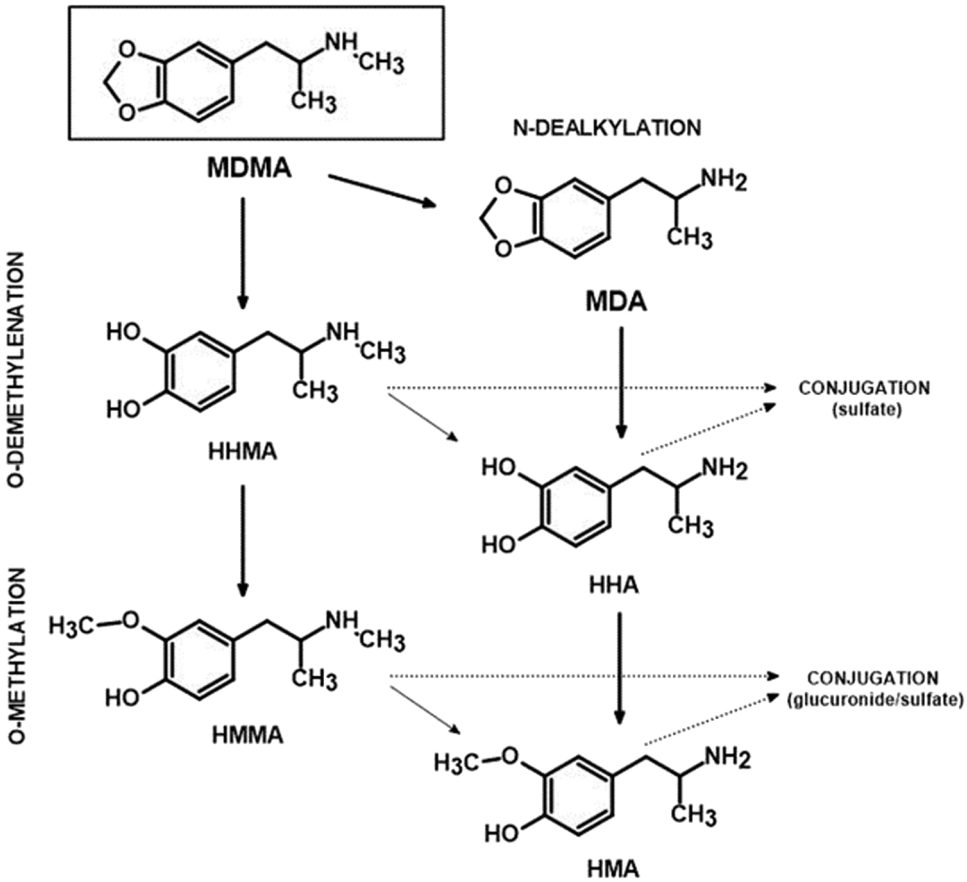

Fig. 1. Structure of MDMA and its major phase-1 metabolites.

The mass spectrometer was operated in scheduled multiple reaction monitoring (MRM) mode, with one transition for HMMA-glucuronide and HMA-glucuronide (HMMA-glu and HMA-glu, respectively), two transitions for MDMA, MDA, HMMA, MDMA-D_5 and MDA-D_5 each and three transitions for HMA (Table 1). Dwell time was set to 0.023 s. ESI conditions were optimized as follows: capillary voltage 3500 V, source temperature 300° C, cone gas flow rate 10 L/min, and desolvation gas flow rate 12 L/min.

Human Samples (plasma, oral fluid and urine) used in the present work originated from a previous clinical study [11] and therefore are not described in detail here. The sample preparation and method validation protocols were the same as described in Ref. [11]. In the previous study we did not attempt the hydrolysis of glucuronides, therefore this procedure is shortly described below: a) To 100 μL urine 10 or 20 μL of concentrated hydrochloric acid (37%) was added and the mixture was incubated at 100° C for 60 minutes. Alternatively, glucuronidase was used to hydrolyze glucuronides according to the following procedure: b) To 100 μL urine 10 μL of an aqueous solution of β-glucuronidase (100, 000–200,000 units/ml) from limpets (Patella vulgata), (Sigma Aldrich, Milan, Italy) was added and incubated at 50° C for 120 minutes before extraction. After hydrolysis of the glucuronides completed, the urine samples were extracted as described in Ref. [11].

3. Results and discussion

3.1. Enantioselective method development and optimization

Firstly, an attempt was made to separate HMA enantiomers on the Lux AMP column that had failed in the previous study [11]. This was achieved by replacing acetonitrile with methanol in combination with 5 mM ammonium bicarbonate buffer of pH 11.0 as the mobile phase (data not shown). Afterwards, the ratio of methanol and ammonium bicarbonate buffer was further optimized in order to achieve the simultaneous separation of enantiomers of all 4 compounds of interest (MDMA, HMA, HMMA and MDA). Acceptable separation was achieved in the mobile phase containing 75% methanol and 25% (v/v) 5 mM ammonium bicarbonate buffer pH 11.0 (Fig. 2a). Under these conditions seven out of eight possible peaks were observed with one instance of coelution. However, as it can be seen from the extracted ion chromatograms (EICs), the enantiomers of each compound were baseline separated from each other. As it can be seen in Fig. 2a, the later eluting enantiomers of HMA and HMMA partially coeluted while the later eluting MDA enantiomer completely coeluted with the later eluting MDMA enantiomer. None of these instances of coelution represents a serious problem for the selective detection of any of these enantiomers in MRM mode given their different *m/z* values. In order to shorten the analysis time, the column with 150 mm length was used instead the column with 250 mm length. Additionally, the mobile phase flow-rate was increased from 1.0 ml/min to 1.5 ml/min. This adjustment of the method enabled to obtain the separation shown in Fig. 2b with the analysis time under 6 minutes. Only 5 peaks were detected as can be seen on this figure. However, as extracted ion chromatograms show, the enantiomers of HMA, HMMA and MDMA were baseline separated while the enantiomers of MDA were close to baseline separated under these conditions. Thus, even this method with the run time under 6 minutes can be used for the enantioselective analysis of MDMA and its major phase-1 metabolites (HMA, HMMA and MDA) in biological fluids. Both of these new methods were applied to human plasma, oral fluid and urine samples. While the results in plasma and oral fluid were discussed in more detail in ref. [11] the major focus below is on urine samples.

Table 1
Mass spectrometry parameters for analytes and internal standards in the positive ionization mode.

Analytes	Molecular mass, g/mol	Precursor ion, *m/z*	Product ion, *m/z*	CE, eV
MDMA	193.2	194.2	163.1	9
			105	25
MDA	179.2	180.2	105.1	25
			77.1	45
HMA	181.2	182.2	165.1	8
			137.1	20
			105.1	24
HMMA	195.2	196.2	165	118
			105.1	118
HMMA-glu	371.1	372.1	196.2	45
HMA-glu	357.2	358.2	182.2	24
MDMA-d_5	198.2	199.2	165.1	13
			107.1	29
MDA-d_5	184.1	185.1	168.1	8
			110.1	24

3.2. Analysis of human urine samples

As it was described in our earlier study [11] cumulative urine samples taken from volunteers after oral intake of 100 mg MDMA tablets were analysed. An example chromatogram generated on a sample taken between 12 and 24 hrs. after MDMA administration is shown in Fig. 3. Interestingly, while one of the metabolites of MDMA, namely HMA was detected only in trace concentrations in plasma, it could be easily detected in the urine of the same volunteer, at the same time interval. The concentration change of *R*(-)-MDMA and *S*-(+)-MDMA in cumulative urine is shown in Fig. 4. As it was stated earlier in several studies and confirmed in our recent study [11] *S*-(+)-MDMA metabolizes at a higher rate compared with *R*-(-)-MDMA. Therefore, the amount of unchanged *S*-(+)-MDMA excreted in urine was as expected lower compared to the amount of unchanged *R*-(-)-MDMA.

One of the reasons for the very low concentration of HMA in plasma could be its fast glucuronidation and excretion in urine. Charged phase-2 metabolites such as glucuronides and sulfates are not likely to be retained on polysaccharide-based chiral columns and therefore cannot be detected with the method proposed here. In order to evaluate the extent of formation of hydroxylated metabolites of MDMA, as well as their further glucuronidation an attempt was made to hydrolyze the glucuronides of HMA and HMMA. Initially, concentrated hydrochloric acid (37%, w/w) was used as catalyst for hydrolysis at two different ratios to urine. The extend of hydrolysis was characterized by the ratio of responses of each enantiomer of metabolites to that of the deuterated internal standard MDMA-d_5 before and after the hydrolysis. The results are summarized in Table 2. As it can be seen from this tabulated data, 10 μl concentrated hydrochloric acid per 100 μl of urine at 100 °C in 60 minutes catalyzes hydrolysis of glucuronides of both, HMA and HMMA sufficiently. The same ratio of glucuronidase solution used in this study was also sufficient for the hydrolysis of glucuronides of both metabolites. Perhaps 20 μl concentrated hydrochloric acid per 100 μl of urine at 100 °C in 60 minutes causes degradation of glucuronides and its use should be avoided. The concentration of HMA in urine increased ca. 5–7 times after the hydrolysis of its glucuronide (Fig. 5a) while the concentration of HMMA increased ca. 13–15 times (Fig. 5b). Although the overall concentration of HMA and HMMA in urine increased significantly after the hydrolysis of glucuronides the ratio of their enantiomers did not change significantly.

4. Conclusions

In this study two HPLC-MS/MS methods were developed for the simultaneous separation of enantiomers of the chiral recreational drug 3,4-methylenedioxymethamphetamine (MDMA) and its major phase-1 metabolites, 4-hydroxy-3-methoxyamphetamine (HMA), 4-hydroxy-3-methoxymethamphetamine (HMMA) and 3,4-methylenedioxyamphetamine (MDA). Both methods with total chromatographic run times of 20 and 6 minutes, respectively, enabled enantioselective quantification

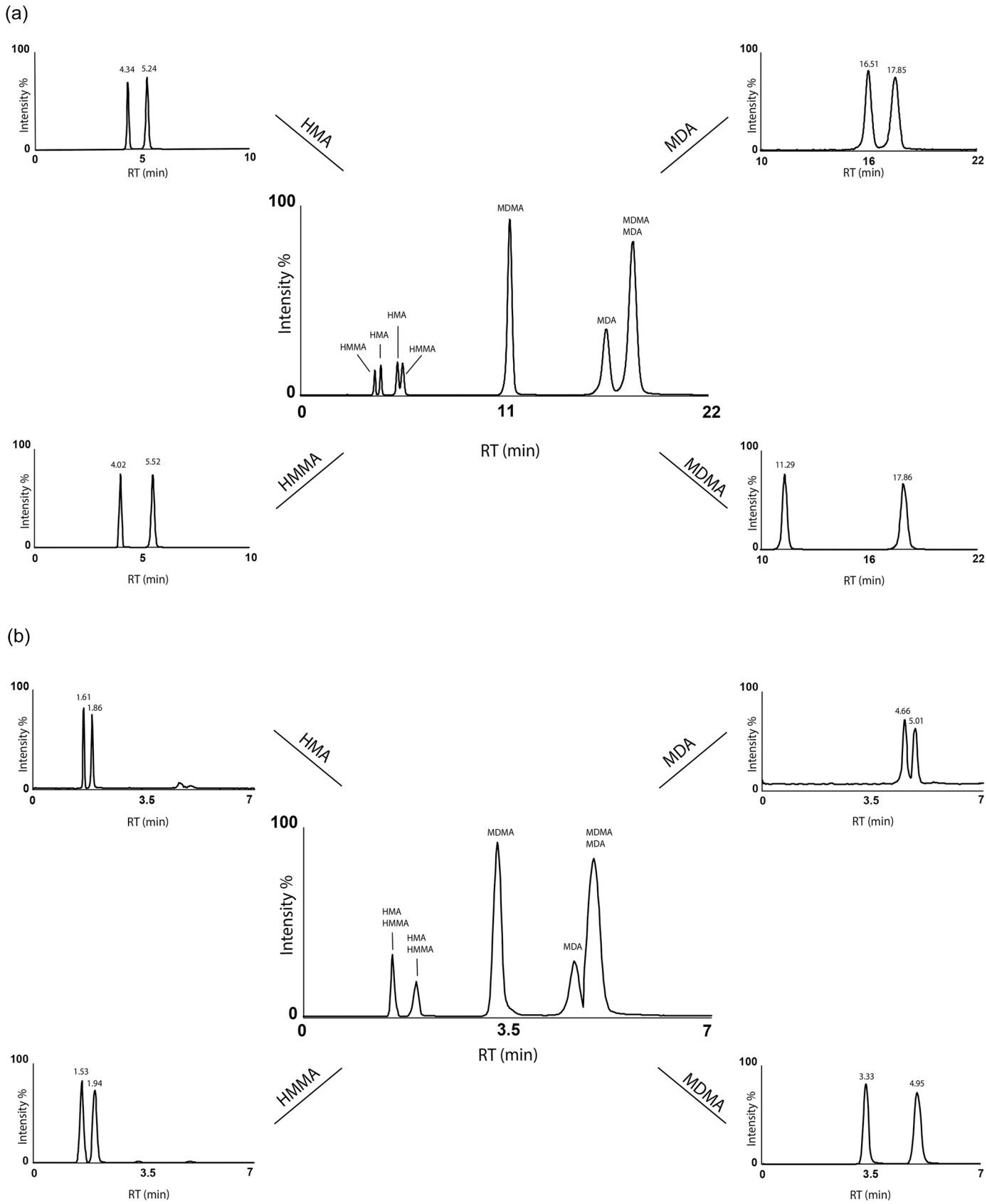

Fig. 2. Simultaneous separation of MDMA, HMA, HMMA and MDA on Lux AMP columns of 25 cm (a) and 15 cm (b) length with the mobile phase containing 75% methanol-25% (v/v) 5 mM ammonium bicarbonate in water pH 11.0. Flow rate: 1 ml/min (a) and 1.5 ml/min (b). Figures include total ion current, as well as EICs of each compound.

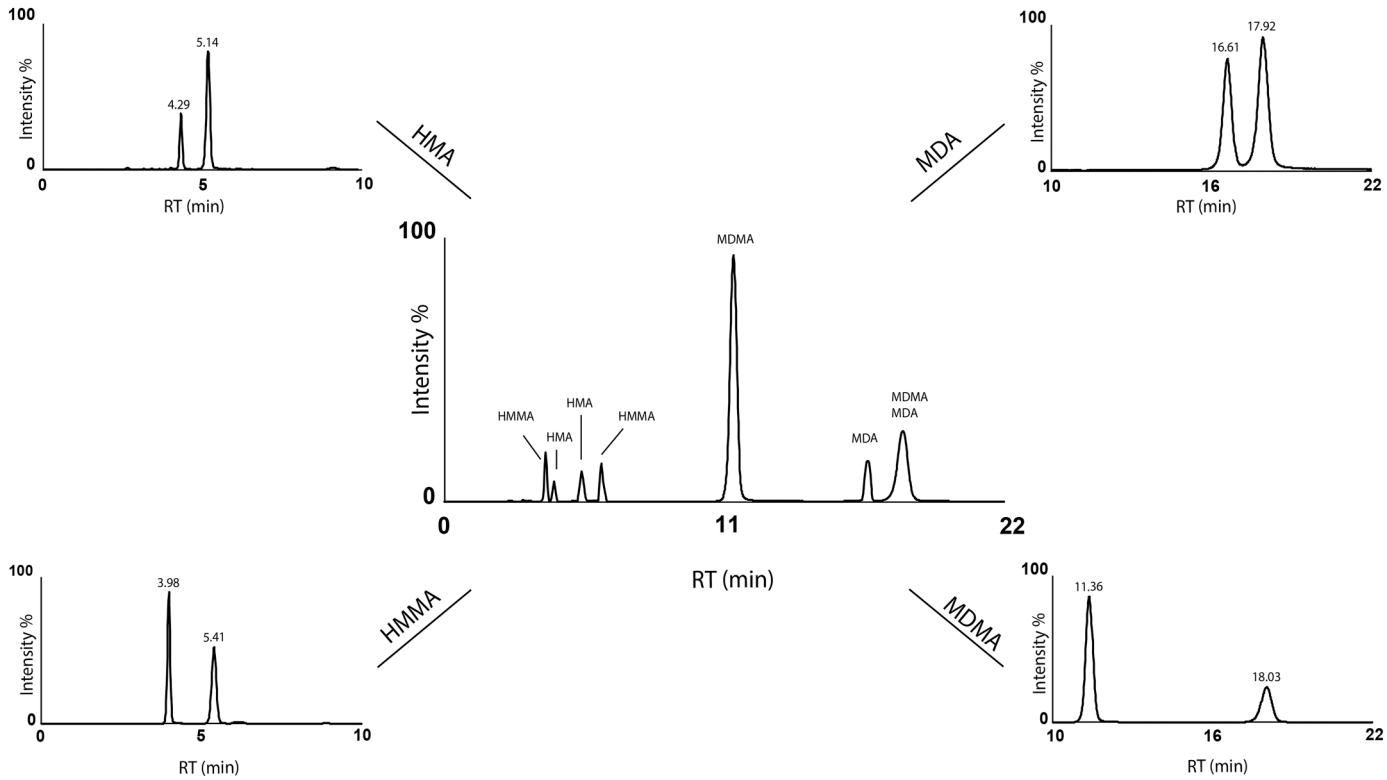

Fig. 3. HPLC-MS/MS chromatogram of a cumulative (12–24 hrs.) urine sample of a volunteer who received 100 mg MDMA orally. The analytical conditions were as described in Fig. 2a and MS/MS detection conditions were as described in Table 1.

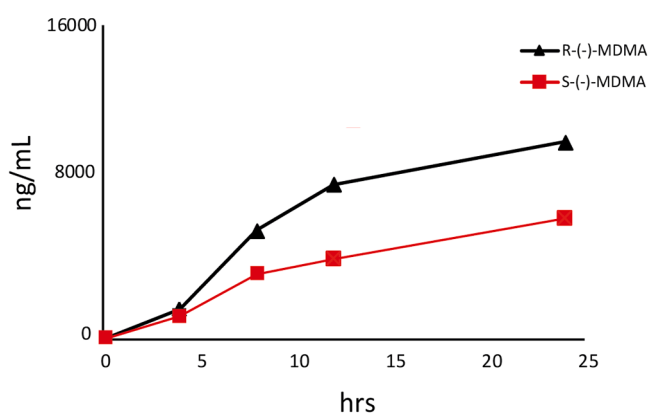

Fig. 4. The concentration change of *R*-(-)-MDMA and *S*-(+)-MDMA in cumulative urine. The separation and detection conditions were as described on Fig. 2a and Table 1, accordingly.

Table 2
Hydrolysis of glucuronides.

Exp.	Catalyst	Ratio of the responses HMA/MDMA-d_5		Ratio of the responses HMMA/MDMA-d_5	
		1st peak	2nd peak	1st peak	2nd peak
1	Before hydrolysis	0.063	0.190	0.94	0.750
2	10 ml HCl	0.290	0.780	14.300	11.900
3	20 ml HCl	0.020	0.090	2.700	3.100
4	10 ml glucuronidase	0.370	1.500	8.900	9.100

of the compounds of interest in the biological samples part of a clinical study. The quantification results in urine samples are reported. It was shown that the hydroxy metabolites of MDMA, such as HMA and HMMA

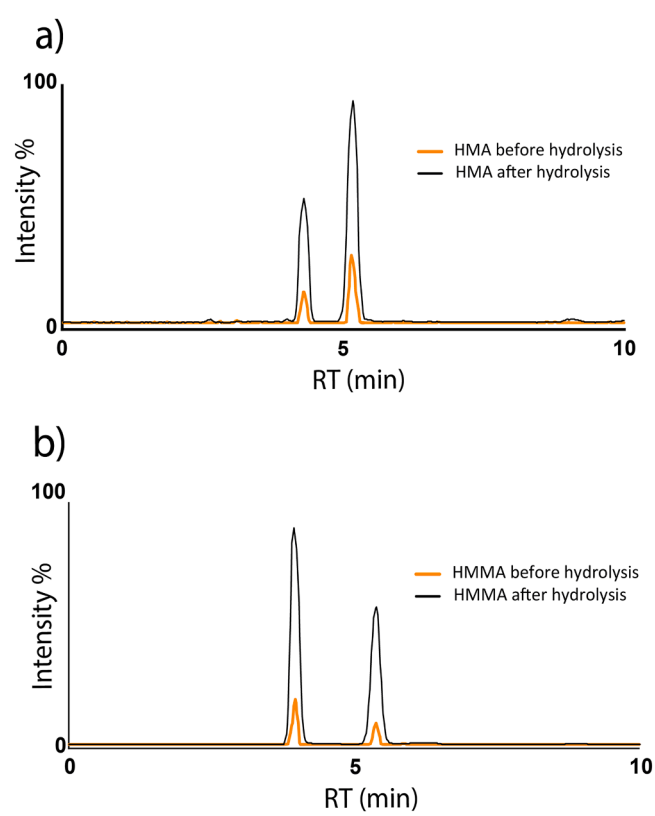

Fig. 5. The EICs of HMA (a) and HMMA (b) before and after hydrolysis of glucuronides in the urine of the volunteer who received 100 mg MDMA. Hydrolysis was performed with 10 microliter concentrated hydrochloric acid per 100 microliter of urine at 100 °C for 60 minutes.

undergo glucuronidation to a significant extent. The glucuronides of the both metabolites can be adequately hydrolyzed either with a properly selected amount of concentrated hydrochloric acid or alternatively, enzymatically with glucuronidase. Although the overall concentration of both metabolites significantly increases following hydrolysis, the enantiomeric ratio remains mostly unaffected.

CRediT authorship contribution statement

Giorgia Sprega: Investigation, Formal analysis. **Giorgi Kobidze**: Investigation, Formal analysis. **Alfredo Fabrizio Lo Faro**: Validation, Supervision, Methodology, Investigation, Formal analysis. **Tidivar Farkas**: Writing - review & editing, Resources. **Anastasio Tini**: Investigation, Formal analysis. **Antonina Mskhiladze**: Investigation, Formal analysis. **Francesco Paolo Busardò**: Writing - review & editing, Supervision, Resources, Investigation, Formal analysis, Conceptualization. **Bezhan Chankvetadze**: Writing - review & editing, Writing - original draft, Supervision, Resources, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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