Journal of Pharmaceutical and Biomedical Analysis 238 (2024) 115768

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Development 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

Alfredo Fabrizio Lo Faro [a], Giorgia Sprega [a], Diletta Beradinelli [a], Anstasio Tini [a], Lourdes Poyatos [b], Esther Papaseit [b], Paolo Berretta [c], Alessandro Di Giorgi [c], Magì Farre [b], Nino Takaishvili [d], Tivadar Farkas [e], Francesco Paolo Busardò [a,*], Bezhan Chankvetadze [d]

[a] Department of Excellence-Biomedical Sciences and Public Health, Università Politecnica delle Marche, 60121 Ancona, Italy
[b] Servei de Farmacologia Clínica, Hospital Universitari Germans Trias i Pujol (HUGTiP, IGTP)- Universitat Aut`onoma de Barcelona, Unitat Docent HUGTiP, Badalona, Spain
[c] National Centre on Addiction and Doping, Istituto Superiore di Sanit`a, Rome, Italy
[d] Tbilisi State University, Institute of Physical and Analytical Chemistry, School of Exact and Natural Sciences, 0179 Tbilisi, Georgia
[e] Phenomenex Inc., 411 Madrid Ave., Torrance 90501, CA, USA

ARTICLE INFO

Keywords:
Chiral HPLC
Enantioselective metabolism
Enantioseparation, MDMA and metabolites

ABSTRACT

In the present study enantioselective high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were developed for the quantitative determination of 3,4-methylenedioxy-methamphetamine (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 (OF) and urine. The simultaneous separation of all these compounds and their respective enantioseparation was accomplished on two polysaccharide-based chiral columns. The Lux AMP column with a proprietary chiral selector enabled baseline separation of the enantiomers of MDMA, HMA and HMMA while MDA enantiomers could not be separated with this column under the experimental conditions used in this study. The Lux i-Amylose-3 column based on amylose tris(5-chloro-3-methylphenylcarbamate) as chiral selector baseline-separated the enantiomers of MDMA, HMMA and MDA while the enantiomers of HMA could not be separated. Thus, the various samples were analyzed by using both columns alternatively in combinations with acetonitrile containing 25% (v/v) 5 mM ammonium bicarbonate buffer at pH 11.0 as mobile phase. Analysis time was less than 4 min with the Lux AMP column and less than 6 min with the Lux i-Amylose-3 column. Both methods were validated and applied to the enantioselective determination of MDMA and its phase-I metabolites in human biological fluids, and enantioselective metabolism of MDMA was confirmed.

1. Introduction

The synthesis of 3,4-methylenedioxy-methamphetamine (MDMA) was first described in 1912 [1]. The use of MDMA as recreational drug of abuse started in the 1970 s after 3,4-methylenedioxyampethamine (MDA) was classified as a schedule one drug under the Comprehensive Drug Abuse and Prevention and Control Act [2]. In humans MDMA promotes positive mood changes, enhances communication and intimacy, increases self-esteem, alertness and endurance, as well as induces a sense of euphoria [3–6]. On the other hand, MDMA administration has also been reported to cause tachycardia, an occasional 'wired' feeling, jaw clenching, nystagmus, a nervous desire to be in motion, transient anorexia, panic attacks, nausea and vomiting, ataxia, urinary urgency, diplopia, insomnia, tremors, inhibition of ejaculation, and rarely, transient hallucinations [1]. Many severe or even fatal intoxications have been reported [3,4,6]. Concerning chronic toxicity, data from animal experiments strongly suggest that intake of MDMA can cause irreversible damage to serotoninergic nerve terminals in the

* Corresponding author.
E-mail address: f.p.busardo@univpm.it (F.P. Busardò).

https://doi.org/10.1016/j.jpba.2023.115768
Received 11 August 2023; Received in revised form 3 October 2023; Accepted 3 October 2023
Available online 5 October 2023
0731-7085/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

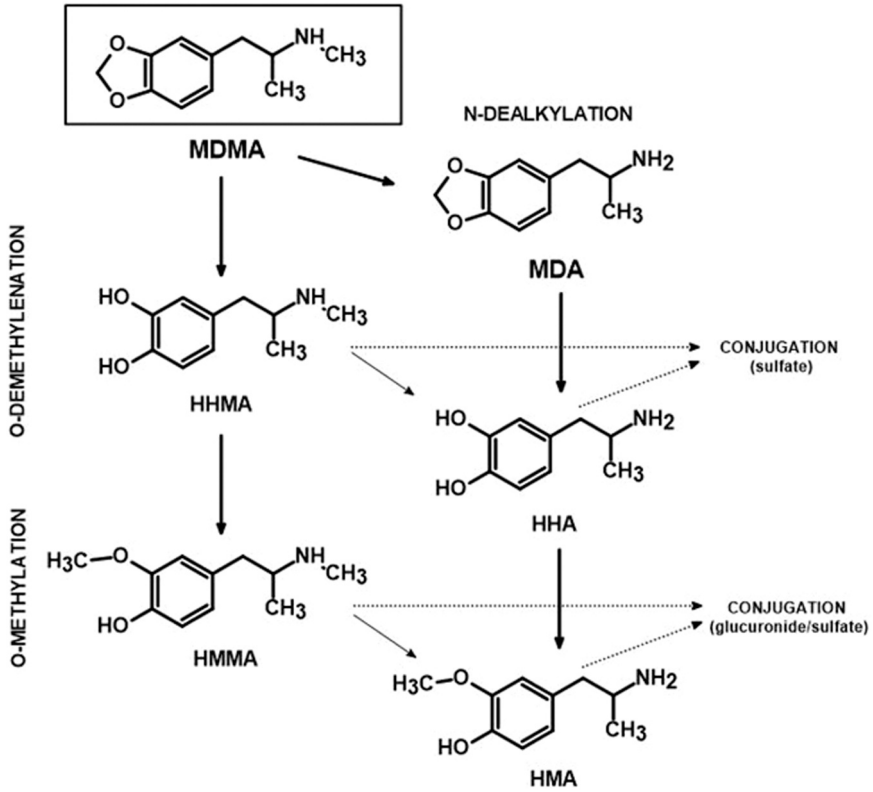

Fig. 1. Phase-1 metabolites of 3,4-methylenedioxymethamphetamine (MDMA).

central nervous system [3–6].

Well characterized metabolites of MDMA include MDA, 4-hydroxy-3-methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA) (Fig. 1). 3,4-Dihydroxyamphetamine (HHA) and 3,4-dihydroxymethamphetamine (HHMA) are metabolic intermediates [7].

MDMA contains an asymmetric carbon and exists (and is commonly used) as a racemic mixture. The S-(+)- isomer of MDMA has been reported to be a more potent neurotoxin than the R-(-)-isomer [8,9]. Also, other pharmacological, toxic, and toxicokinetic properties of MDMA enantiomers are known to differ considerably [4,6,8–12]. Whereas S-(+)-MDMA has been described as the more potent stimulant, R-(-)-MDMA seems to exhibit more mescaline-like effects [4]. Furthermore, elimination of the S-(+) enantiomer is faster than elimination of the R-(-) enantiomer [4,6,9–12].

The report on enantioselective formation of MDA due to metabolism of MDMA was published in 1989 [2], while the first quantitation and differential distribution of the enantiomers of MDMA and its MDA metabolite in a fatal poisoning following insufflation of MDMA, cocaine and heroin was reported in 1996 [1]. Between 1989 and 2009 mostly GC-MS methods were reported for the enantioselective analysis of MDMA and some of its metabolites. GC analysis of the compounds under this study in their intact form is problematic due to their low volatility. Thus, some form of derivatization is required in order to transform the native compounds into derivatives which are suitable for GC analysis. If derivatization is performed with an enantiomerically pure chiral derivatizing reagent, then the enantiomers get converted to diastereomers which can be separated on (less expensive) achiral columns. With the same derivatization one may also improve the detection sensitivity of the applied detector (mostly MS). Such indirect enantioselective methods have potential disadvantages such as possible racemization during the derivatization step, interference with analytes (i.e. coelution and potentially ion-suppression) from the excess of derivatizing reagent or shorter column lifetime. Furthermore, any enantiomeric impurity present in the derivatizing reagent may affect analytical performance, specifically the limit of detection and quantification of a minor analyte enantiomer. In 2016 Ward and co-authors compared GC-MS and HPLC-MS methods for the enantioselective determination of methamphetamine and showed preferential bias of a GC method for producing error rates in the range of 8–19%. The HPLC–MS method produced less than 2% deviation errors. Additionally, the GC method failed to return 100% D- or L-isomer purity results for enantiomerically pure standards. A higher rate of D- and L-methamphetamine isomer racemization was observed in samples analyzed by GC–MS using N-trifluoroacetyl-L-prolyl chloride as derivatizing agent. Such racemization was not experienced when the samples were tested by HPLC-MS. Thus, the authors concluded that a more accurate method of enantioselective analysis was provided by direct HPLC–MS [12].

Simultaneous separation and enantioseparation of MDMA, HMMA and MDA without derivatization has been reported by Thormann and co-workers using capillary electrophoresis (CE) [13]. Later, de la Torre and co-workers used quite similar CE method to the one reported in ref. 15 and followed the pharmacokinetics of MDMA and some of its major metabolites [14,15]. In addition to its miniaturized format and high efficiency, CE offers an obvious advantage over GC since it does not require analyte volatility (often achieved by derivatization). However, CE-based methods developed with randomly substituted cyclodextrin derivatives (as reported in references 13–15) may suffer from poor reproducibility. Also, in case of using UV detection, method sensitivity, may be limited, especially for minor metabolites.

Since 2008 HPLC-based separation methods for the enantiomers of MDMA and its metabolites were reported [16–22]. Maurer and co-workers used a cyclodextrin-based chiral HPLC column for the fractionation of MDMA enantiomers. The obtained R-(-)-MDMA and S-(+)-MDMA were used for in vitro metabolic studies and enantioselectivity was observed for N-demethylation and demethylenation by CYP2C19 with a preference for the S-enantiomer. In addition, CYP2D6 showed preference also for S-MDMA in the case of demethylenation.

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
			105.0	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	165.0	118
			105.1	118
HMMA-glu	371.1	372.1	196.2	45
HMA-glu	357.2	358.2	182.2	24
MDMA-d5	198.2	199.2	165.1	13
			107.1	29
MDA-d5	184.1	185.1	168.1	8
			110.1	24

Abbreviations: MDMA, 3,4-methylenedioxy-methamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; HMA, 4-hydroxy-3-methoxyamphetamine; MDA, 3,4-methylenedioxyamphetamine; HMMA-glu, 4-hydroxy-3-methoxymethamphetamine-glucoronate; HMA-glu, 4-hydroxy-3-methoxyamphetamine-glucoronate, CE, collision energy.

Analyses were performed by achiral GC-MS [16]. Later the same group stated that "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" [17]. The authors proposed a combination of 3 different methods [17]. Since that time several LC-based methods were reported for the separation of MDMA enantiomers for micro preparative purpose [18–20], in spiked urine [21], reference standard [22] or seized street drug samples [23]. In addition, indirect HPLC methods based on sample derivatization with a chiral derivatizing reagents have been also described [24,25]. However, at least to the best of our knowledge no direct HPLC-MS method for the simultaneous separation of enantiomers of MDMA and its major phase-1 metabolites has been reported in the literature to date. Therefore, the goal of the present study was to develop a direct HPLC-MS/MS method for the simultaneous separation and enantioseparation of MDMA and its major phase-1 metabolites and to apply this method to human biological fluids such as plasma, urine, oral fluid (OF) and sweat.

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), Ammonium bicarbonate (98.5% purity) and hydrochloric acid (37% w/w aqueous solution) were purchased from Honeywell Fluka™ (Morristown, NJ, USA).

2.2. Chiral columns

Nine polysaccharide-based chiral columns incorporating the following chiral selectors cellulose tris(3,5-dimethylphenylcarbamate) (Lux Cellulose-1), cellulose tris(3-chloro-4-methylphenylcarbamate) (Lux Cellulose-2), cellulose tris(4-methylbenzoate) (Lux Cellulose-3), cellulose tris(4-chloro-3-methylphenylcarbamate) (Lux Cellulose-4),

Table 2
Method validation parameters and Mandel's fitting test (p-value and Fcrit95%) for analytes under investigation in OF, plasma, urine and sweat samples.

		Linear range ng/mL	r^2	p-value	$F_{crit95\%}$	LOD ng/mL	LOQ ng/mL	QC ng/mL			Accuracy (%)			Intra-day precision CV (%)			Inter-day precision CV (%)			Recovery (%)			Matrix effect (%)		
								L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
Plasma	MDMA	5–5000	0.9973	0.345	0.154	1	5	15	2000	4000	101	98	108	4.6	6.8	3.7	4.6	5.3	7.0	103	101	96	-5.6	-3.6	0.7
	HMMA	0.5–500	0.9982	0.698	0.289	0.1	0.5	1.5	200	400	123	93.9	99.5	2.9	5.9	6.2	2.3	5.9	3.7	106	99	98	-1.8	7.0	1.6
	MDA	0.5–500	0.9985	0.873	0.106	0.1	0.5	1.5	200	400	97	103	98.4	2.5	5.0	1.6	4.1	7.2	2.2	117	97	103	1.7	1.5	4.8
OF	MDMA	5–10000	0.9993	0.915	0.992	1	5	15	4000	8000	104	98	103	1.5	6.0	3.1	0.9	6.4	1.7	99	108	97	-3.9	6.8	5.2
	HMMA	0.5–1000	0.9899	0.425	1.652	0.1	0.5	1.5	400	800	95	103	105	3.8	4.5	2.9	4.3	2.4	6.1	102	101	101	0.4	4.5	-1.5
	MDA	0.5–1000	0.9985	0.143	0.981	0.1	0.5	1.5	400	800	97.9	99	97.3	2.9	4.2	7.1	6.4	3.1	4.8	97	97	95	3.2	0.9	6.5
Urine	MDMA	5–10000	0.9977	0.701	1.436	1	5	15	4000	8000	102	104	96.4	4.1	1.9	4.2	5.1	1.6	4.2	98	103	99	4.2	5.1	3.6
	HMMA	0.5–1000	0.9892	0.358	0.387	0.1	0.5	1.5	400	800	99.2	98	101	5.9	2.4	7.2	2.8	3.7	5.3	105	104	106	1.4	-4.4	2.8
	MDA	0.5–1000	0.9986	0.430	1.452	0.1	0.5	1.5	400	800	98.5	97	103	6.7	7.2	2.1	3.4	7.0	7.3	99	101	108	3.5	1.3	-6.8
Sweat	MDMA	0.05–500	0.9977	0.224	0.627	0.025	0.05	0.15	200	400	101	103	99.3	5.2	3.7	2.1	4.2	4	5.2	101	97	101	4.7	3.1	2.7
	HMMA	0.005–50	0.9899	0.463	0.198	0.003	0.005	0.015	20	40	94.8	97.4	98.8	0.7	2.7	5	1.7	2.8	2.0	98	97.9	96	0	-2.5	-0.5
	MDA	0.005–50	0.9985	0.478	1.032	0.003	0.005	0.01	20	40	108	94.8	107	1.3	2.1	2.9	3.3	7.1	3.8	103	99.3	101	-2.1	1.6	-6.0

Abbreviations: MDMA, 3,4-methylenedioxy-methamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; LOD, Limit of detection; LOQ, Limit of quantification; L, low quality control; M, medium quality control; H, high quality control; CV, coefficient of variation.

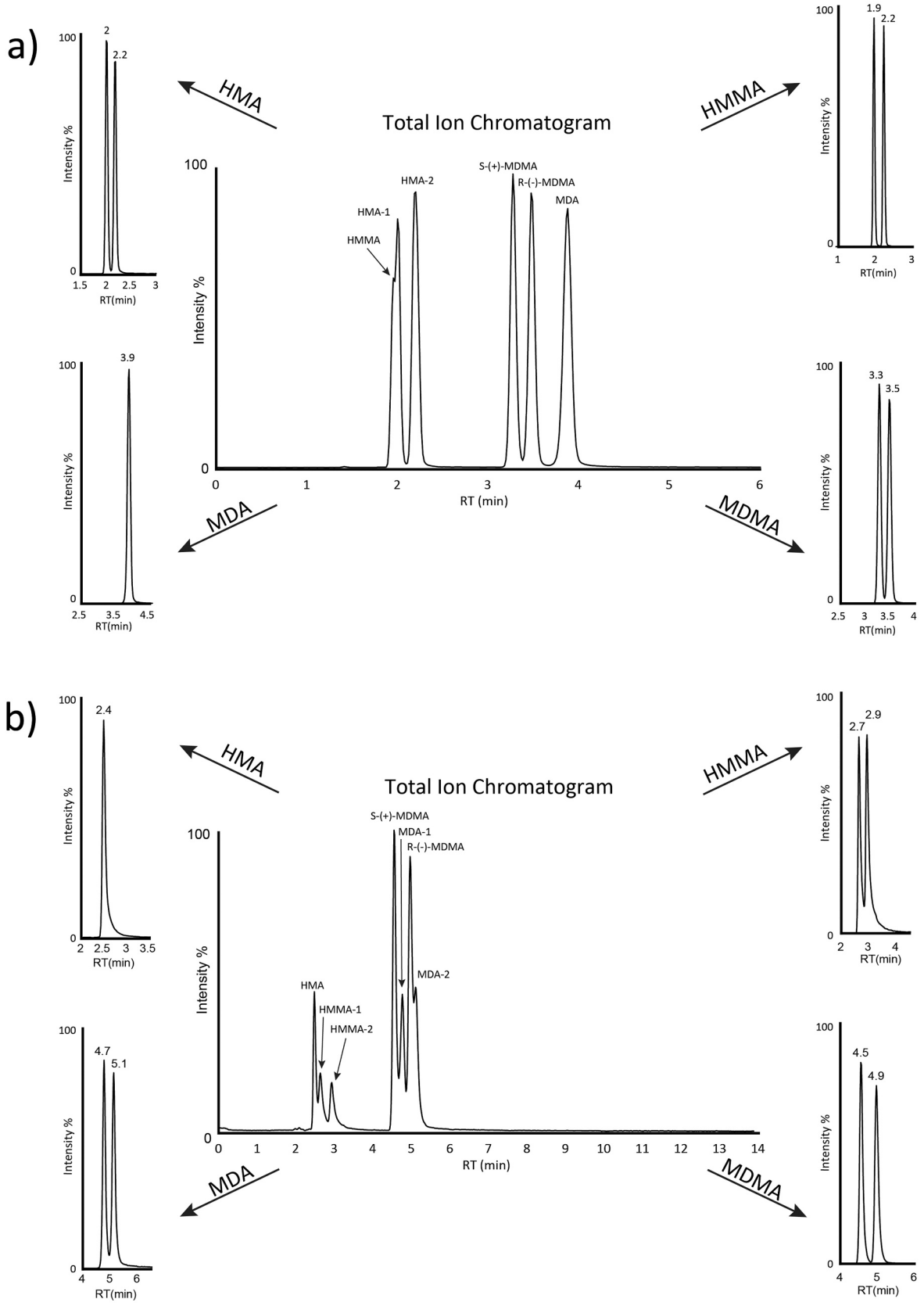

Fig. 2. Simultaneous enantioseparation of MDMA and its major phase-1 metabolites on Lux AMP (a) and Lux i-Amylose-3 columns (b). Mobile phase was acetonitrile with 25% (v/v) 5 mM ammonium bicarbonate buffer pH= 11.0 with the flow rate 1 mL/min. For MS conditions see Table 1.

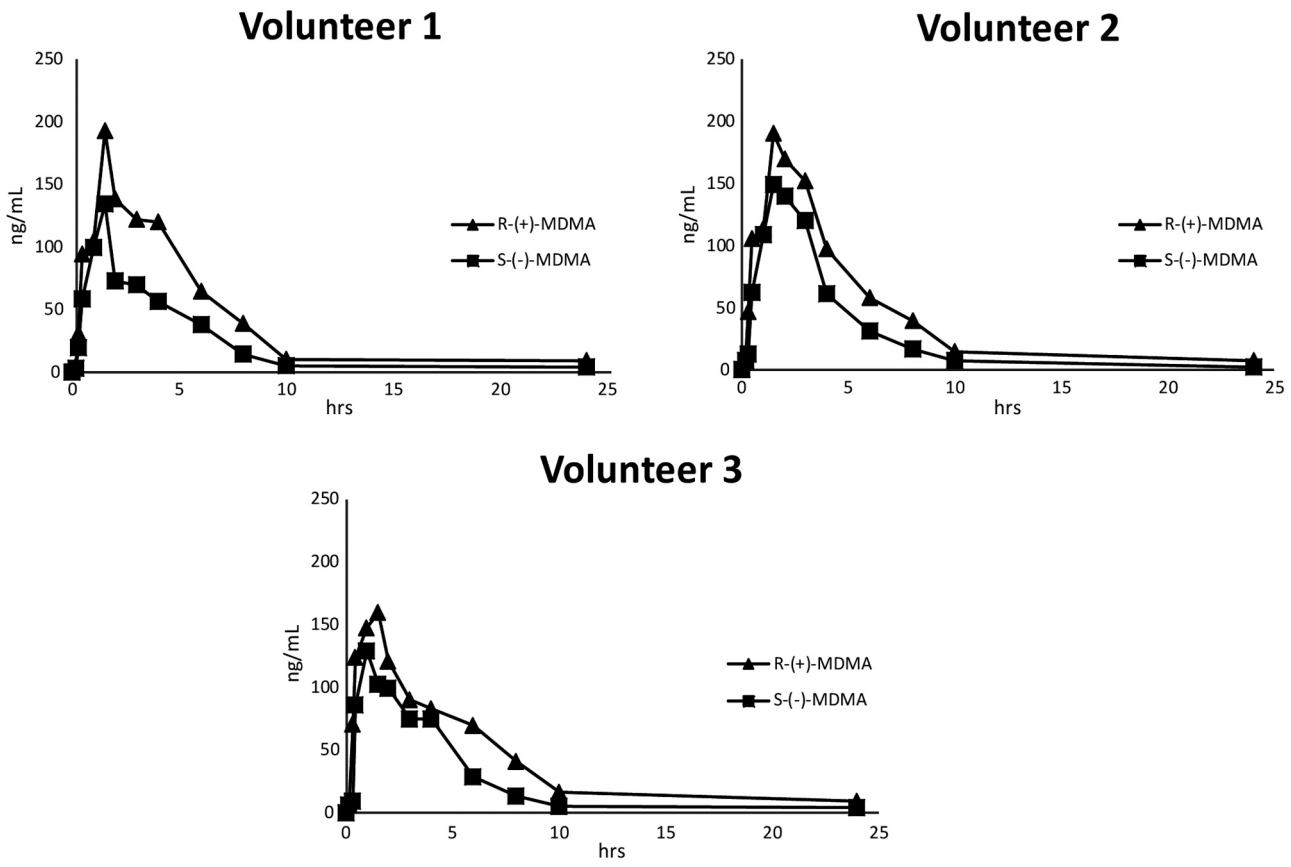

Fig. 3. C-t curves for MDMA in plasma for 3 volunteers after receiving 100 mg dose of racemic MDMA. The plasma samples were analyzed on Lux AMP column under the conditions mentioned in the legend to Fig. 2 and in Table 1.

cellulose tris(3,5-dichlorophenylcarbamate) (Lux i-Cellulose-5), amylose tris (3,5-dimethylphenylcarbamate) (Lux Amylose-1), amylose tris(5-chloro-2-methylphenylcarbamate) (Lux Amylose-2), amylose tris (3-chloro-5-methylphenylcarbamate) (Lux i-Amylose-3) and Lux AMP were used. All the columns of the Lux series were provided from Phenomenex Inc. (Torrance, CA, USA). The columns were of 250 × 4.6 mm dimension and packed with 5 μm particles except for the Lux AMP column which had 150 × 4.6 mm dimensions and was packed with 3 micrometer particles. Mobile phases consisting of methanol, aqueous methanol, acetonitrile and aqueous acetonitrile were tested to find suitable conditions for the enantioseparation of MDMA and its metabolites.

2.3. 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 "MassHunter Optimizer" tool provided by Agilent and manually confirmed. After an initial screening of all chiral columns mentioned in subsection 2.2 the simultaneous separation of MDMA, its metabolites and their enantiomers was performed on 2 chiral columns: Lux i-Amylose-3 and Lux AMP. Analysis time was less than 6 min on both columns with an isocratic mobile phase composed of acetonitrile containing 25% (v/v) 5 mM ammonium bicarbonate buffer pH 11.0 at a flow rate of 1.0 mL/min. Autosampler and column oven temperatures were maintained at 10 °C and 20 °C, respectively. The mass spectrometer operated in scheduled multiple reaction monitoring (MRM) mode, with one transition for HMMA-glucoronide and HMA-glucoronide (HMMA-glu and HMA-glu, respectively), two transitions for MDMA, MDA, HMMA, MDMA-D5 and MDA-D5 each and three transitions for HMA (Table 1). Scan speed (dwell time) was 0.023 s. ESI conditions were optimized as follows: capillary voltage 3500 V, source temperature 300 °C, cone gas nitrogen flow rate 10 L/min, desolvation gas (nitrogen) flow rate 12 L/min.

2.4. Human samples

A randomized, cross-over, placebo-controlled, double-blind study was conducted with 3 male volunteers (mean age 23 years old [range 22–24], mean weight 70.2 [range 60.4–87.0 kg]) at the Hospital Universitari German Trias i Pujol, Institut d'Investigatiò en Ciènces de la Salut Germans Trias i Pujol, in Badalona, Spain. All participants have recreational experience with common drugs of abuse (psychostimulants as cocaine, amphetamines, MDMA and synthetic cathinones). Prior to study sessions, the participants were submitted to a general medical examination including blood and urine chemistry. Measures of pharmacological effects were collected (not presented in this manuscript). The clinical trial was approved by the local human research ethics committee (CEI-HUGTiP ref. PI-19–082) to investigate the potential for abuse and human pharmacology of MDMA. The study was registered in ClinicalTrials.gov (number NCT05488171). It was conducted according to the Declaration of Helsinki recommendations and Spanish rules for clinical investigation. All participants were informed, both orally and in writing, about of the scope of the study and signed an informed consent prior inclusion.

Capsules contained 50 mg MDMA, 100 mg MDMA or placebo and participants received the same number of capsules per session (n = 3), in order to administer 100 mg MDMA and to blind the dose for participants

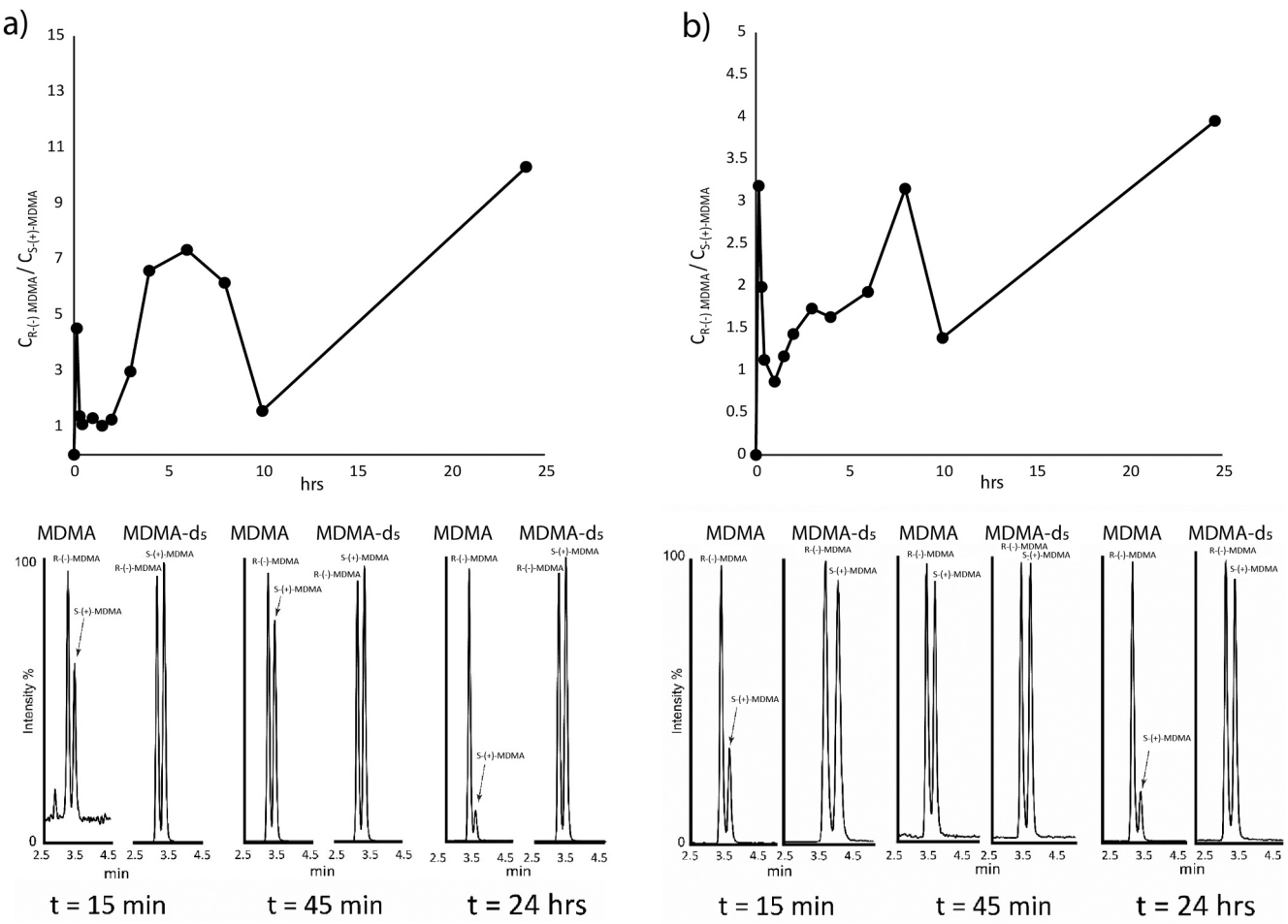

Fig. 4. Enantioselective MRM chromatograms of MDMA enantiomers after 15 and 45 min and 24 hrs. of oral administration of 100 mg racemic MDMA and concentration ratio of MDMA enantiomers within 15 min to 24 hrs time range in plasma (a) and oral fluid (b).

and clinical staff. Biological samples were collected in plastic tubes before and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10 and 24 h after dosing and immediately stored at − 20 °C until the analysis.

2.5. Sample preparation

2.5.1. Plasma, oral fluid and urine

10 μL of the 100 ng/mL internal standard (MDMA-d_5 and MDA-d_5) and 2 μL 2% NH_3 in H_2O (pH 9), and 2 mL chloroform: ethyl acetate 9:1 (v/v) mixture were added to 100 μL biological sample such as plasma, OF or urine. Sample tubes were stirred on a roller mixer for 10 min and centrifuged at 3500 rpm for 5 min. Organic phase was transferred into clean tubes, 100 μL acidic methanol (1% HCl) was added to prevent evaporative losses, and samples were dried under nitrogen for approximately 30 min. Samples were reconstituted in 100 μL mobile phase (acetonitrile containing 25% (v/v) 5 mM ammonium bicarbonate buffer at pH 11.0) and transferred into autosampler vials prior to injection of 2 μL into the chromatographic system.

2.5.2. Sweat

Patches were fortified with 50 μL of the 200 ng/mL internal standard (MDMA-d_5 and MDA-d_5) and left to dry for 30 min at room temperature. 2 mL of methanol were then added to the shredded patches and, after 1 h at room temperature, 100 μL of the solution were injected into the chromatographic system.

2.6. Method development and validation

The forensic toxicology international guidelines [26] were followed to validate the experimental procedures used in this study. The sample preparation methods described in previously published articles [27–30] were used to extract the analytes of interest from biological matrices. MDMA and its metabolites' calibration curves were linear with coefficients of determination greater than 0.99 regardless of the matrix used in sample preparation. The covered range is shown in Table 2 and the best fit calibration model was a linear least squares regression with $1/x^2$ weighting, as confirmed by Mandel test coefficient [31]. The LOD ranged from 0.1 to 1 ng/mL for plasma, OF and urine and 0.003–0.025 ng/mL for sweat samples. For plasma, OF and urine the LOQ ranged from 0.5 to 5.0 ng/mL while for sweat samples the LOQ ranged from 0.005 to 0.05 ng/mL (Table 2). The analytical recovery of MDMA was determined using three quality control samples (15 ng/mL, 2000 ng/mL, and 4000 ng/mL for plasma, 15 ng/mL, 4000 ng/mL, and 8000 ng/mL for OF and urine and 0.15 ng/mL, 200 ng/mL, and 400 ng/mL for sweat) [32] and ranged from 95% to 117%, while matrix effects ranged from − 6.8–7%. Recovery and matrix effect results for metabolites are shown in Table 2. The dilution controls fell within the acceptable range (± 15%) for method validation, indicating that the assay was reliable. Analytes stability tests in all biological matrices were performed using 4 replicates at QC1, QC2 and QC3 concentrations. stored at room temperature and − 20 °C. Freezing and thawing stability, short term (0, 1, 2, 4, 24 and 48) and medium term (1 month) stability and stability in the reconstitution solvent for 24 h after extraction at + 10 °C were evaluated and the observed concentrations were all within

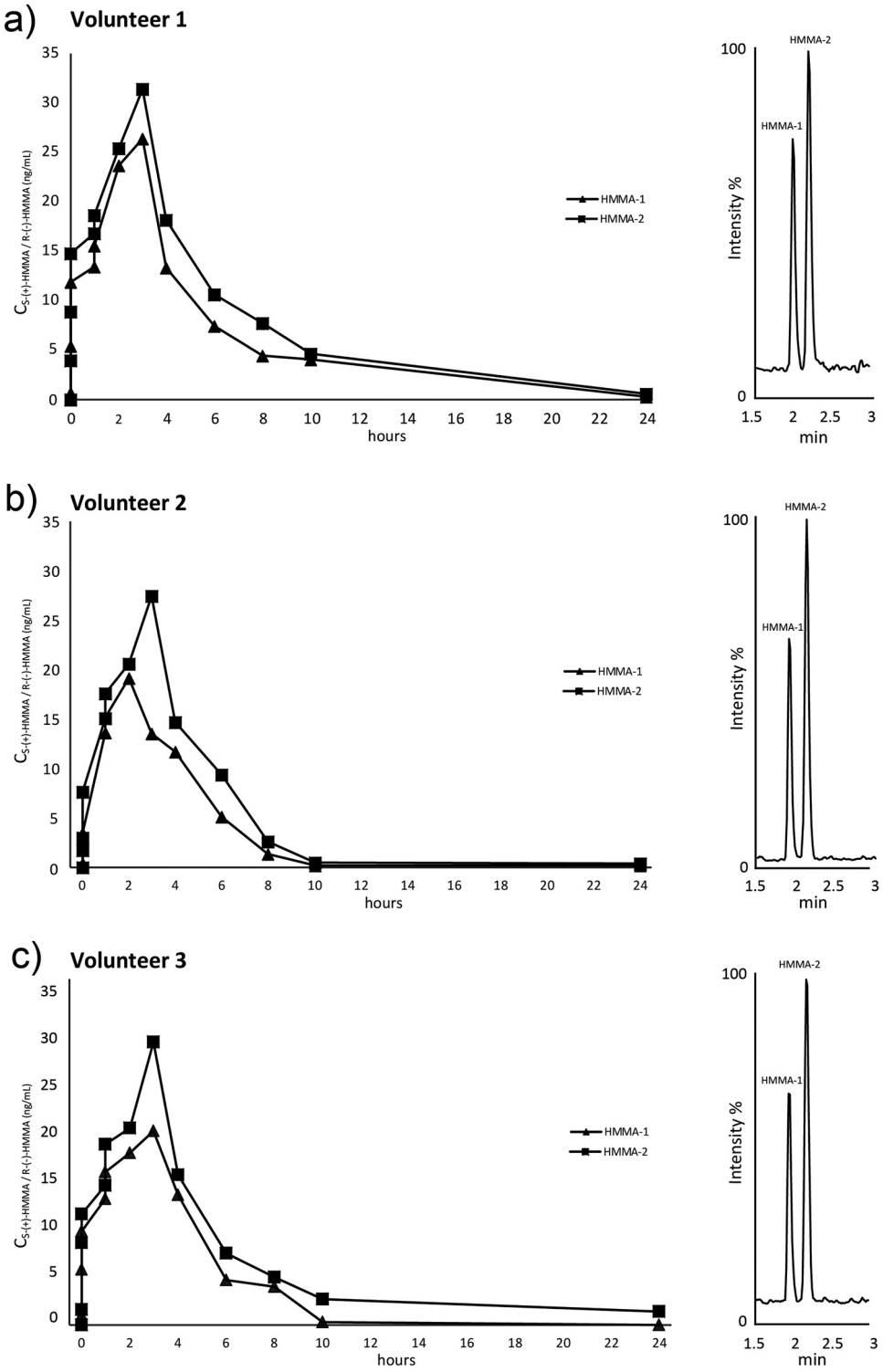

Fig. 5. C-t curves for HMMA in plasma for 3 volunteers after receiving 100 mg dose of racemic MDMA and MRM chromatograms for HMMA after 15 min of MDMA administration. The plasma samples were analyzed on Lux AMP column under the conditions mentioned in the legend to Fig. 2 and in Table 1.

± 20% of the target concentration. Moreover, in this study, there were no interfering peaks in any of the biological matrices analyzed.

3. Results and discussion

3.1. Enantioselective method development (chiral column and mobile phase selection)

Polysaccharide-based columns mentioned in the subsection 2.2 were tested for the separation of enantiomers of MDMA and its major pharmacologically relevant commercially available metabolites HMA,

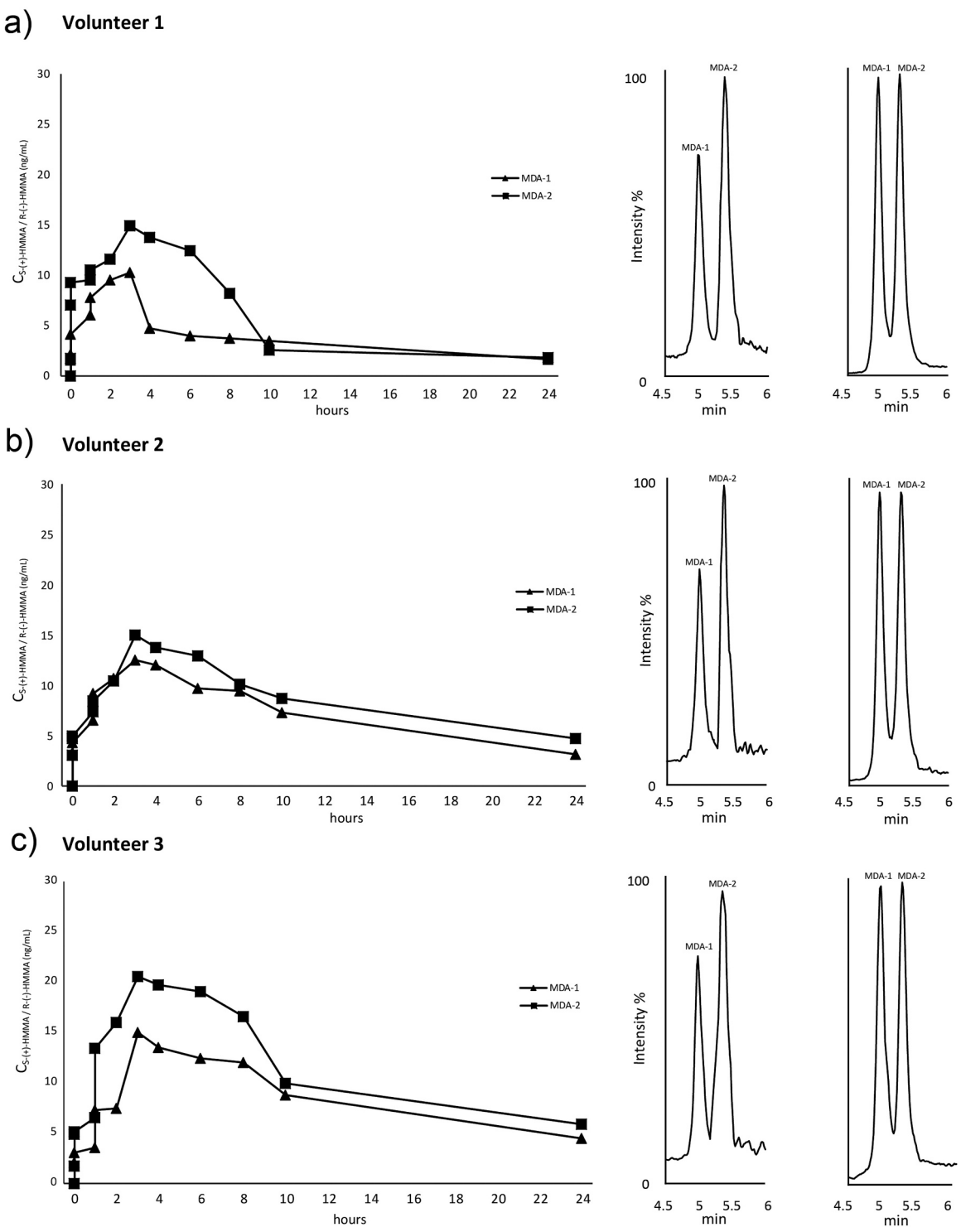

Fig. 6. C-t curves for MDA in plasma for 3 volunteers after receiving 100 mg dose of racemic MDMA and MRM chromatograms for MDA after 24 hrs of MDMA administration. The plasma samples were analyzed on Lux i-Amylose-3 column under the conditions mentioned in the legend to Fig. 2 and in Table 1.

HMMA and MDA. Cellulose-based columns in combination with any of the MS-compatible mobile phases tested in this study did not show any promising enantiorecognition. An example of total ion current (TIC) of a standard mixture of MDMA, HMA, HMMA and MDA separated on the amylose-based Lux AMP column is shown on Fig. 2a. Four well separated and one marginally separated peaks were observed. As it can be seen from MRM chromatograms, the enantiomers of HMA, HMMA and MDMA are baseline resolved on this column while the enantiomers of MDA are not separated at all. While the enantiomers of HMA and HMMA are almost completely coeluted this is not a significant problem since the response of both analytes can be extracted separately in MRM mode (although some ionization suppression/competition for available charge cannot be excluded). The fully-resolved MDMA enantiomers are not overlapping with any component of the mixture and the unresolved enantiomers of MDA coelute as the last peak at 3.75 min. The TIC of the same standard mixture of MDMA, HMA, HMMA and MDA on Lux i-Amylose-3 column is shown on Fig. 2b. On this column, 7 peaks could be counted but some of them were severely overlapped. As it can be seen

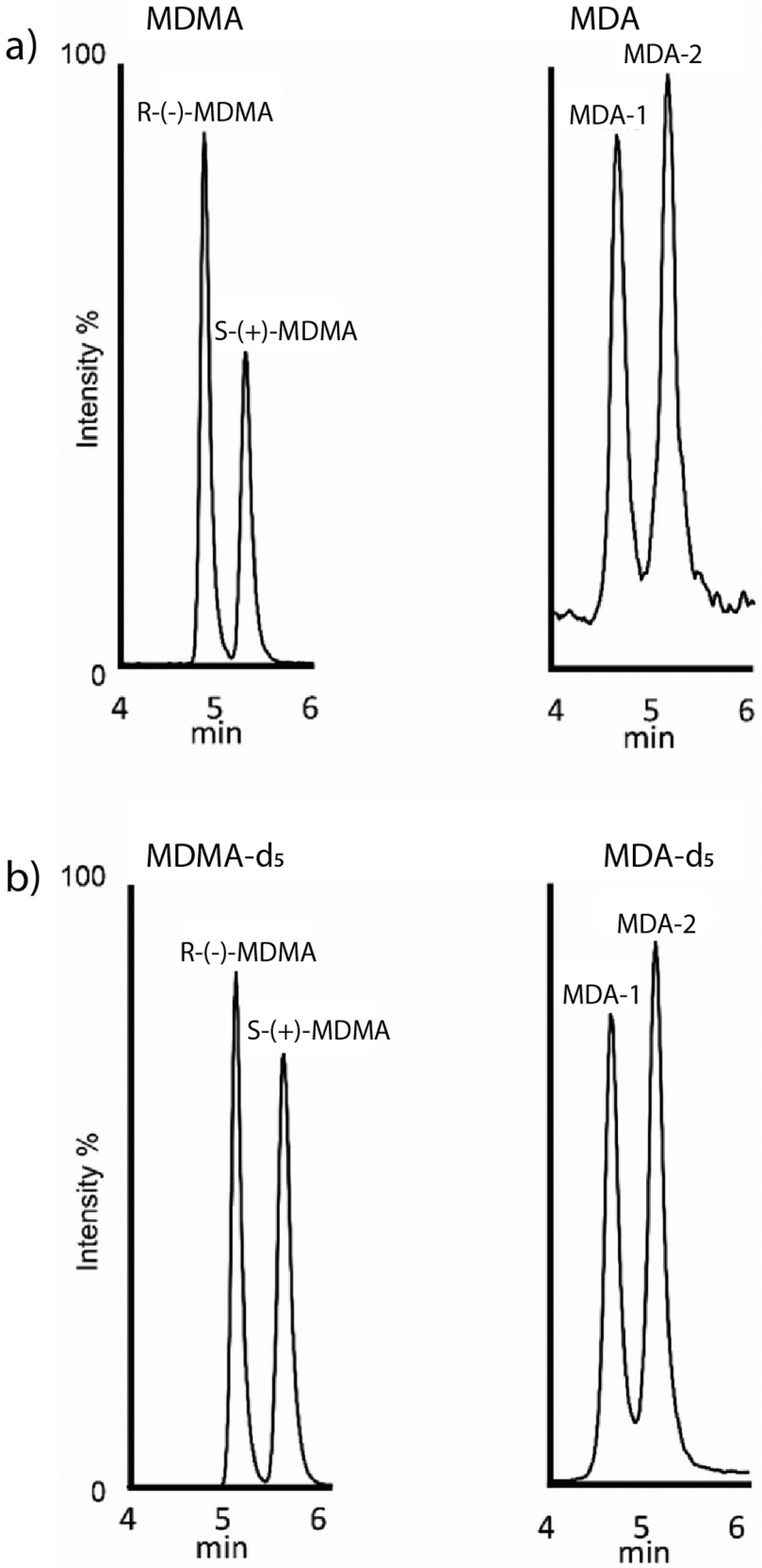

Fig. 7. Chromatograms of MDMA (a) and MDA (b) in the 0–10 hrs. cumulative sweat (patch) samples which were analyzed on Lux i-Amylose-3 column under the conditions mentioned in the legend to Fig. 2 and in Table 1. MDMA-d_5 and MDA-d_5 were used as internal standards.

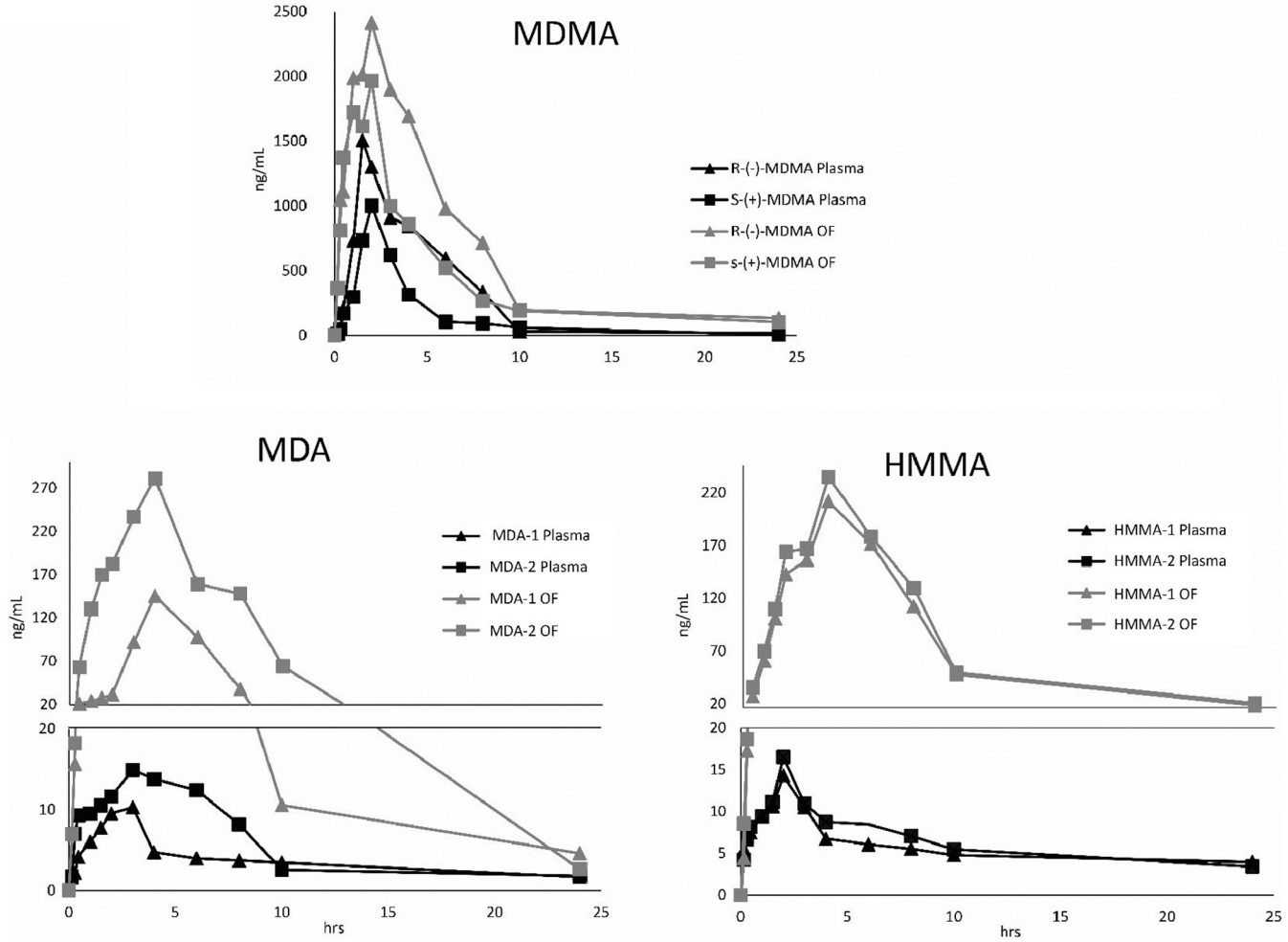

Fig. 8. Comparative C-t curves for plasma and OF for MDMA (a), MDA (b) and HMMA (c) enantiomers for a volunteer who received 100 mg MDMA.

from extracted ion chromatograms (EIC), the enantiomers of MDMA were baseline separated, the enantiomers of HMMA and MDA were almost baseline separated, while the enantiomers of HMA were not separated at all. In addition, the unresolved HMA enantiomers partially coeluted with the first eluting HMMA enantiomer. At the same time, enantioselectively well resolved MDMA enantiomers coeluted with also enantioselectively well resolved MDA enantiomers. Coelution of specific HMMA and HMA enantiomers, as well as MDMA and MDA enantiomers is not a significant problem since MRM mode allows for the deconvolution of their respective response. In summary, neither selected chiral columns was able to resolve all 4 pairs of enantiomers in one single chromatographic separation. Since the behavior of Lux AMP and Lux i-Amylose-3 columns was quasi-complementary an attempt was made to use them serially connected in an attempt to simultaneously resolve the enantiomers of all 4 studied compounds. Although the enantiomers of MDMA were baseline separated and the enantiomers of HMMA were close to baseline separated on the serially connected two columns, the enantiomers of both, HMA and MDA were only partially separated (data not shown). This approach was considered unsuccessful, and it was decided to proceed with using both columns alternatively for the analysis at hand. This approach enabled to study the enantioselective separation of MDMA and HMMA with two alternative chiral columns and that of HMA and MDA with one chiral column each.

3.2. Application to biological samples

3.2.1. Enantioselective metabolism and pharmacokinetics of MDMA

As mentioned above, no direct HPLC-MS/MS method has been published on simultaneous separation of enantiomers of MDMA and its major phase-1 metabolites in biological fluids to date. However, several early studies dealt with the enantioselective metabolism and pharmacokinetics of MDMA by using indirect methods based on derivatization with chiral reagents and analyses of the resulting diastereomers by GC or HPLC-MS/MS [4,6,9–11,24,25,27]. In one such study, HPLC was used for the micro preparative fractionation of MDMA and then purified enantiomers were used for in vitro metabolic studies [16]. This is an acceptable approach. However, it does not mimic the real administration of racemic MDMA when two enantiomers interact in a living body in a competitive manner. The fact that MDMA undergoes enantioselective metabolism with preference for S-(+)-MDMA is well established [4,6,9–11,24,25]. Our experimental results on all studied biological fluids (plasma, OF, sweat and urine) are in agreement with this earlier finding (see representative results for plasma shown in Fig. 3). Another interesting observation made in early studies was that the enantiomeric excess for MDMA increased over time following racemic drug administration. Our results generated in plasma samples did not exactly support this conclusion for the initial hours after drug administration. In fact, a significant enantioselectivity with the concentration ratio of enantiomers $C_{R\text{-}(-)\text{-MDMA}} / C_{S\text{-}(+)\text{-MDMA}} = 4.5$ was observed already in the first measurement of enantiomers after 15 min from drug administration, then this ratio decreased reaching a minimum at around 45 min after

Table 3
Some pharmacokinetic data of MDMA, HMMA, and MDA in human OF and plasma.

Patient/ Volunteer	Dose mg	Matrix	MDMA						HMMA						MDA					
			t_{max}, hr		C_{max}, ng/mL		$T_{1/2}$, hr		t_{max}, hr		C max, ng/mL		$T_{1/2}$, hr		t_{max}, hr		C_{max}, ng/mL		$T_{1/2}$, hr	
			R-(-)	S-(+)	R-(-)	S-(+)	R-(-).	S-(+)	1st-Enant.	2-nd-Enant.	1st-Enant.	2-nd-Enant.	1st-Enant.	2-nd-Enant.	1st-Enant.	2-nd-Enant.	1st-Enant.	2-nd-Enant.	1st-Enant.	2-nd-Enant.
1	100	OF	2	2	2414.1	1966.1	5.2	6.1	4	4	215.5	238.7	6.6	6.8	4	4	145.1	281.1	4.5	3.3
2	100	OF	1.5	1	2921.6	2087.7	6.9	7.2	3	3	297.4	306.1	6.5	6.7	3	4	291.1	375.1	4.1	6.8
3	100	OF	1.5	1	2250.9	2007.3	6.4	7.0	4	4	302.1	313.1	8.0	9.4	4	4	215.3	368.8	6.0	3.2
1	100	Plasma	1.5	1.5	193.2	134.4	11.4	4.5	3	3	26.4	31.4	3.3	3.9	3	3	10.3	14.9	13.2	6.8
2	100	Plasma	1.5	1.5	190.1	148.9	11.7	4.9	2	3	19.1	27.5	3.1	3.8	3	3	12.5	14.9	10.2	12.7
3	100	Plasma	1.5	1	159.2	128.9	11.3	4.4	3	3	19.6	28.6	2.8	5.6	3	3	14.9	20.5	12.5	11.4

Abbreviations: MDMA, 3,4-methylenedioxy-methamphetamine; HMMA, 4-hydroxy-3-methoxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; Cmax, maximum concentration; Tmax: time to reach maximum concentration; T1/2, elimination half-life.

administration of racemic MDMA, then increased, decreased and increased again reaching its highest value at the final sampling of this study (24 hrs.) (Fig. 4a). In Fig. 4 the MRM chromatograms of MDMA and its deuterated standard (MDMA-d_5) are shown after 15 min, 45 min and 24 hrs of racemic MDMA administration. As mentioned above the deuterated standards were added on the sample preparation step. The chromatograms of the racemic deuterated standard confirm that enantiodifferentiation originates from the biological system (living body) and it is not caused by any analytical bias. This quite unexpected observation for plasma samples was also confirmed in OF of the same patient (Fig. 4b).

Due to the low concentrations of MDMA in biological fluids at the initial and terminal steps of drug administration and the relatively large concentration range the C-t curve has to cover, a typical C-t curve does not adequately visualize enantioselectivity in drug metabolism and pharmacokinetics at these steps. This is also the case for the C-t curves shown on Fig. 3. Based on these dependences one may conclude minimal or no enantioselectivity at the initial and terminal steps of MDMA administration. Therefore, in order to better visualize these differences on Fig. 4a and 4b together with chromatograms, the dependence of the concentration ratio R-(-)-MDMA / S-(+)-MDMA was plotted function of the time passed since administration of racemic MDMA. Two important conclusions can be drawn from Fig. 4a and 4b: firstly, there is significant enantioselectivity in metabolism and pharmacokinetics of MDMA present immediately after administration of the drug, as well as of course on the terminal stage of the drug administration and secondly, the results for plasma and OF correlate qualitatively quite well.

3.2.2. *Phase-1 metabolites of MDMA*

Out of phase-1 metabolites of MDMA we could detect just traces of HMA in plasma samples after 45 min of drug administration and it remained detectable after 24 hrs. Although HMA can be formed from MDMA through two different pathways (Fig. 1), due to its low concentration in plasma samples discussing enantioselectivity of its formation or its further conjugation does not seem reasonable.

In contrast to HMA the presence of HMMA could be detected in plasma already at the first sampling point (after 15 min from drug administration) and it remained detectable after 24 hrs. HMMA was formed enantioselectively and the ratio of enantiomer concentrations varied in the range of 1.2–2.5 over the time range 15 min-24 hrs from drug administration (Fig. 5). Since HMMA also undergoes further metabolism to HMA as well as conjugation, the overall enantioselectivity observed in plasma is a sum of its enantioselective formation and apparently also enantioselective further transformations, as well as of its clearance. The MRM chromatograms of this metabolite for 3 volunteers after 15 min of MDMA administration are shown in the same figure (Fig. 5).

The enantiomers of another metabolite of MDMA, specifically MDA, were clearly detectable in plasma after 45 min of MDMA administration and were still detectable after 24 hrs. At the same time, the enantiomers of this metabolite were present in plasma in unequal concentrations. Based on its structure MDA cannot undergo conjugation. However, its enantioselective formation from MDMA, enantioselective transformation to HHA, as well as its clearance is of course possible. The C-t curves shown on Fig. 6 for this metabolite also contain the MRM chromatograms of MDA, as well as its deuterated internal standard MDA-d_5 in the plasma sample taken after 24 hrs from MDMA administration. In overall, our results indicate that the concentration ratio of MDA enantiomers in plasma is closer to 1 compared to the concentration ratio of HMMA enantiomers shown on Fig. 5.

We did not have access to enantiomerically pure HMMA and MDA, as well as could not directly confirm stereochemistry of their enantiomers based on spectroscopic and computational tools. However, based on earlier publication by Steuer and co-authors [25] we can assume that for both discussed metabolites HMMA and MDA, the enantiomer with higher concentration has S-stereochemical configuration.

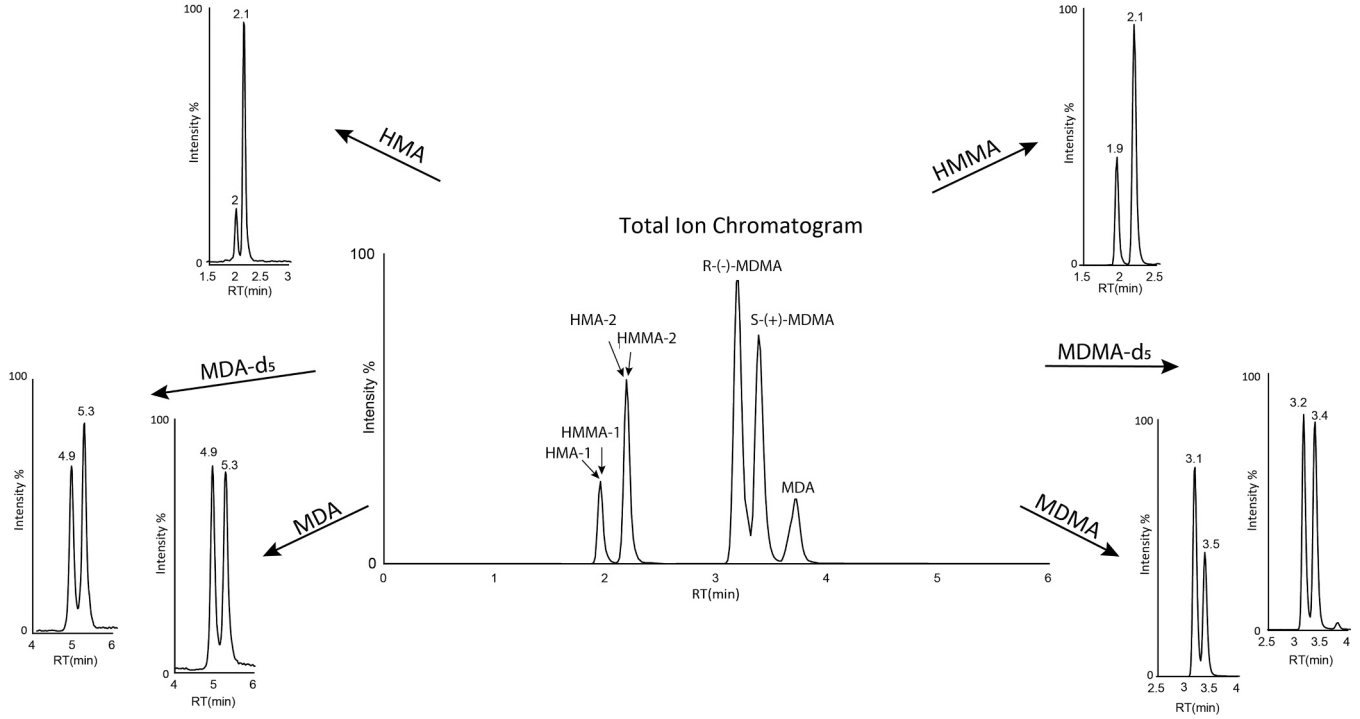

Fig. 9. TIC chromatogram of cumulative (0–4 hrs.) urine sample and MRM chromatograms of MDMA, HMA, HMMA and MDA for a volunteer who received 100 mg MDMA. The sample was analyzed on Lux AMP column under the conditions mentioned in the legend to Fig. 2 and in Table 1. MDA and MDA-d_5 were analyzed on Lux i-Amylose-3 column under the conditions mentioned in the legend to Fig. 2 and in Table 1. MDMA-d_5 and MDA-d_5 were used as internal standards.

It is also to be mentioned that some of the MDMA metabolites detected in the present study were not detected either in plasma or urine in earlier study [25].

3.2.3. *Comparison of results obtained from plasma, oral fluid, sweat and urine*

Various biological matrices have their advantages and disadvantages in following the fate of exogenous compounds in the living body. Blood, plasma and serum have the advantage of showing the actual concentration still in circulation in the body and thus responsible for pharmacodynamic effects. The disadvantage of using these matrices is that they have to be collected in an invasive way. OF may represent a better alternative from this point of view. However, in contrast to circulating blood, OF is collected in a (so called) open system and analyte concentrations may intentionally or unintentionally be affected by intake of liquids, mouthwash and other factors. Sweat is not easy to be adequately collected and processed but may provide valuable information. Urine is a well-accepted matrix in clinical analysis in general, especially in doping and narcotics analyses. The disadvantage of analyzing urine is that the drug to be detected in this matrix has already left the circulatory system and thus the results are mostly post-factum (although sometimes very valuable). The qualitative and quantitative correlations between these matrices is of a certain interest and addressed in several studies in the last decade [33–35].

In addition to the correlation between plasma and OF shown in Figs. 4a and 4b, qualitative correlations were observed also between other matrices, although one has to consider that the sampling method was different between plasma and OF on one side and urine and sweat on the other one. Actually, in the latter 2 cases cumulative samples were taken and the results cannot be plotted as typical C-t curves shown in Figs. 4a and 4b. In particular, the patches used for collecting sweat samples were attached to the body shortly before MDMA administration and removed one by one after certain time intervals from drug administration. Typical analysis results are shown in Fig. 7. Only unchanged MDMA and its metabolite MDA were detected in sweat samples while HMA and HMMA were not detected. The concentration of R-(-)-MDMA was higher in sweat compared to that of S-(+)-MDMA similar to results observed in other matrices while for MDA almost no preference could be observed for any enantiomer (Fig. 7). Significantly higher concentrations for both MDMA enantiomers, as well as their metabolites was observed in OF compared to plasma (Fig. 8, Table 3). This, at the first glance unexpected observation, is in agreement with earlier achiral studies on the same compound and its metabolites [33]. Possible reasons of this unusual result are also mentioned in the same ref. 33.

The most complete picture about MDMA metabolism could be obtained from urine sample. HMA, not detected in any other studied matrices, could be detected in urine samples along with HMMA, MDA and non-metabolized MDMA. As already noticed in several earlier studies, as well as confirmed by enantioselective analysis of plasma, OF an sweat samples, out of the two MDMA enantiomers the S-(+)-enantiomer metabolizes faster and to a higher degree. Therefore, the concentration of R-(-)-MDMA is higher in all matrices analyzed, among them also in urine. Accordingly, the content of the second eluted enantiomers of all metabolites, which presumably are also of S-configuration, is higher compared to the first eluted enantiomers. Based on our results the route of metabolism via O-demethylenation (apparently the major pathway leading to HMMA and HMA) seems to be more enantioselective compared to the route of N-demethylation (leading to MDA). As evident in Fig. 9. the response ratio for the two MDA enantiomers (formed in the body via MDMA metabolisms) in urine and the response ratio of their deuterated analogues used as internal standards are almost the same, while significantly different in the case of MDMA enantiomers and their deuterated analogs used as internal standards (as shown in the same figure).

4. Conclusions

Enantioselective isocratic HPLC-MS/MS methods were developed for the first time for the simultaneous analysis of MDMA and its phase-1 metabolites HMA, HMMA and MDA in various biological fluids. The

methods require the alternative use of two amylose-based chiral columns, namely Lux AMP and Lux i-Amylose-3. The developed methods were validated and used with plasma, OF, urine and sweat samples of volunteers who were administered a single 100 mg dose of MDMA. The enantioselective metabolism of MDMA was confirmed. Namely, its S-(+) enantiomer was metabolized faster and to a higher degree. Of metabolites, mostly HMMA and MDA were detected in plasma while in addition to these two and unchanged MDMA, HMA was also detected in urine samples. In sweat only unchanged MDMA and MDA were detected while HMA was present only in trace amounts in plasma and OF. When detected, the enantiomers of MDMA, HMMA and HMA were present in significantly different concentrations, while for MDA some enantiomeric preference was found in plasma and OF while almost no difference could be observed in urine concentrations of its enantiomers. Demethylenation pathway of MDMA seems to be more enantioselective compared to the N-demethylation pathway. An earlier observation about higher concentrations of MDMA and its metabolites in OF compared to plasma made in a non-enantioselective study was confirmed also in the present enantioselective study for both enantiomers and their related metabolites.

CRediT authorship contribution statement

Alfredo Fabrizio Lo Faro: Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Validation, Writing – review & editing. **Giorgia Sprega:** Formal analysis, Investigation, Writing – review & editing. **Diletta Berardinelli:** Formal analysis, Investigation. **Anastasio Tini:** Formal analysis, Investigation, Writing. **Lourdes Poyatos:** Formal analysis, Investigation, Writing – review & editing. **Esther Papaseit:** Formal analysis, Investigation, Writing – review & editing. **Paolo Berretta:** Conceptualization, Methodology, Supervision. **Alessandro Di Giorgi** Formal analysis, Investigation. **Magì Farre:** Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Nino Takaishvili:** Formal analysis, Investigation. **Tivadar Farkas:** Writing – review & editing. **Francesco Paolo Busardò:** Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Writing – review & editing**. Bezhan Chankvetadze:** Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Validation, Writing – original draft, Writing – review & editing.

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.

Acknowledgements

Bezhan Chankvetadze thanks Department of Excellence-Biomedical Sciences and Public Health, Università Politecnica delle Marche for providing financial support for his stay in Ancona as Visiting Professor. He also thanks Shota Rustaveli National Science Foundation of Georgia for a partial support of this study through the grant N№ FR-22–971 for fundamental research. This investigation was partially supported by grants from the Instituto de Salud Carlos. III (ISCIII, Fondo de Investigación en Salud (FIS)-Fondo Europeo de Desarrollo Regional (FEDER), grant numbers PI17/01962 and PI20/00879; predoctoral PFIS fellowship grant number FI18/00179; Plataformas ISCIII de apoyo a la I + D + I en Biomedicina y Ciencias de la Salud grant number PT20/00018).

References

[1] K.A. Moore, A. Mozayani, M.F. Fierroc, A. Poklis, Distribution of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) stereoisomers in a fatal poisoning, Forensic Sci. Int 83 (1996) 111–119, https://doi.org/10.1016/s0379-0738(96)02025-7.

[2] R.L. Fitzgerald, R.V. Bianke, J.A. Rosecrans, R.A. Glennon, Stereochemistry of the metabolism of MDMA to MDA, Life Sci. 45 (1989) 295–301, https://doi.org/10.1016/0024-3205(89)90138-0.

[3] K.M. Hegadoren, G.B. Baker, M. Bourin, 3,4-Methylenedioxy analogues of amphetamine: defining the risks to humans, Neurosci. Biobehav Rev. 23 (1999) 539–553, https://doi.org/10.1016/s0149-7634(98)00046-3.

[4] H. Kalant, The pharmacology and toxicology of "ecstasy" (MDMA) and related drugs, CMAJ 165 (2001) 917–928. ⟨https://pubmed.ncbi.nlm.nih.gov/11599334/⟩.

[5] R. De la Torre, M. Farre, P.N. Roset, N. Pizarro, S. Abanades, M. Segura, Human pharmacology of MDMA: pharmacokinetics, metabolism, and disposition, Ther. Drug Monit. 26 (2004) 137–144, https://doi.org/10.1097/00007691-200404000-00009.

[6] T.D. Steele, U.D. McCann, G.A. Ricaurte, 3,4-Methylenedioxymethamphetamine (MDMA, "Ecstasy"): pharmacology and toxicology in animals and humans, Addiction 89 (1994) 539–551, https://doi.org/10.1111/j.1360-0443.1994.tb03330.x.

[7] C.J. Schmidt, Neurotoxicity of the psychedelic amphetamine, methylenedioxymethamphetamine, J. Pharmacol. Exp. Ther. 240 (1987) l–7. ⟨https://pubmed.ncbi.nlm.nih.gov/2433425/⟩.

[8] T. Kraemer, H.H. Maurer, Toxicokinetics of amphetamines: Metabolism and toxicokinetic data of designer drugs, of amphetamine, methamphetamine and their N-alkyl derivatives, Ther. Drug Monit. 24 (2002) 277–289, https://doi.org/10.1097/00007691-200204000-00009.

[9] J.K. Fallon, A.T. Kicman, J.A. Henry, P.J. Milligan, D.A. Cowan, A.J. Hutt, Stereospecific analysis and enantiomeric disposition of 3,4-methylenedioxymethamphetamine (Ecstasy) in humans, Clin. Chem. 45 (1999) 1058–1069. ⟨https://pubmed.ncbi.nlm.nih.gov/10388483/⟩.

[10] R.L. Fitzgerald, R.V. Blanke, R.A. Glennon, M.Y. Yousif, A. Poklis, Determination of 3.4-methelenedioxyamphetamine and 3,4-methylenedioxymethamphetamine enantiomers in whole blood, J. Chromatogr. B 490 (1989) 59–69, https://doi.org/10.1016/s0378-4347(00)82761-9.

[11] R.L. Fitzgerald, R.V. Blanke, A. Poklis, Stereoselective pharmacokinetics of 3,4–methylenedioxymethamphetamine in the rat, Chirality 2 (1990) 241–248, https://doi.org/10.1002/chir.530020409.

[12] L.F. Ward, J.R. Enders, D.S. Bell, H.M. Cramer, F.N. Wallace, G.L. McIntire, Improved chiral separation of methamphetamine enantiomers using CSP-LC–MS-MS, J. Anal. Toxicol. 40 (2016) 255–263, https://doi.org/10.1093/jat/bkw005.

[13] M. Lanz, R. Brenneisen, W. Thormann, Enantioselective determination of 3,4-methylenedioxymethamphetamine and two of its metabolites in human urine by cyclodextrin-modified capillary zone electrophoresis, Electrophoresis 18 (1997) 1035–1043, https://doi.org/10.1002/elps.1150180628.

[14] N. Pizarro, J. Ortuno, M. Farre, C. Hernlindez-Lopez, M. Pujadas, A. Llebaria, J. Joglar, P.N. Roset, M. Mas, J. Segura, J. Carol, R. de la Torre, Determination of MDMA and its metabolites in blood and urine by gas chromatography-mass spectrometry and analysis of enantiomers by capillary electrophoresis, J. Anal. Toxicol. 26 (2002) 158–165, https://doi.org/10.1093/jat/26.3.157.

[15] N. Pizarro, R. De la Torre, M. Farre, J. Segura, A. Llebaria, J. Joglar, Synthesis and capillary ellectrophoresis analysis of enantiomerically enriched reference standards of MDMA and its main metabolites, Bioorg. Med. Chem. 10 (2002) 1085–1092, https://doi.org/10.1016/s0968-0896(01)00367-4.

[16] M.R. Meyer, F.T. Peters, H.H. Maurer, The role of human hepatic cytochrome p450 isozymes in the metabolism of racemic 3,4-Methylenedioxy-Methamphetamine and its enantiomers, Drug Metab. Dispos. 36 (2008) 2345–2354, https://doi.org/10.1124/dmd.108.021543.

[17] A.E. Schwaninger, Markus R. Meyer, M.A. Huestisb, H.H. Maurer, 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, J. Mass Spectrom. 46 (2011) 603–614, https://doi.org/10.1002/jms.1929.

[18] T.C. Lourenco, G.C. Bósio, N.M. Cassiano, Q.B. Cass, R.L.M. Moreau, Chiral separation of 3,4-methylenedioxymethamphetamine (MDMA) enantiomers using batch chromatography with peak shaving recycling and its effects on oxidative stress status in rat liver, J. Pharm. Biomed. Anal. 73 (2013) 13–17, https://doi.org/10.1016/j.jpba.2012.01.025.

[19] J.C. Barreiro, M.W. Paixão, T.C. Lourenco, Q.B. Cass, T. Venaancio, A high-resolution magic angle spinning NMR study of the enantiodiscrimination of 3,4-Methylenedioxymethamphetamine (MDMA) by an immobilized polysaccharide-based chiral phase, Plos One 26 (2016) 1–11, https://doi.org/10.1371/journal.pone.0162892.

[20] A.R. Costa, V. Gonçalves, B.B. Castro, J.S. Carrola, I. Langa, A. Pereira, A. R. Carvalho, M.E. Tiritan, C. Ribeiro, Toxicity of the 3,4-methylenedioxymethamphetamine and its 2 enantiomers to Daphnia magna, after isolation by semipreparative chromatography, Molecules 28 (2023) 1457, https://doi.org/10.3390/molecules28031457.

[21] W.-J. Chen, R.-J. Wu, H.-H. Yang, A.-C. Lua, Y.-J. Tzeng, S.-H. Tuan, L. Chen, T.-Y. Chou, A New Method for Enantiomeric Determination of 3,4-Methylenedioxy-methamphetamine and p-Methoxymethamphetamine in Human Urine, Chemosensors 10 (2022) 50, https://doi.org/10.3390/chemosensors10020050.

[22] M.G. Schmid, J.S. Hägele, Separation of enantiomers and positional isomers of novel psychoactive substances in solid samples by chromatographic and electrophoretic techniques -a selective review, J. Chromatogr. A 2020 (1624), 461256, https://doi.org/10.1016/j.chroma.2020.461256.

[23] M. Losacker, S. Zörntlein, B. Schwarze, S. Staudt, J.R.öhrich C. Hess, Determination of the enantiomeric composition of amphetamine, methamphetamine and 3,4-methylendioxy-N-methylamphetamine (MDMA) in seized street drug samples from

southern Germany, Drug Test. Anal. 14 (2022) 557–566, https://doi.org/10.1002/dta.3118.
[24] K. Nakanishi, M. Katagi, K. Zaitsu, N. Shima, H. Kamata, A. Miki, H. Kato, K. I. Harada, H. Tsuchihashi, K. Suzuki, Simultaneous enantiomeric determination of MDMA and its phase I and phase II metabolites in urine by liquid chromatography tandem mass spectrometry with chiral derivatization, Anal. Bioanal. Chem. 404 (2012) 24–27, https://doi.org/10.1007/s00216-012-6385-9.
[25] A. Steuer, C. Schmidhauser, Y. Schmid, A. Rickli, M.E. Liechti, T. Kraemer, Chiral plasma pharmacokinetics of 3,4-Methylenedioxymethamphetamine (MDMA) and its phase I and II metabolites following controlled MDMA administration to humans, Drug Metab. Dispos. 43 (2014) 1864–1871, https://doi.org/10.1124/dmd.115.066340.
[26] K.M. Yang, Organization of Scientific Area Committees (OSAC) for Forensic Science Standard Practices for Method Development. https://www.nist.gov/osac.
[27] A.F. Lo Faro, D. Berardinelli, G. Sprega, A. Tini, J. Carlier, T. Farkas, F.P. Busardò, B. Chankvetadze, Development of an enantioselective high-performance liquid chromatography-tandem mass spectrometry method for the quantitative determination of methorphan and its O-demethylated metabolite in human blood and its application to post-mortem samples, J. Pharm. Biomed. Anal. 15 (2023) 230–115384, https://doi.org/10.1016/j.jpba.2023.115384.
[28] L. Poyatos, A.F. Lo Faro, D. Berardinelli, G. Sprega, S. Malaca, S. Pichini, H. A. Huestis, E. Papaseit, C. Pérez-Mañá, F.P. Busardò, M. Farré, Methylone and MDMA pharmacokinetics following controlled administration in humans, Int. J. Mol. Sci. 23 (2022) 23–14636, https://doi.org/10.3390/ijms232314636.
[29] A. Di Giorgi, G. Sprega, L. Poyatos, E. Papaseit, C. Pérez-Mañá, A. Di Trana, M. R. Varì, F.P. Busardò, S. Pichini, S. Zaami, A.F. Lo Faro, M. Farré, Sweat testing for the detection of methylone after controlled administrations in humans, Int. J. Mol. Sci. 17 (2023) 24–7395, https://doi.org/10.3390/ijms24087395.
[30] G. Sprega, A. Di Giorgi, L. Poyatos, E. Papaseit, C. Pérez-Mañá, A. Tini, S. Pichini, F.P. Busardò, A.F. Lo Faro, M. Farré M, Usefulness of oral fluid for measurement of methylone and its metabolites: correlation with plasma drug concentrations and the effect of oral fluid pH, Metabolites 24 (2023) 13–468, https://doi.org/10.3390/metabo13040468.
[31] J. Mandel, The statistical analysis of experimental data, Biom. J. 10 (1968) 231–279, https://doi.org/10.1002/bimj.19680100407.
[32] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030, https://doi.org/10.1021/ac020361s.
[33] M. Navarro, S. Pichini, M. Farre, J. Ortun, P.N. Roset, J. Segura, R. de la Torre, Usefulness of saliva for measurement of 3,4-Methylenedioxymethamphetamine and its metabolites: correlation with plasma drug concentrations and effect of salivary pH, Clin. Chem. 47 (2001) 1788–1795, https://doi.org/10.1093/clinchem/47.10.1788.
[34] N. Samyn, G. de Boeck, M. Wood, C.T.J. Lamers, D. De Waard, K.A. Brookhuis, A. G. Verstraete, W.J. Riedel, Plasma, oral fluid and sweat wipe ecstasy concentrations in controlled and real life conditions, Forensic Sci. Int. 128 (2002) 90–97, https://doi.org/10.1016/S0379-0738(02)00157-3.
[35] A.T.M. da Silva, C.D.B. Bessa, W.S. Borges, K.B. Borges, Bioanalytical methods for determining ecstasy components in biological matrices, Trends Anal. Chem. 108 (2018) 323–346, https://doi.org/10.1016/j.trac.2018.08.001.