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Challenges encountered in the enantioselective analysis of new psychoactive substances exemplified by clephedrone (4-CMC)

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ABSTRACT

In this study we report on efforts to develop an enantioselective method for the detection of the drug of abuse clephedrone (1-(4-chlorophenyl)-2-(methylamino)-1-propanone (4-chloromethcathinone, also known as 4-CMC or *para*-chloro-methcathinone)) and its phase-1 metabolites in human biological fluids. The major goal is not to only report results, but primarily to emphasize the various challenges encountered when developing a reliable analytical method for the detection and quantification of novel psychoactive substances (NPS) and their metabolites in the matrix of interest. Such challenges start with the lack of chemical stability of some NPS in biological matrices. Additionally, most often metabolites are unavailable in pure form to serve as analytical standards, just as deuterated standards for native drugs and metabolites are frequently not commercially available. Furthermore, if the NPS is chiral, enantiomerically pure standards with known absolute stereochemistry are required, as well as a stereochemical stability of a drug and its metabolites becomes an issue. In addition, the chirality of a NPS significantly increases the number of species to be detected in the sample and thus challenges the development of an adequate separation method. These issues are shortly addressed, and some solutions offered in this manuscript.

1. Introduction

As new psychoactive substances (NPS) are swamping illicit drug markets, police departments, as well as analytical and research laboratories specialized in the forensic analysis and clinical toxicology fields are challenged to keep up. This puts pressure on the analytical staff responsible for the development of methods which can be used for reliable (selective) detection and accurate quantification of not only the specific NPS but also its phase-1 and, if possible, also phase-2 metabolites. There are quite many challenges in this process which need to be clearly identified and addressed to ensure the quality and reliability of the strategies and analytical methods used for the detection of NPS in seized materials and in biological matrices. This is a primary requirement to avoid false positive and/or false negative analytical results, given their legal and therapeutic implications.

1-(4-Chlorophenyl)-2-(methylamino)-1-propanone (4-chloromethcathinone, 4-CMC, *para*-chloro-methcathinone) is known on the illicit drug market under the name clephedrone. This drug, as NPS, part of the cathinone family, was first identified on the European drug market in June 2014 [1]. Since that time 4-CMC is one of the most persistent drugs of abuse in Europe regularly found in clandestine laboratories, in seized materials and identified in biological specimens of abusers, even though it now has a controlled drug status in many countries. The use of 4-CMC is causing various health problems and accidents, and ultimately, death in case of overdosing [2]. In March 2020, the United Nations Commission on Narcotic Drugs decided to include 4-CMC in Schedule II of the

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Convention on Psychotropic Substances of 1971 [1–3]. There are only a few analytical methods available for the quantitative determination of 4-CMC in seized [4] and biological materials [2,5,6]. In 2014 Schmid and co-workers have used NMR spectroscopy to confirm the chemical identity of 4-CMC purchased on-line, as well as capillary electrophoresis and indirect GC-MS methods to confirm that the chiral compound 4-CMC was present in the acquired material in racemic form [4]. Several analytical methods based on GC, among them also one based on trimethylsilylation enabling differentiation between the positional isomers such as 2-, 3-, and 4-CMC have been recently described [5,6]. In some of these studies selected metabolites, such as diastereomeric dihydro-4-CMC, have been also quantitatively determined [2]. These authors also paid attention to the lack of chemical stability of 4-CMC especially in blood samples and recommended the use of its more stable dihydro-metabolite as a reliable biological marker of 4-CMC consumption [2]. GC-based methods for the quantification of 4-CMC in biological matrices involve its derivatization with PFPA prior the analysis. Few HPLC-MS-based methods for the identification and quantification of 4-CMC together with other synthetic cathinones or other drugs of abuse in biological samples have been also reported [7,8]. In most cases these GC- and HPLC-based analytical methodologies have been reported in relation to real forensic cases. Only in few studies the metabolites of 4-CMC have been also mentioned, mostly from the viewpoint of avoiding false-negative results due to rapid metabolism [7] or perhaps the instability of 4-CMC in blood and some other biological fluids [2, 8–12]. A dedicated study on the *in vitro* metabolism of 4-CMC with human liver microsomes and the determination of some of its metabolites was published in 2023 [13]. Some clinically relevant metabolites of 4-CMC are also mentioned in a recently published observational clinical study [14]. Spalovska et al. reported the semipreparative separation of 4-CMC enantiomers on a polysaccharide-based chiral column and determined the absolute stereochemical configuration to be (S)-4-CMC for the first eluted enantiomer and (R)-4-CMC for the second eluted enantiomer under their experimental conditions [15]. To the best of our knowledge no method has been published on the enantioselective determination of 4-CMC and its phase-1 metabolites in human biological fluids and no data is available about possible enantioselective metabolism and pharmacokinetics neither *in-vitro* nor *in-vivo*.

In this regard, the development of a reliable HPLC-MS/MS method for the enantioselective quantification of 4-CMC and its metabolites in biological matrices faced the following challenges: 1) 4-CMC along with its positional isomers, 2-CMC and, especially 3-CMC are available on the illicit market. As already mentioned above, a GC-method based on trimethylsilylation can differentiate between the positional isomers 2-, 3-, and 4-CMC. However, no such method based on HPLC is known to us; 2) 4-CMC, just like its positional isomers, is a chiral molecule with one chiral center. The pure enantiomers of 4-CMC are not commercially available; 3) Based on published literature [2,7,13,14] and our own experimental results, 4-CMC undergoes intense metabolism in the living body generating at least 7 phase-1 metabolites (Fig. 1). Of all these metabolites a standard compound is available only for two of them. This makes the quantification of the other metabolites impossible. 4) All phase-1 metabolites are chiral and several of them contain 2 chiral centers due to the reduction of the carbonyl moiety located in the β-position in relation to the aromatic ring. Thus, a large number of stereoisomers and enantiomers are present in biological samples; 5) As reported in previous studies and confirmed in the present one, 4-CMC and its positional isomers are chemically unstable, especially in blood and some other biological matrices [6].

On this basis, the goals of the present study were the development of a method based on high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) for the enantioselective determination of 4-CMC and its phase-1 metabolites, and its application to human oral fluid and urine samples part of a controlled clinical study. Furthermore, given the difficult background underlying the present study, some specific aspects will be highlighted, i.e. what could be achieved in terms of analytical method performance as well as other issues of general interest in this field and not specifically related to 4-CMC.

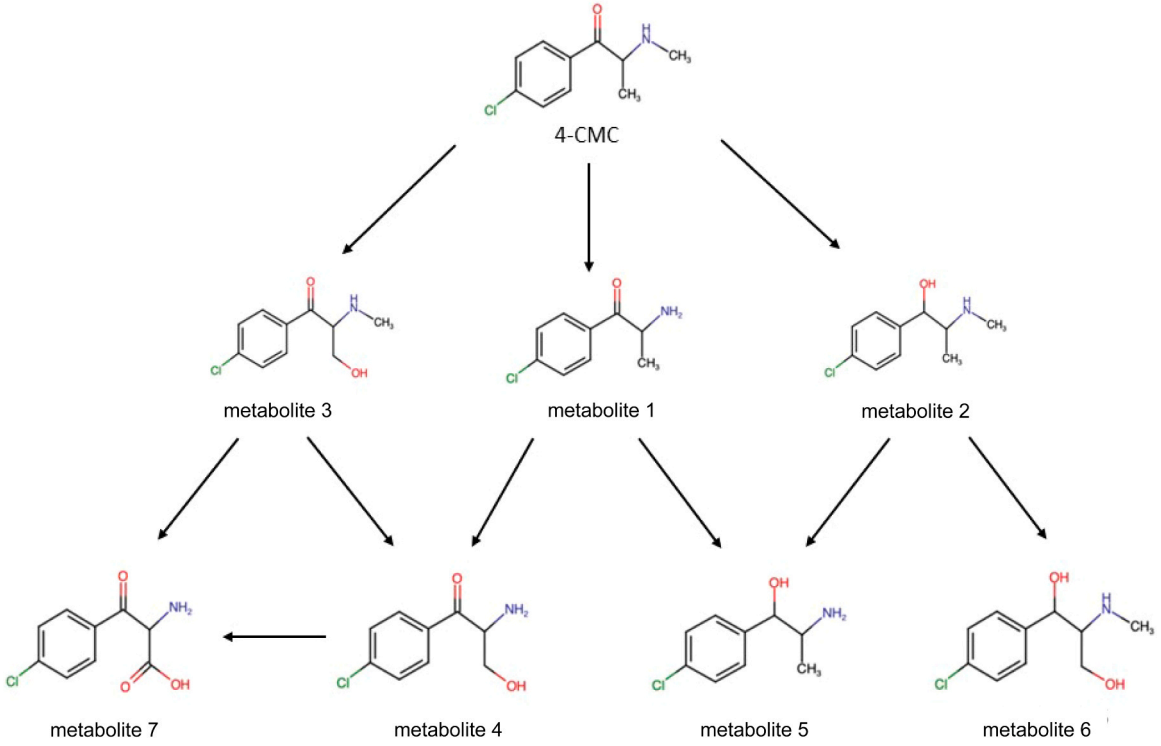

Fig. 1. Structure of 4-CMC and its major phase-1 metabolites.

2. Experimental part

2.1. Reagents and standards

Standards of clephedrone and N-ethylpentedrone were sourced from Cayman Chemical (Ann Arbor, MI, USA). Standards were stored at −20° C until analysis. LC-MS grade water, methanol, acetonitrile, as well as formic acid, chloroform, ammonium hydroxide (25 % w/w aqueous solution), hydrochloric acid (37 % w/w aqueous solution), ammonium formate, ammonium acetate and ethyl acetate were obtained from Carl Roth GmbH (Karlsruhe, Germany). Hepatocytes were sourced from Lonza (Basel, Switzerland), while HEPES, Williams medium E, β-glucuronidase, ammonium acetate and LC-grade acetic acid were from Sigma Aldrich (Milan, Italy).

2.2. Chiral columns

Nine polysaccharide-based chiral columns of the Lux series manufactured by Phenomenex Inc. (Torrance, CA, USA) were screened for selectivity. These columns are based on 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), cellulose tris(3,5-dichlorophenylcarbamate) (Lux i-Cellulose-5), amylose tris (3,5-dimethylphenylcarbamate) (Lux Amylose-1 and Lux i-Amylose-1), amylose tris(5-chloro-2-methylphenylcarbamate) (Lux Amylose-2), amylose tris (3-chloro-5-methylphenylcarbamate) (Lux i-Amylose-3) and the specialty column with a proprietary chiral selector Lux AMP. In addition, an experimental column based on cellulose tris(3-chloro-5-methylphenylcarbamate) immobilized on silica made in our laboratory was also tested. All columns were of 250 ×4.6 mm dimensions and packed with 5 μm particles.

2.3. Identification of 4-CMC metabolites

4-CMC metabolites were identified in 10-donor-pooled human hepatocytes following our in-house protocol [16–22]. Briefly, 10 μmol/L 4-CMC standard (250 μL) was incubated with 10^6 hepatocytes/ml in 20 mmol/L HEPES in Williams' medium E at 37° C for 3 h. The metabolic reactions were quenched with ice-cold acetonitrile. Following protein precipitation, supernatant evaporation, and reconstitution in HPLC mobile phases, the incubates were analyzed by HPLC-high-resolution tandem mass spectrometry (HPLC-HRMS/MS) composed of LC (UltiMate 3000, Dionex) and HRMS (Q Exactive, Thermo Scientific, Waltham, MA, USA) using a Kinetex Biphenyl column (150 ×2.1 mm, 2.6 μm) from Phenomenex at 37° C in gradient elution mode with a mobile phase composed of water and acetonitrile containing 0.1 % formic acid. Software-assisted raw data mining was performed with Compound Discoverer (Thermo Scientific) using a dual workflow for targeted and non-targeted analysis.

4-CMC-positive urine samples collected at diverse time points after administration to human subjects were analyzed following the same HPLC-HRMS/MS and data-mining protocol after protein precipitation, supernatant evaporation, and reconstitution in 100 μL 0.1 % formic acid in water:0.1 % formic acid in acetonitrile 90:10 (v/v). The samples were previously prepared with or without incubation with β-glucuronidase originated from limpets at 37° C and pH 5.0 for 3 h.

2.4. High-performance liquid chromatography– tandem mass spectrometry (HPLC–MS/MS) analysis

Chromatographic separations were performed on an HPLC 1290 Infinity II (Agilent Technologies, Waldbronn, Germany) instrument coupled to a mass spectrometer (G6410A Triple Quadrupole LC-MS) equipped with an electrospray ionization source (ESI) operated in positive ionization mode. Autosampler and column oven temperatures were set to 25° C. Data was acquired with MassHunter® Workstation Qualitative Analysis B.04.00 Software (Agilent Technologies). MS/MS-detection optimization was conducted in part automatically and in part manually. MS parameter settings were optimized by ramping cone voltage and collision energy. Mass spectrometric parameters for the detection of 4-CMC and its phase-1 metabolites are summarized in Table 1. Scan speed (dwell time) was 0.93 sec. ESI conditions were optimized as follows: capillary voltage 3500 V, source temperature 300° C, cone gas flow rate 10 L/min, desolvation gas flow rate 12 L/min.

2.5. Human samples

Oral fluid (OF), sweat and urine samples were obtained from an observational-naturalistic study. The study was conducted in 8 volunteers (6 females and 2 male, mean age 36 years old (range 30–54), mean weight 64.55 kg (range 46–87.0 kg) at the Hospital Universitari German Trias I Pujol, Institut d'Investigatiò en Ciènces de la Salut Germans Trias i Pujol, in Badalona, Spain. The observational session took place at a private club with ambient music. The ambient temperature in the private club was around 24 °C. The sessions started at 3:00 p.m. and finished at 9:00 p.m. All the doses were self-administered and were also self-selected by each participant, based presumably on their previous experience. The substance samples were tested by Energy Control (https://energycontrol.org/), a harm reduction organization that provides a drug checking service for users. All participants had recreational experience with common drugs of abuse (psychostimulants as cocaine, amphetamines, 3,4-methylenedioxymethamphetamine (MDMA), and synthetic cathinones). Prior to study sessions, the participants were submitted to a general medical examination including rapid urine drug analysis and declared nor consuming cathinones in the last weeks. 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). It was conducted according to the Declaration of Helsinki recommendations and Spanish rules about clinical investigation. All the participants were informed, both orally and in writing, and signed an informed consent prior to inclusion. They received monetary compensation for their participation.

Capsules contained 100 mg (in case of 3 females, 1 male) or alternatively 150 mg (in case of 3 females, 1 male) of 4-CMC were self-administered. OF samples were collected in Salivette^R plastic tubes

Table 1
MRM parameters for 4-CMC, its phase-1 metabolites and N-ethyl-pentedrone.

Analytes	Molecular mass, g/mol	Precursor ion, *m/z*	Product ion, *m/z*	CE, eV
4-CMC	197.6	198.1	180.1	12
			158.1	12
			145.1	18
N-Ethyl-pentedrone	205.3	206	161.3	12
			146.2	30
Metabolite 1	183.6	184.6	131.2	18
			112.8	18
			102	18
Metabolite 2	199.6	200.6	182.1	10
			145.2	20
Metabolite 3	213.6	214.6	186.2	20
			157.1	30
			131.2	30
Metabolite 4	198.6	199.6	158.1	14
			100.1	12
			98.8	14
Metabolite 5	185.6	186.6	168	10
			116	20
Metabolite 6	215.6	216.6	125.9	15
			98	30
			70	30

before (pre-dose) and at 0.5, 1, 1.5, 2, 3, 4, and 5 hours after dosing and immediately centrifuged and stored at −20 °C until the analysis.

2.6. Sample preparation

2.6.1. OF and urine

Ten μL of the 100 ng/ml internal standard (N-ethylpentedrone) and 5 ml chloroform: ethyl acetate 9:1 (v/v) mixture were added to 100 μL biological sample such as oral OF or urine. Sample tubes were stirred for 5 min and centrifuged at 6000 rpm for 12 min. Organic phase was transferred into clean tubes, 100 μL acidic methanol (1 % HCl) was added to prevent evaporative losses while samples were dried under nitrogen for approximately 30 min. Dry samples were reconstituted in 100 μL methanol and transferred into autosampler vials prior to injection of 5 μL into the chromatographic system.

2.7. Stability study of 4-CMC is blood, oral fluid and urine

Two sets of experiments were performed in order to examine the stability of 4-CMC in human blood, OF and urine. In the first set of experiments 0.5 ml whole blood, OF or urine were placed in 10 ml plastic tubes. 10 samples of each matrix were prepared and spiked with 50 ng 4-CMC and 50 ng N-ethylpentedrone (IS) each. The tubes were covered and 5 of the tubes of each matrix were kept at the room temperature (23 °C) and 5 in the freezer at −20 °C. The first sample treatment and HPLC-MS/MS analysis was performed immediately after spiking the matrixes. The next analyses of blood samples were performed after 1, 3, 12, 25, 29 and 33 h after spiking and 1, 5 and 15 days after spiking OF and urine samples.

In another set of stability experiments each of 5 ml blood OF or urine were dispensed into 2 different glass tubes of 10 ml volume (2 ×3 tubes in total). Each matrix was spiked with 4-CMC and N-ethylpentedrone (as internal standard) at 100 ng/ml concentration level. The tubes were covered and 3 of them (one of each matrix) were kept at room temperature (23 °C) while the other 3 were placed in the freezer (-20 °C). Immediately after spiking, the tubes were well shaken manually, and 2 ×0.2 ml matrix were withdrawn from each tube. The samples were prepared separately (2 samples of each matrix at each temperature and at the given time point) and analyzed by HPLC-MS/MS (each sample was injected twice). In separate experiments deionized water was used as a matrix instead of the abovementioned biological fluids. In addition, in one set of samples the internal standard was added not from the very beginning but just after incubation of 4-CMC and immediately before sample preparation for HPLC-MS/MS analysis. This last experiment was performed in order to examine the stability of the internal standard itself in the studied matrices.

3. Results and discussion

3.1. Identification of phase-1 metabolites of 4-CMC

As mentioned above, several studies reported the presence of some metabolites of 4-CMC in incubations with human liver microsomes [13], in samples from forensic cases [2], or from controlled clinical studies [14]. However, a comprehensive identification of all potential phase-1 metabolites in human samples has not published yet. In the analytical part covering forensic cases [2] the attention was focused on the dihydro-metabolite of 4-CMC (Hydroxy-4-CMC; Metabolite 2 in Fig. 1) which was proposed as a reliable biomarker of 4-CMC consumption while other potential metabolites were not investigated. In the recently published report on a controlled clinical study [14], in addition to the above mentioned dihydro-metabolite and its N-demethylation product (Hydroxy-nor-4-CMC) (metabolite-5), the N-demethylation product of intact 4-CMC were detected along with their phase-2 metabolites (glucuronides). In addition to these 3 phase-1 metabolites, the hydroxymethyl derivative at the chiral center was reported in human liver microsome incubates in ref. 13 (Metabolite 3 on Fig. 1). The identification of any possible 4-CMC phase-1 metabolites in the OF and urine of one of the volunteers, part of this study, led to the detection of the metabolites shown in Fig. 1. Thus, 3 additional metabolites, specifically metabolite **3**, metabolite **6** and metabolite **7** were identified. Of these metabolites, metabolite **1** - **3** seem to be the major metabolites while the metabolites **4**, **5, 6** and **7** were detected in minor amounts or not detected at all in the studied OF sample. Of all the compounds shown in Fig. 1, standards were commercially available only for metabolite **1** (which is in fact 4-chlorocathinone) and metabolite **5**. Unfortunately, these standards were not available to us early on when the project was initiated. Thus, this preliminary enantioselective method for the analysis of 4-CMC and its metabolites was developed by using real biological samples and a standard was only available for the native compound, 4-CMC.

3.2. Development of an enantioselective HPLC-MS/MS method for the separation of 4-CMC and its phase-1 metabolites

Of the 8 compounds depicted on Fig. 1, 5 compounds contain 1 center of chirality and 3 contain 2 centers of chirality. Thus, if all metabolites and in all possible stereochemical configurations were formed one should expect 22 individual species (excluding any phase-2 metabolites). Thus, such a sample is quite complex and challenging to successfully and completely analyze. As mentioned above, the separation of 4-CMC enantiomers has been reported by Schmid and co-workers using capillary electrophoresis and GC (following derivatization with a chiral reagent) [4]. In 2021 Spalovska et al. published HPLC separation of 4-CMC enantiomers with alkane-alcohol-type mobile phases [15]. In the same study the enantioseparation of one of the phase-1 metabolites of 4-CMC, namely 4-chloro-cathinone (or 4-chloro-normethcathinone) was reported (metabolite **1** in Fig. 1). The authors also performed the isolation of enantiomers of 4-CMC and based on electronic- and vibrational circular dichroism spectroscopy and density-functional theory calculations assigned the absolute stereochemical configuration for the first and the second eluted chromatographic peaks of the amylose tris(3, 5-dimethylphenylcarbamate)-based column(s) in the used mobile phase to be (S)- before (R)-4-CMC [15]. The alkane-alcohol-based mobile phases used in ref. 15 are unsafe to be used in HPLC-MS analyses, given their high flammability. Therefore, in the present study the chiral columns described in subsection 2.2 were initially screened with mobile phases compatible with MS-detection for the separation of 4-CMC enantiomers. Baseline separation of these enantiomers was obtained on two columns, namely Lux i-Amylose-3 (Fig. 2a and c) and Lux AMP (Fig. 2b and d). With both columns, the enantiomer elution order was (R)- before (S)-4-CMC in mobile phase containing bicarbonate buffer at pH=11.0 and (S)- before (R)-4-CMC in the mobile phase containing ammonium acetate, respectively. Thus, with both columns a reversal of the enantiomer elution order was observed in the mobile phases specified above.

As already mentioned, of the possible 7 different phase-1 metabolites of 4-CMC none were available for this study. Thus, a possible solution to his problem was to generate 4-CMC metabolites *in-vitro* or just try their identification in clinical or forensic samples. This last approach is in principle valid for the identification of metabolites, but it does not enable their quantification. Still, the identification of metabolites proved challenged in this particular case due the instability of 4-CMC and potentially of some of its metabolites in particular biological matrices (see the subsection 3.3 below).

Thus, in order to screen chiral columns for their ability to resolve the enantiomers and stereoisomers of 4-CMC and of its phase-1 metabolites the OF collected from one of the volunteers after 5 h of oral administration of 4-CMC was used as sample. No extensive mobile phase optimization was attempted, but only a few variations of the mobile phase composition were explored. Only positive results are discussed in this subsection and the numbering of metabolites is the same as in Fig. 1. The

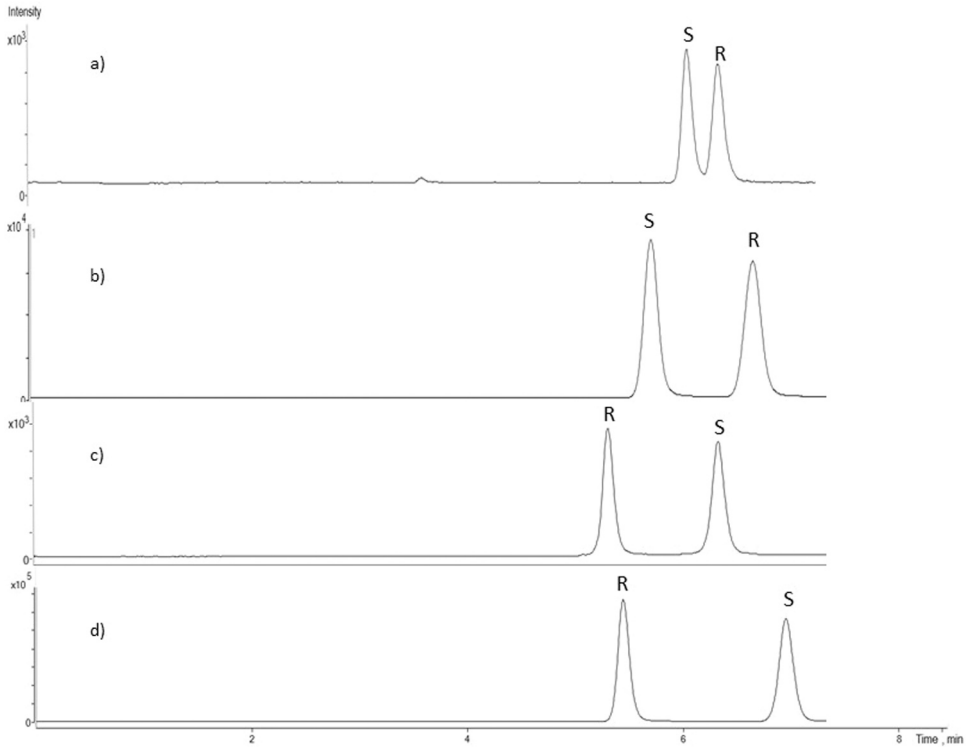

Fig. 2. HPLC-MS/MS chromatogram of 4-CMC enantiomers separated on Lux i-Amylose-3 (a, c) and Lux AMP (b, d) columns. The mobile phases were ACN + 3 % H_2O + 5 mM ammonium acetate (a, b) and methanol with 5 % (v/v) aqueous 5 mM ammonium bicarbonate pH=11.0 (c, d). MS/MS detection conditions were as described in Table 1.

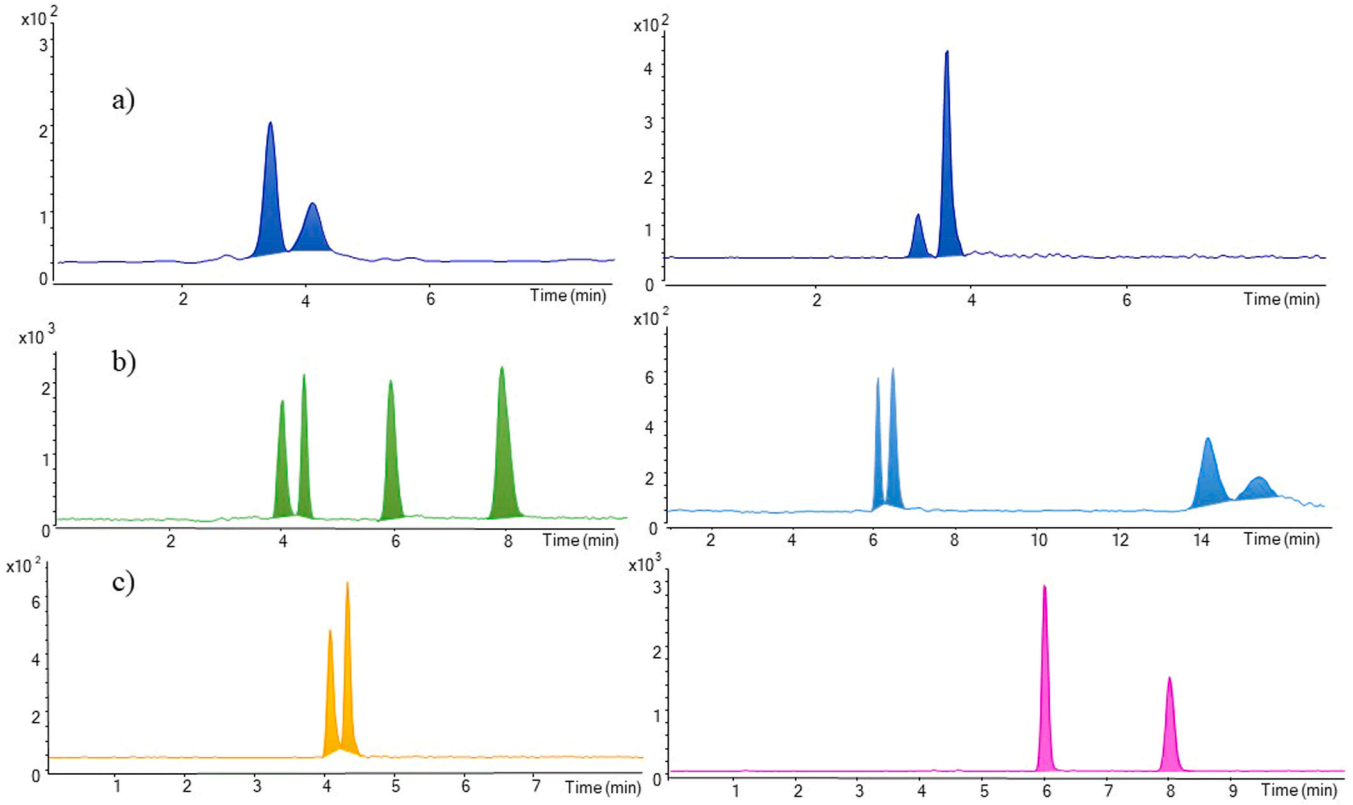

Fig. 3. Separation of the stereoisomers of metabolite **1** of 4-CMC (a), on Lux AMP and Lux Cellulose-1 (a) on the left and right, respectively, of metabolite **2** on Lux AMP and Lux i-Amylose-3 (b) on the left and right, respectively, and of metabolite **4** on Lux Cellulose-1 and Lux Cellulose-3 (c) on the left and right, respectively. Mobile phases were methanol with 5 % (v/v) 5 mM ammonium bicarbonate with pH=11.0 with Lux AMP and ACN + 3 % H_2O + 5 mM ammonium acetate with all other columns. MS/MS detection conditions were as described in Table 1.

enantiomers of metabolite **1** of 4-CMC containing one chiral center seem to be separated by the Lux AMP column in methanol-containing mobile phase (Fig. 3a, left), as well as by Lux Cellulose-1 (Fig. 3a, right), Lux Cellulose-3 and Lux i-Cellulose-5 columns (data not shown). The observed enantiomer elution order was opposite on the Lux AMP and on the Lux Cellulose-1 columns under the studied conditions (Fig. 3a). For 4-CMC metabolite **5** having 2 chiral centers and therefore four possible stereoisomers only 2 partially separated peaks could be observed with the Lux AMP column and 2 baseline separated peaks on the experimental column containing cellulose tris(3-chloro-5-methylphenylcarbamate) as chiral selector (data not shown). For the 4-CMC metabolite **2** also having 2 chiral centers and thus 4 stereoisomers, 4 well separated peaks could be observed with the Lux AMP and the Lux i-Amylose-3 columns (Fig. 3b, left and right, respectively). Metabolite **2,** considered to be one of the major metabolites of 4-CMC appears to be stable in biological fluids and is therefore recommended as biomarker of 4-CMC consumption [2,23]. Thus, baseline separation of all of its stereoisomers is useful for following any potential stereoselective biotransformation of 4-CMC. The diastereomer elution order of metabolite **2** was opposite to each other on Lux AMP and Lux i-Amylose-3 columns.

The enantiomers of 4-CMC metabolite **3** having a single chiral center were partially separated on Lux AMP and Lux i-Amylose-3 columns, close to baseline separated on the Lux Cellulose-1 column (Fig. 3c, left) and very well separated on the Lux Cellulose-3 column (Fig. 3c, right). For the 4-CMC metabolite **4** having one chiral center only a single peak was observed on all columns under the studied conditions (Data not shown). For 4-CMC Metabolite **6** having 2 chiral centers only 2 partially resolved peaks were observed on Lux AMP while these two peaks were baseline separated on Lux i-Amylose-3 (Data not shown).

Preliminary method validation was performed by using N-ethylpentedrone as internal standard (since deuterated 4-CMC was not available to our knowledge, to serve as internal standard).

3.3. Stability of CMC in human blood, OF and urine

The instability of synthetic cathinones and specifically of 4-CMC is quite well known [2,5–12,15] with Romanczuk et al. recently emphasizing the interpretative problems associated with the instability of cathinone derivatives in blood [23]. In order to cope with this problem in clinical toxicology and forensic analysis some authors recommended using its dihydrometabolite (metabolite **2**) as a biological measure of 4-CMC consumption [2,23]. Alternatively, converting 4-CMC and its nor-metabolite (4-chlorocathinone) in hydrochlorides was recommended in order to improve their stability after sampling [15]. In the early stages of this project, we observed the absence of 4-CMC in OF within 0–1.0 h (in some cases even after 1.5 h) after its administration, its fast disappearance from blood and its relatively longer persistence in OF and urine. Based on these observations we decided to devote part of the study to the stability of 4-CMC in blood, OF and urine. Various stability experiments were undertaken with more consistent results obtained with the experimental setup described in Section 2.6. The initial sample concentration in all 3 studied matrices were 100 ng/ml with one set of samples kept in a freezer at −20 °C and the other set kept at a room temperature (23 °C). The signal of 4-CMC disappeared from the blood sample kept at 23 °C within 33 h (Fig. 4a). Based on the detection limit of our method and the initial 4-CMC concentration in the stability samples, its half-life time when part of whole blood samples seems to be about 8 h at 23 °C. The half-life of 4-CMC under physiological conditions could be significantly shorter. This means that, whenever possible, blood samples must be immediately frozen after sampling. Otherwise, data generated for the identification or quantification of 4-CMC in blood samples should be only used with reservations in clinical-toxicological or forensic studies (or not be used at all). 4-CMC was more stable in OF and urine although significant degradation was also observed in these matrices within a few days (Fig. 4b). Our finding of higher stability of 4-CMC and of the closely related 3-CMC in urine compared to blood is in agreement with earlier studies [6].

The degradation rate of 4-CMC in water was strongly pH-dependent. The highest stability was observed at pH 3.0. Its stability decreased at pH 6.5 and it was the lowest at pH 10.3 (data not shown). 4-CMC was stable in methanol and quite stable in both mobile phases used in the present study (acetonitrile: water 97: 3 (v/v) +5 mM ammonium acetate, and methanol: 5 mM ammonium bicarbonate (pH-11.0) 90: 10 (v/v)). The stability of 4-CMC in the latter mobile phase seems quite surprising given its low stability in water under alkaline conditions as mentioned above. In order to examine the effect of the human serum albumin (HSA) on the potential adsorption/binding and chemical transformation of 4-CMC, the whole blood was replaced with serum as matrix. The results indicate that 4-CMC is more stable in serum compared to whole blood (data not shown). As expected, degradation rates of 4-CMC in the samples stored frozen were significantly lower in all studied matrixes compared to samples stored at room temperature. For instance, in blood samples spiked with 100 ng/ml 4-CMC and stored at −20 °C 4-CMC could still be detected after 150 h while in the same type of sample stored at the room temperature it fell under the limit of detection after about 33 h.

These stability studies are reported here in order to underline the challenges faced when trying to generate pharmacokinetic and metabolism data for 4-CMC. It is worth noting that the metabolites **2** and **3** of 4-CMC could not be detected by HPLC-MS/MS in any of the matrices of interest in this study and under any storage conditions. Thus, anytime these 2 metabolites get detected in the living body most likely they cannot originate from the degradation of 4-CMC, while information on any of the other metabolites has to be interpreted with great care.

3.4. Application of the analytical method to oral fluid

As mentioned above, 4-CMC is more stable in OF and urine rather

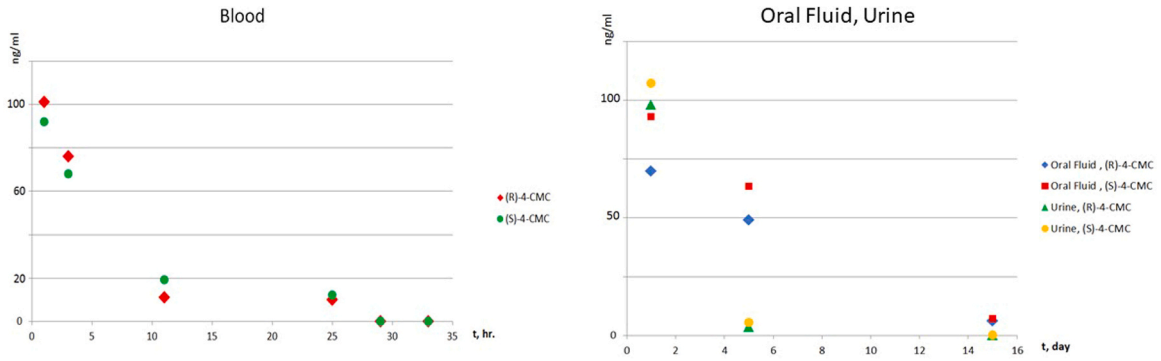

Fig. 4. C-t Dependences of 4-CMC incubated with whole blood (a), OF and urine (b) at 23 °C. The separation and detection conditions were as mentioned in the legend to Fig. 3 and in Table 1.

than in whole blood, plasma, or serum. Therefore, the use of OF and urine for the study of the pharmacokinetics and metabolism of 4-CMC seems more reliable. The C-t dependence of 4-CMC levels in OF of 3 volunteers is shown in Fig. 5. Interestingly, within the first 1 h after its administration 4-CMC cannot be detected in OF (Fig. 6a,b). Apparently, on the initial step of administration the entire dose of 4-CMC, absorbed into blood, gets rapidly transformed. After 2 h from 4-CMC administration, the intact drug and all the 4 stereoisomers of metabolite **2** are detectable in the OF (Fig. 6d). In order to confirm these results, the chromatograms for the OF sample after 5 h from drug administration are shown on both, Lux i-Amylose-3 (Fig. 7a) and Lux AMP (Fig. 7b) columns in Fig. 7. The following important conclusions can be drawn based on the pharmacokinetics study of 4-CMC in OF: 1) Within the first hour since ingestion 4-CMC does not seem detectable in OF and most likely also in blood. This observation is of significant forensic relevance and must be carefully considered; 2) Using the dihydro-metabolite of 4-CMC (metabolite **2**) as a biomarker of 4-CMC consumption may be acceptable but not absolutely reliable, since in the time interval (induction period) when 4-CMC is not detectable (0–1 h after administration), also its metabolite **2** is undetectable at least with the method proposed in the present study; 4) Of two quite contradictory opinions on the metabolic stability and clearance of 4-CMC recently published [7,14] perhaps its fast metabolism [7] is more plausible rather than its high metabolic stability and its low clearance [14]. 5) Based on the results of the present study 4-CMC's metabolism and pharmacokinetics in the human body does not seem to be enantioselective to a significant degree. However, we shall emphasize that our preliminary experiments on the stereochemical stability of 4-CMC showed that it is stereochemically extremely unstable. Thus, the results observed in the present study may be severely affected by after-sampling transformations/racemization.

3.5. Application of the method to urine samples

Cumulative urine samples collected over the time periods 0–2 and 2–5 h from drug administration were analyzed in the case of two volunteers. Both volunteers had received 150 mg single doses of 4-CMC. The data looked qualitatively similar in these two cases and therefore the urine samples of one of the two volunteers are discussed in more details below. The concentration of 4-CMC was lower in the cumulative urine sample collected within the 0–2 h period compared to the 2–5 h period. A small but reliable difference was observed in the concentration levels of 4-CMC enantiomers. This difference was confirmed with 2 chiral columns that provided a reversal in the elution order of 4-CMC enantiomers (Fig. 8). However, the observed results are too preliminary (considering all the challenges mentioned above) for drawing any conclusions on the enantioselective metabolism of 4-CMC and its clearance. It has to be mentioned that one of the stereoisomers of metabolite **2** was detected at significantly lower concentration with both chiral columns (Fig. 8). Thus, marked stereoselectivity from this point of view seems to be present. A significant increase in the response of 4-CMC enantiomers, as well as of the enantiomers and stereoisomers of Metabolite **2** were observed following the acid hydrolysis of urine samples. This finding indicates that not only O-glucuronides but apparently N-glucuronides get also formed since at least for 4-CMC only N-glucuronide is possible based on its structure. It is interesting to note that in the OF of the same volunteer, 4-CMC cannot be detected between 0 and 1 h from its administration while it is easily detectable in the cumulative urine sample collected between 0 and 2 h after 4-CMC administration. Unfortunately, the urine sample within the 0–1 h time period was not available from the present clinical study in order to conclude if 4-CMC can be detected in the urine collected in the first hour from its consumption while it is not detectable in OF.

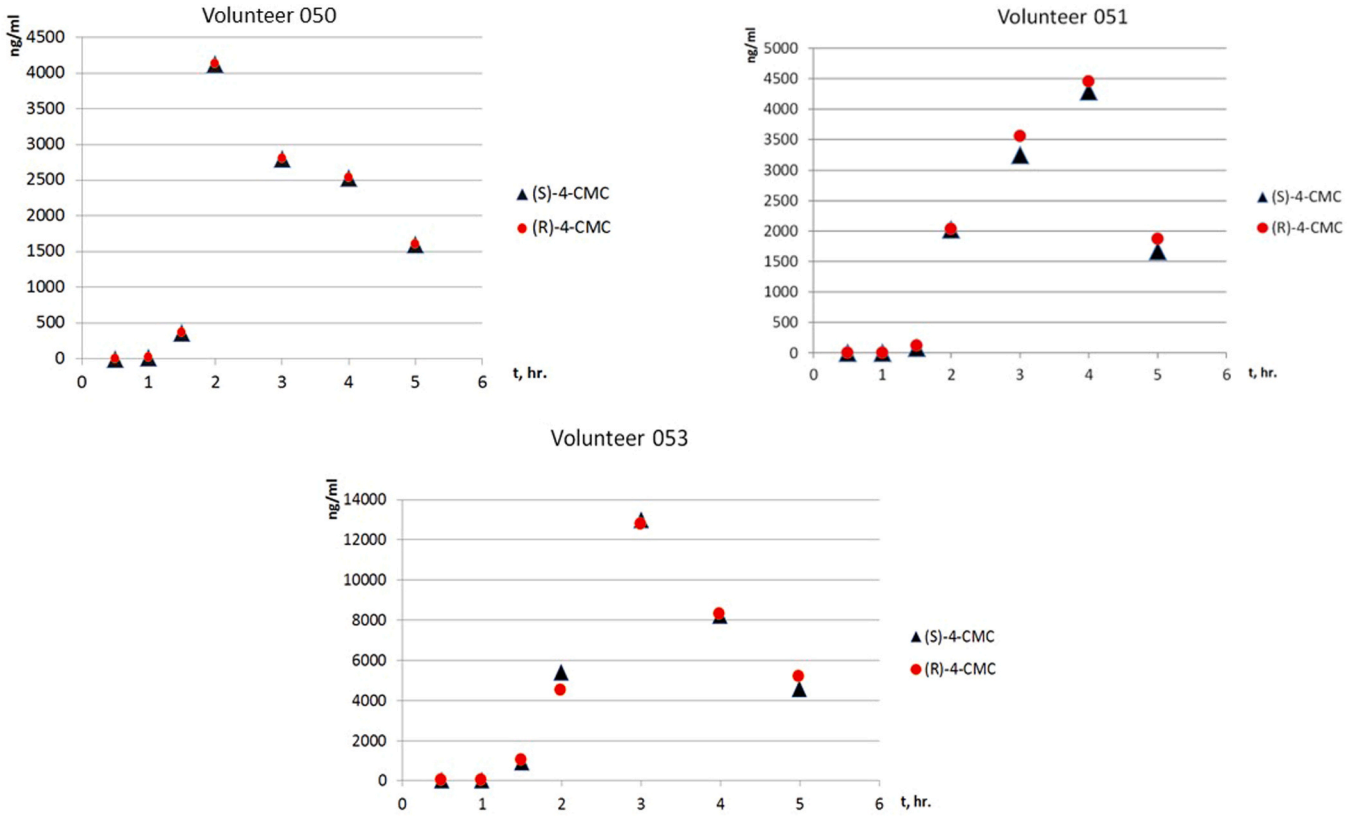

Fig. 5. C-t Dependences for enantiomers of 4-CMC in the OF of 3 volunteers. The samples were analyzed on both chiral columns Lux AMP and Lux i-Amylose-3 and the averaged concentrations were plotted vs. time after administration.

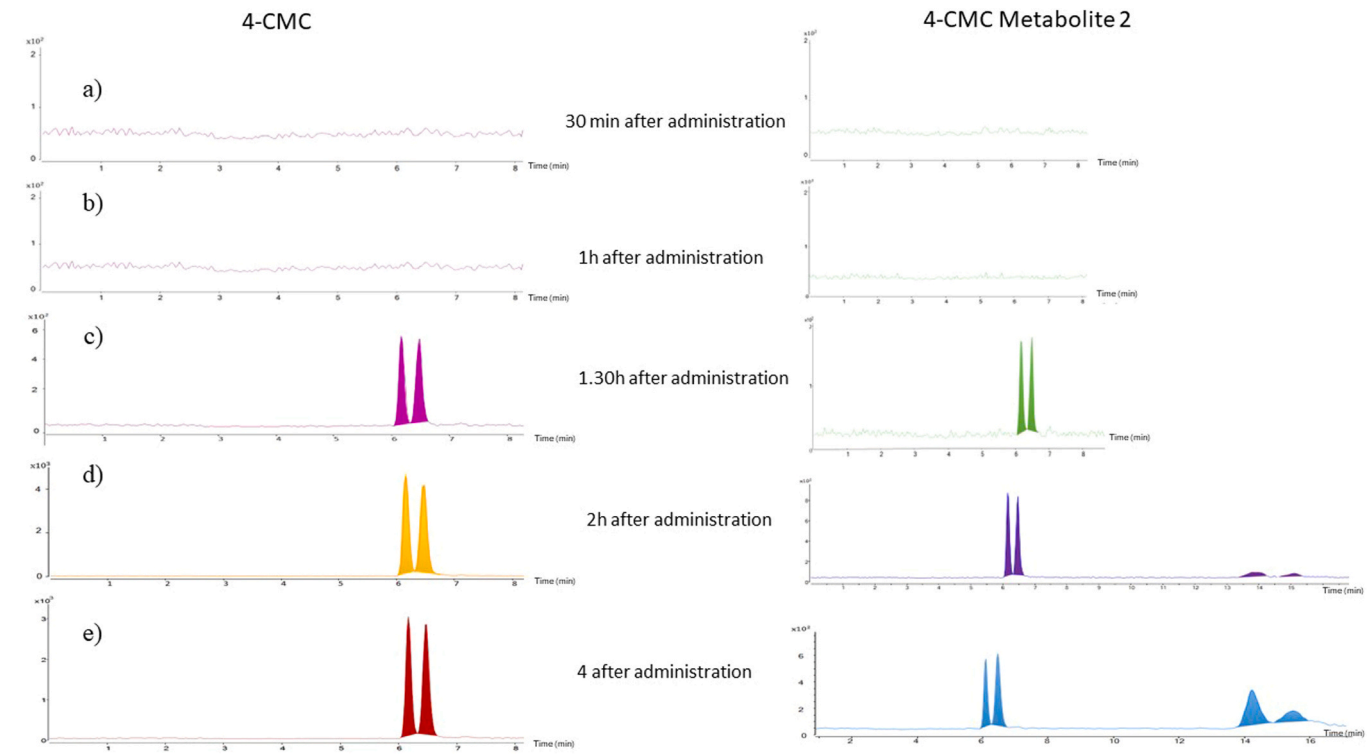

Fig. 6. Selected ion chromatograms for 4-CMC and its metabolite **2** after different times of drug administration on Lux i-Amylose-3 column. The separation and detection conditions were as mentioned in the legend to Fig. 3 and in Table 1.

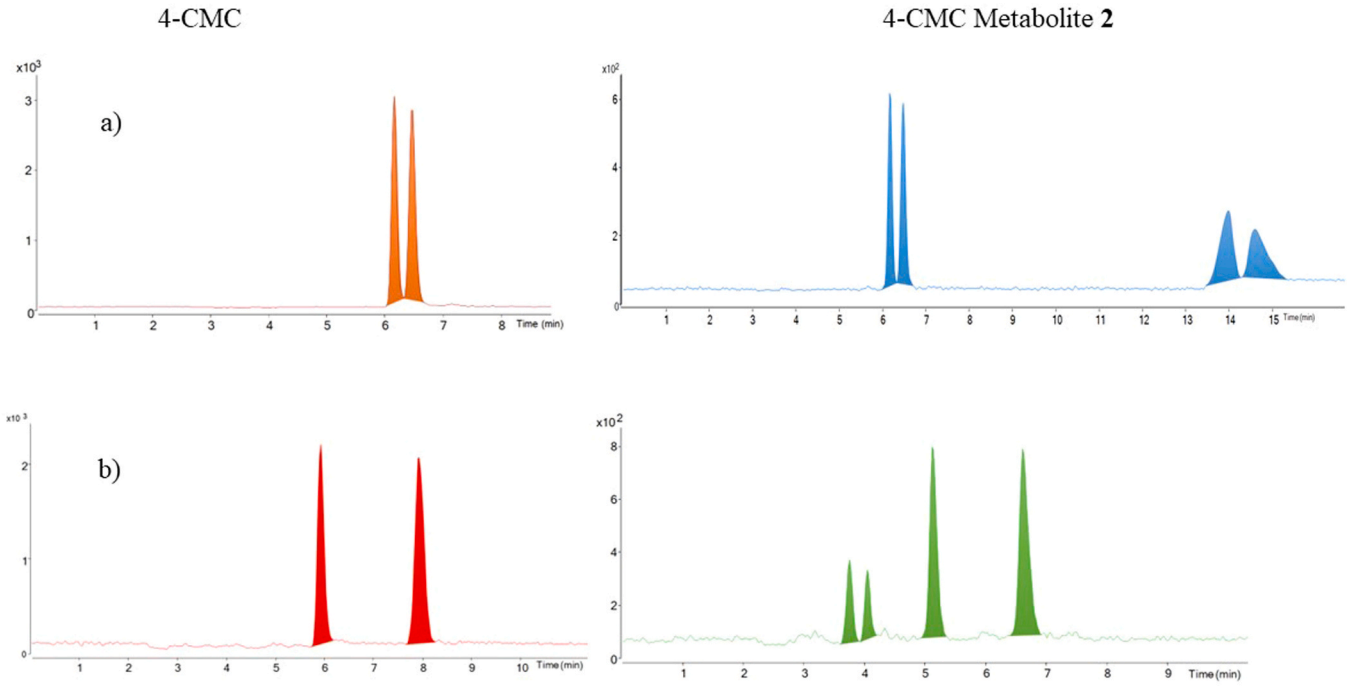

Fig. 7. Selected ion chromatograms for 4-CMC and its metabolite **2** after 5 h of drug administration on Lux i-Amylose-3 (a) and Lux AMP (b). The separation and detection conditions were as mentioned in the legend to Fig. 3 and in Table 1.

4. Conclusions

Bioanalysis of new psychoactive substances (NPS) faces significant challenges due to absence of related standards, especially for NPS metabolites. When NPS is chiral then additional challenges are raised by the absence of enantiomerically pure standards, as well as due to the significant increase in the number of targeted species (enantiomers and stereoisomers) to be separated. If the NPS is a synthetic cathinone, then the lack of stability of the parent compound and its metabolites in biological matrices and solvents used for sample preparation and chromatographic separation creates additional problems. All of these issues have to be carefully considered in order to develop a reliable

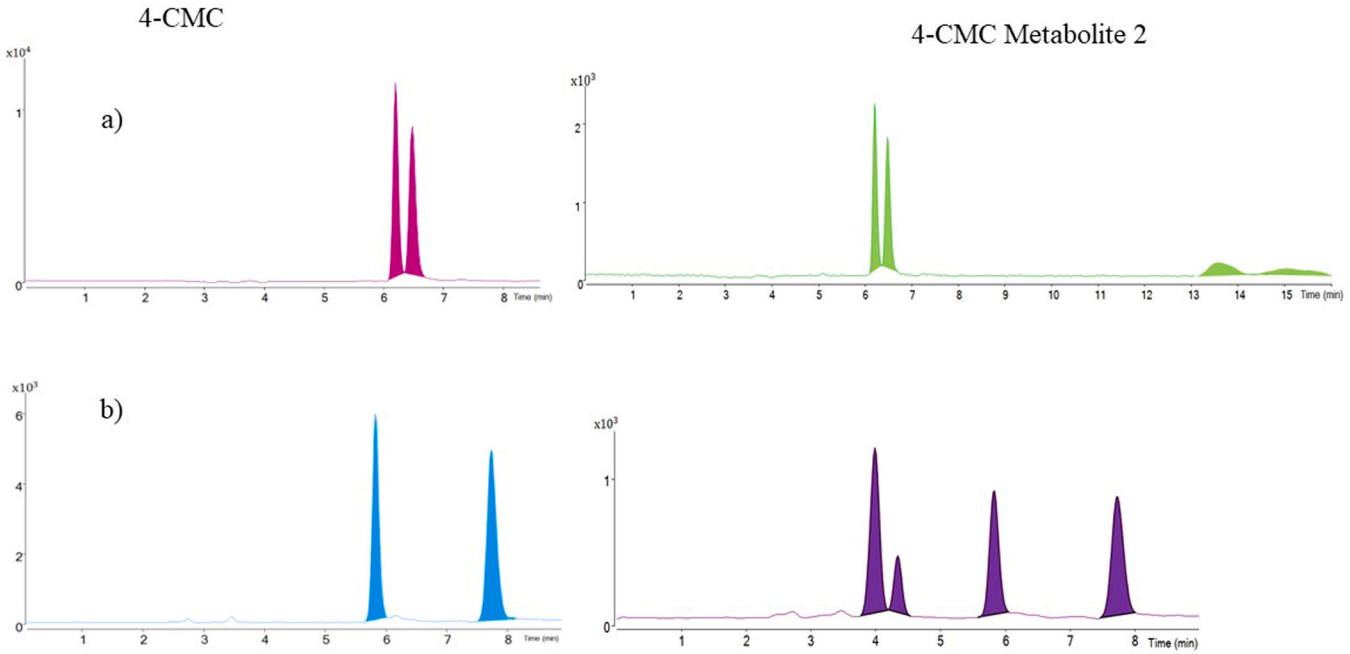

Fig. 8. Selected ion chromatograms of urine samples of a volunteer collected within the 0–2 h period after administration of a 150 mg dose of 4-CMC analyzed on Lux i-Amylose-3 (a) and Lux AMP (b). The separation and detection conditions were as mentioned in the legend to Fig. 3 and in Table 1.

enantioselective bioanalytical method for the simultaneous analysis of a (chiral) NPS and its pharmacologically relevant metabolites in biological matrixes. Some of these problems were highlighted in the present work based on the example of 4-CMC and in some cases possible solutions to these challenges are discussed. The HPLC-MS/MS method described in this study enables simultaneous enantioselective quantitative determination of 4-CMC and its phase-1 metabolites in biological fluids. If one decides to extend such a study to phase-2 metabolites, then apparently even more efficient methods, such as capillary electromigration techniques coupled with MS or two-dimensional chromatography coupled with MS have to be used.

CRediT authorship contribution statement

Saba Jorbenadze: Validation, Supervision, Methodology, Investigation, Formal analysis. **Tamar Khatiashvili:** Validation, Supervision, Methodology, Investigation, Formal analysis. **Lasha Giunashvili:** Validation, Supervision, Methodology, Investigation, Formal analysis. **Aluda Tchelidze:** Validation, Supervision, Methodology, Investigation, Formal analysis. **Alfredo Fabrizio Lo Faro:** Validation, Supervision, Methodology, Investigation, Formal analysis. **Simona Pichini:** Resources, Methodology, Conceptualization. **Magi Farré:** Writing – review & editing, Supervision, Resources, Methodology, Investigation. **Esther Papaseit:** Writing – review & editing, Resources, Methodology, Investigation. **Melani Nuñez-Montero:** Writing – review & editing, Resources, Methodology, Investigation. **Jeremy Carlier:** Validation, Investigation, Formal analysis. **Tivadar Farkas:** Writing – review & editing, Resources. **Francesco Paolo Busardo:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis. **Bezhan Chankvetadze:** Writing – review & editing, Writing – original draft, Supervision, 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.

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