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Metabolic profile of N-ethylhexedrone, N-ethylpentedrone, and 4-chloromethcathinone in urine samples by UHPLC-QTOF-HRMS

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ABSTRACT

Forensic laboratories are constantly required to identify new drugs and their metabolites. N-ethylhexedrone (NEH, HEXEN), N-Ethylpentedrone (NEP), and 4-Chloromethcathinone (4-CMC, clephedrone) are synthetic substances structurally related to natural cathinone, alkaloid present in the leaves of the Catha edulis (Khat) plant. These synthetic cathinones (SC) are members of the heterogenous family of new psychoactive substances (NPS) that raised major concerns in scientific and forensic communities over the past years due to their widespread consumption. In this context, we investigated their metabolic profile using of UHPLC-QTOF-HRMS to elucidate the distribution of the parent drug and its metabolites in urine samples over time. Initially, both male and female volunteers were divided into three groups and eight subjects of each group were administered intranasally or orally with one SC (20–40 mg of NEH or NEP intranasal, 100–150 mg of 4-CMC oral). Urine samples were collected at 0–2 and 2–4 or 2–5 h. Urine (50 µL) was diluted 1:2 with acetonitrile/methanol (95:5) and injected into the UHPLC-QTOF-HRMS. Phase-I and phase-II metabolites were identified on the basis of fragmentation patterns and exact masses. Several phase-I and glucuronide-phase-II metabolites were identified in urine samples. Keto group reduction, hydroxylation and dealkylation were the common metabolic pathways identified for all cathinones and the presence of NEH-glucuronide, NEP-glucuronide and 4-CMC-glucuronide was also relevant. Significant is the slower metabolite formation for 4-CMC, which was detected at high concentrations in its original form even 5 h after administration, due to its long half-life and low intrinsic clearance compared to the other SCs. UHPLC-QTOF-HRMS demonstrated a considerable capability to semi-quantify the three synthetic cathinones and identify the target metabolites with high reliability. The introduction of new target compounds improves the efficiency of toxicological screening analysis on real samples and extends the window of detection of the SCs in biological matrices.

1. Introduction

Nowadays, novel psychoactive substances (NPS) abuse in U.S [1] and Europe is proliferating at unprecedented rate and represents an increasing challenge to the established national and international drug policies. At the end of 2022, the EMCDDA was monitoring around 930 new psychoactive substances[2] and 41 were first reported in Europe in 2022. Generally, the NPS are categorized as synthetic cannabinoids, stimulants, depressant (benzodiazepines and opioids), and hallucinogens, but this classification does not express adequately the variety and complexity of their potency, combined effects, and risk profiles that intersect categories and often differentiate compounds belonging to the same category [3]. After synthetic cannabinoids, synthetic cathinones (SCs) were the second-largest category of NPS monitored by the EU Early Warning System and at the end of 2021 the EMCDDA monitored 162 cathinones [4]. SCs appeared in drug markets in the mid-2000 s.

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Since their first appearance on the recreational drug market, these synthetic substances have changed their chemical structure mainly in an attempt to evade law enforcement. In recent years, both the US Drug Enforcement Agency and Council of the European Union have implemented control measures to prohibit their use and there has subsequently been a decline in their use [5].

Most of the cathinone seizures are powder, together with pills and similar products. These compounds have also been found as adulterants in "classical" illegal drugs such as cocaine or MDMA, suggesting that their prevalence of consumption could be underestimated [6], [7]. Their consumption represents an important public health problem, according to the most recent report from the United Nations Office on Drugs and Crime[1]. In addition to the data obtained by seizure analysis, the public health problem related to cathinones is also illustrated by numerous intoxication cases related to these substances, and even some fatalities [8–10]. When the first generation synthetic cathinones (methylone, mephedrone, MDPV) became illegal, a second and third generation emerged and a lack of information exists on their molecular structure and metabolic pathway, making their identification in biological samples challenging. This is critical in the case of cathinones that undergo extensive/fast metabolic degradation, for which the identification of metabolites in urine constitutes the only possible way of attesting their consumption, in forensic and clinical contexts [11]. Therefore, understanding the mechanisms of action of these new drugs and the knowledge of their short- and long-term effects, their pharmacokinetic properties, and the correlation between the concentration in biological fluids and their activity has become crucial for public health reasons.

The main objective of the study presented here was indeed to develop a method for the investigation of the main metabolic pathways of the latest generation synthetic cathinones (N-ethylhexedrone, N-ethylpentedrone and 4-chloromethcathinone) in urine of both male and female human volunteers. Furthermore, the simultaneous semi-quantification of these synthetic cathinones was carried out in order to observe the variation of their concentration simultaneously with the formation of the metabolites. High performance of ultra-high-pressure liquid-chromatography (UHPLC) was combined with quadrupole time-of-flight high-resolution mass spectrometry (QTOF-HRMS) allowing the prediction of the metabolic profile on the basis of similar studies on analogous synthetic cathinones. Using this approach, it has been possible to hypothesize and confirm the structures of the main phase I and II metabolites, their exact masses, and their fragmentation patterns. Several studies on the metabolism of these three cathinones can be found in the literature; however, none of them studied the actual metabolic pattern in human urine but only attempt to reproduce the phenomenon in mice [12], human liver microsomes [13] or in vitro [14]. In the latter studies, the formation of metabolites was observed following reduction, glucuronidation, hydroxylation and combined N-deethylation and N-acetylation, N-deethylation and succinic conjugation and N-deethylation and adipic conjugation reactions. The presence of these metabolites, however, was observed after many hours (even 24 h) of exposure to the substances.

2. Materials and methods

2.1. Reagents and standards

All chemicals, including HPLC grade methanol, formic acid, and acetonitrile, were purchased from Sigma-Aldrich (Milan, Italy). Ultrapure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). For UHPLC-QTOF-HRMS semi-quantitative analysis, mephedrone-D_3 was purchased from LGC Promochem (Milan, Italy) (purity >99%, concentration 1 mg/mL). N-ethylhexedrone (NEH), N-Ethylpentedrone (NEP), and 4-Chloromethcathinone (4-CMC) were kindly provided Cayman Chemical (Ann Arbor, Michigan, USA) (methanolic solution at a 0.1 mg/ mL concentration, purity provided by the supplier >99%). All working solutions were prepared in methanol at 1 μg/mL and stored at − 20 °C until used.

2.2. Study protocol

The urine samples used in this study were obtained from, 13 women (mean age: 32.2 years (range 24–54) and 11 men (mean age: 31.0 (range 23–40) enrolled at the Hospital Universitari Germans Trias i Pujol (HUGTiP-IGTP), in Badalona, Spain. The participants were recruited by word of mouth and snowball sampling through the harm reduction, non-governmental organization Energy Control (ABD). The protocol to investigate the potential for abuse and the human pharmacology of substances of abuse, including synthetic cathinones and cannabinoids, was approved by the local human research ethics committee (CEI-HUGTiP ref. PI-18–267). The study was conducted according to the Declaration of Helsinki recommendations (Fortaleza, 2013 and Spanish law on clinical investigation). All the participants were informed, both orally and in writing, and signed an informed consent prior to inclusion. The participants received monetary compensation for their participation. All participants had past recreational experience with cocaine, amphetamines, MDMA and synthetic cathinones, but they declared no recent use of the three cathinones under study. The 24 participants were divided into 3 groups of 8 subjects each: G1, self-administration of NEH intranasally, G2, self-administration of NEP intranasally and, G3, self-administration of 4-CMC orally. Each subject participated in only one session and so they only consumed one of the three cathinones under study. The doses and routes of administration of these substances were chosen on the basis of information obtained from internet forums, which is the main channel used to obtain this type of information for the time being and were in the range of those recommended in risk reduction organizations.

The study design was observational, naturalistic, and prospective, with minimal intervention. The sessions took place on three different days, one for each substance, 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 8:00–9:00 p.m. All the doses were self-administered and were also self-selected by each participant, based presumably on their previous experience. The drug samples were tested by Energy Control, a harm reduction organization that provides a drug checking service for users. Measures of the pharmacological effects were collected (data not presented in this manuscript). The study

Table 1
Characteristics six volunteers participating in the study and dosages administered.

Group	Compound	Volunteer	Sex	Dosage (mg)
G1	**NEH**	33	Female	30 mg
		34	Male	40 mg
		35	Male	30 mg
		36	Male	40 mg
		37	Female	30 mg
		38	Male	40 mg
		39	Male	30 mg
		40	Female	20 mg
G2	**NEP**	41	Male	40 mg
		42	Male	40 mg
		43	Female	20 mg
		44	Male	30 mg
		45	Male	40 mg
		46	Female	30 mg
		47	Female	30 mg
		48	Female	40 mg
G3	**4-CMC**	49	Female	150 mg
		50	Male	100 mg
		51	Female	100 mg
		52	Female	150 mg
		53	Male	150 mg
		54	Female	100 mg
		55	Female	100 mg
		56	Female	150 mg

methodology was similar to that of other previously published studies [15], [16]. Table 1 shows the characteristics of the study volunteers. Urine samples were collected at two ranges of time: G1 and G2 at 0–2 h and 2–4 h while for G3 at 0–2 and 2–5 h after consumption. All samples were collected in 1.5-mL tubes and stored at − 20 °C before the analysis. The evolution of the three synthetic cathinones and their metabolites over time was monitored, noting the differences between male and female metabolic pathways and observing how they formed and evolved in the two-time ranges studied. Regarding the influence of the age factor on metabolism, the age groups of the volunteers were narrow (range 25–41 years, only one subject was 51 years old) and preliminary results did not indicate any age-related differences. Furthermore, there are no articles in the literature focusing on the effect of age in the metabolism of cathinone or similar compounds. There are several studies [17], [18] defining age as a key factor influencing the expression of cytochromes P450. Cytochromes involved in phase I metabolism. We will take advantage of this suggestion to investigate in the future.

2.2.1. Statistical data processing

The collected data were evaluated in the form of boxplots [19], comparing the time ranges of 0–2 h vs. 2–4 h or 2–5 h. Different boxplots were created in order to observe possible differences in the presence of the cathinones and their metabolites in urine samples as a function of dosage and gender. Boxplots are valuable tools in data analysis, which allow easy comparison of multiple groups and identification of data skewness and potential outliers within a dataset. In addition, Wilcoxon signed-rank test [20] was employed as non-parametric statistical test used to determine whether there was a significant difference between the paired observations in a dataset, in terms of sex and dosage, by comparing the two-time ranges examined. Wilcoxon test is an alternative to the paired t-test and is particularly useful when the data do not meet the assumptions required for parametric tests, such as when the data are not normally distributed or when the sample size is small, as in this case.

2.3. Urine samples collection and preparation

Briefly, 50 μL of the urine samples were initially added with the internal standard (mephedrone-D3 at final concentration of 100 ng/mL), and then they were diluted 1:2 with a frozen acetonitrile/methanol (95:5) mixture and vigorously stirred for 5 min. After centrifugation for 5 min at 13,000 g, 5 μL of the supernatant was directly injected into the UHPLC system. No enzymatic hydrolysis of glucuronides was performed on phase II metabolites. In order to semi-quantify the possible presence of the three synthetic cathinones in the urine samples, and as the study did not include validation of the method used, a urine matrix previously tested as negative was used for the preparation of the calibration curve. The negative matrix was, therefore, fortified at five concentration levels (100, 500, 1000, 5000, 10,000 ng/mL) with each of the NEH, NEP and 4-CMC working solution and mephedrone-D3 was used as the internal standard (ISTD). The choice of calibration levels was based on the first publication concerning this experiment relating to the administration of cathinones to the healthy volunteers and the first investigation of their levels in real samples. [21].

2.4. Instrumental conditions

UHPLC separation was performed on the SCIEX ExionLC™ AC system (Sciex, Darmstadt, Germany) using a Phenomenex Kinetex C18 column (100 ×2.1 mm, 1.7 μm) maintained at 45 °C. The mobile phase was a mixture of water (A) and acetonitrile (B), both with 0.01% of formic acid. The LC flow rate was set at 0.5 mL/min and the mobile phase eluted under the following linear gradient conditions: (A:B, v-v) isocratic elution at 95:5 for 0.5 min, from 95:5 to 5:95 in 7.5 min, isocratic elution at 5:95 for 0.5 min and final re-equilibration for 2.5 min to the initial condition. The total run time was 10 min. All analyses were performed using a quadrupole/time-of-flight SCIEX X500R QTOF mass spectrometer (Sciex, Darmstadt, Germany) equipped with a Turbo VTM ion source operating in positive-ion electrospray ionization mode (full MS and MS/MS parameters are available in Table S1). Data acquisition involved a preliminary TOF-MS high-resolution full scan followed by a SWATH™ acquisition protocol which used a variable window setup (18 windows covering mass range from *m/z* 100.0 to 500.0 at 0.025 resolving power), resulting in a final cycle time of 0.933 s. The variable windows technique allows the reduction of the size of the Q1 window in order to further improve the quality of the SWATH acquisition data, while maintaining a complete coverage of the mass range and optimal cycle times. In this case it was decided to use 22-Da windows as they allowed an optimal acquisition of the peaks, improving the specificity and reducing interference from possible co-eluting analytes. The qualitative identification of the target analyte (NEH, NEP and 4-CMC) was based on the coincidence of its retention times, precursor ion and characteristic fragment ion *m/z* values, while the tentative metabolites were identified by their fragmentation patterns and the exact masses of both their precursor and fragment ions (accepted mass error <5 ppm). To ensure the reliability of the data acquired by the instrument, an automatic calibration was set up every three samples using a solution of calibrators supplied by SCIEX. Data were acquired using the SCIEX OS 1.5 Software and raw data files were processed using the MarkerViewTM software from Sciex.

3. Results and discussion

3.1. Metabolism of NEH and NEP

In-house accurate mass library was built, considering the metabolic pathways already known for other structurally similar cathinones. NEH and NEP metabolites were investigated based on mephedrone (4-MMC) metabolism due to the similarity of its molecular structures [22–24]. Thus, similar metabolic reactions can be assumed, including reduction, hydroxylation, N-dealkylation and di-hydrogenation, similarly to 4-MMC. Figs. 1 and 2 shows the expected phase I metabolites of NEP and NEH compounds. The conjugation reactions with glucuronic acid (phase II metabolism) were expected and these metabolites' structures were confirmed by the high-resolution mass spectra corresponding to new chromatographic peaks appearing in the urine samples collected after NEH and NEP administration. Candidate metabolites were singled out from the chromatographic profile of the full-scan analysis by checking the exact mass of the corresponding protonated molecular ion. Then, the elemental composition of the relative fragment ions and the rationality of its fragmentation pattern was checked in the MS/HRMS spectra to confirm the tentative metabolite's identification (Supplementary Figs. S1 and S2). In accordance with the literature [25], [26], a total of six metabolites (4 phase I and 2 phase II metabolites) for NEH and five for NEP (3 phase I and 2 phase II metabolites) were identified in urine samples, including phase I and glucuronated phase II metabolites. In detail, 1) at *m/z* 192.1382, nor-NEH, the metabolite formed by dealkylation of the ethyl chain, which subsequently affords hydroxy-nor-NEH upon reduction of α-keto group, observed 2) at *m/z* 194.1539. 3) at *m/z* 222.1852, hydroxy-NEH, stemming for reduction of the NEH keto group; 4) at *m/z* 238.1801, dihydroxy-NEH following hydroxylation of the aromatic ring. Finally, 5) NEH-Gluc and 6) NEH-nor-Gluc, respectively, by conjugation with glucuronic acid and subsequent reduction of the α-keto group The same metabolites were identified for NEH and NEP, except for the di-hydroxylated form, which was only observed for NEH. This similarity in the metabolic pattern is due to the similarity of the two structures. In fact, NEH has only one more methyl in the alkyl chain than NEP. No sulfated metabolites were found (Table 2). This conjugation reaction represents a metabolic step that seems to be important in humans compared to glucuronidation and for this reason further experiments will be considered to confirm or exclude the presence of the sulfate conjugates in humans.

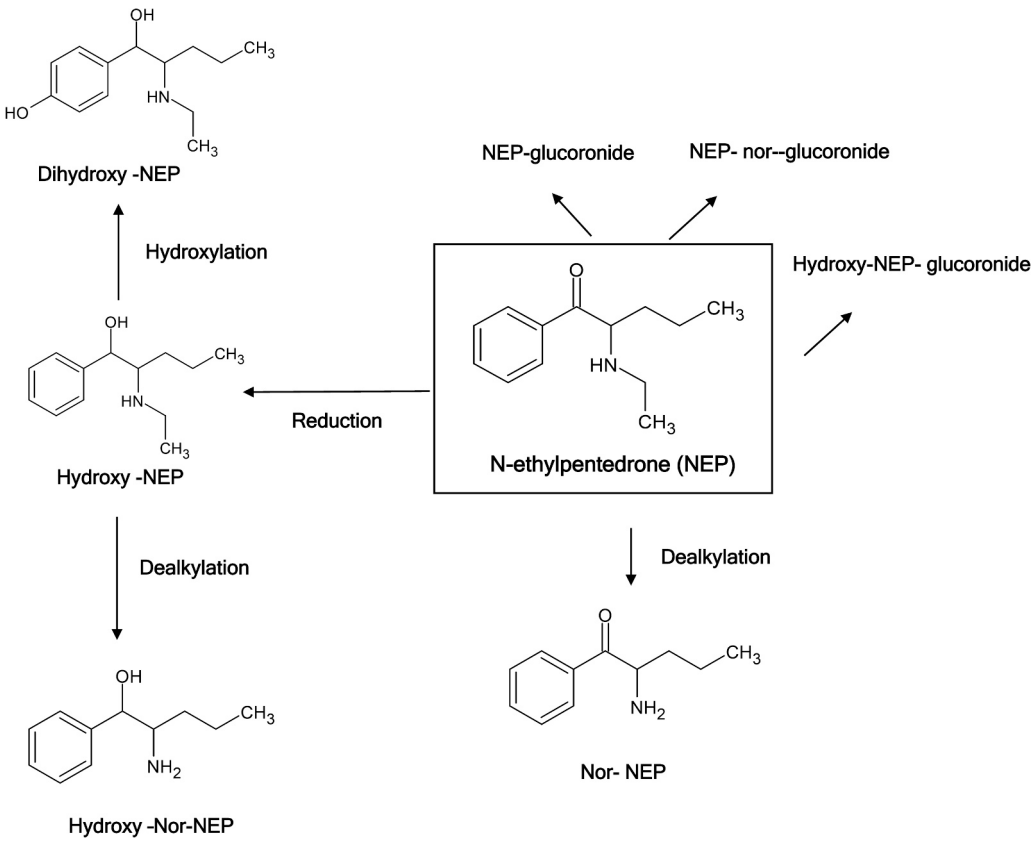

Fig. 1. Expected metabolic reactions of NEP.

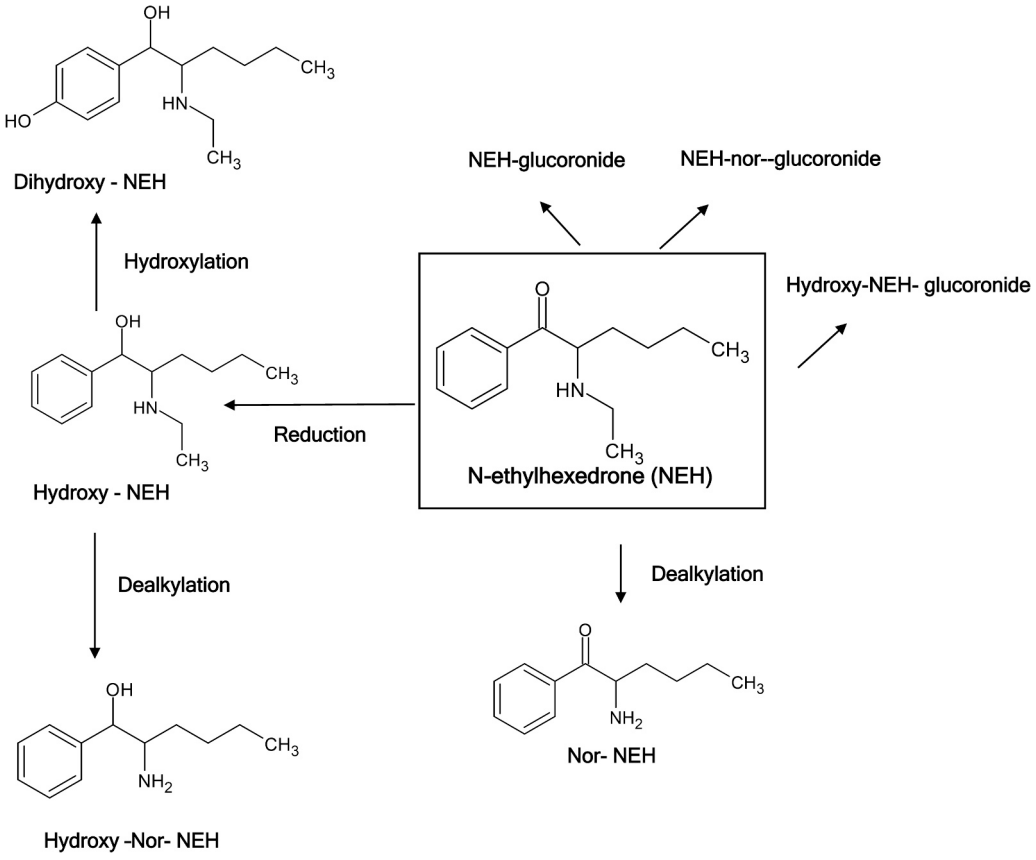

Fig. 2. Expected metabolic reactions of NEH.

Table 2
Name, elemental composition, exact (theoretical) protonated mass, mass error found and retention time of the synthetic cathinones and their hypothesized metabolites in UHPLC-QTOF-HRMS.

ID Compound	Elemental composition	$[M+H]^+$	t_r (min)	Found in urine 0-2 h (error mass <± 5 ppm)	Found in urine 2-4 or 2/5 h (error mass <± 5 ppm)
4-CMC	$C_{10}H_{12}ClNO$	198.0681	2.2	yes	yes
Nor-4-CMC	$C_9H_{10}ClNO$	184.0523	2.8	no	yes
Hydroxy-nor-4-CMC	$C_9H_{12}ClNO$	186.0680	0.4	no	yes
Hydroxy-4-CMC	$C_{10}H_{14}ClNO$	200.0836	2.2	yes	yes
Nor-4-CMC-Gluc	$C_{15}H_{20}ClNO_7$	362.1001		no	no
4-CMC-Gluc	$C_{16}H_{22}ClNO_7$	376.1157	7.8	yes	yes
N-Ethylhexedrone (NEH)	$C_{14}H_{21}NO$	220.1695	2.3	yes	yes
Nor-NEH	$C_{12}H_{17}NO$	192.1382	2.4	yes	yes
Hydroxy-NEH	$C_{14}H_{23}NO$	222.1852	2.6	yes	yes
Hydroxy-nor-NEH	$C_{12}H_{19}NO$	194.1539	2.3	yes	yes
Dihydroxy-NEH	$C_{14}H_{23}NO_2$	238.1801	2.5	yes	yes
NEH-Gluc	$C_{20}H_{31}NO_7$	398.2173	2.3	yes	yes
NEH-nor-Gluc	$C_{18}H_{27}NO_7$	370.1860	8.4	yes	yes
Hydroxy-NEH-Gluc	$C_{20}H_{31}NO_7$	398.2173		no	no
N-Ethylpentedrone (NEP)	$C_{13}H_{19}NO$	206.1539	2.3	yes	yes
Nor-NEP	$C_{11}H_{15}NO$	178.1226	2.2	yes	yes
Hydroxy-NEP	$C_{13}H_{21}NO$	208.1695	2.3	yes	yes
Hydroxy-nor-NEP	$C_{11}H_{17}NO$	180.1382	2.3	yes	yes
Dihydroxy-NEP	$C_{13}H_{21}NO_2$	224.1645		no	no
NEP-Gluc	$C_{19}H_{29}NO_7$	384.2016	3.3	yes	yes
NEP-nor-Gluc	$C_{17}H_{25}NO_7$	356.1703	2.4	yes	yes
Hydroxy-NEP-Gluc	$C_{19}H_{29}NO_7$	384.2016		no	no

Information on the concentration of NEH and NEP, their presence together with their metabolites in urine, the trends with which they occur in the two different time ranges (0–2 and 2–4 h) and their possible different expression in male and female samples are shown in Table 3, Fig. 3 and in supplementary Figs. S3-S9. Concerning NEP, the trends of variation (evidenced by the trend line joining the same samples analysed in the two-time ranges) in the abundances of the compound and its metabolites can be observed in Fig. 3 (30 mg dose) and Fig. S3 (40 mg dose). Particularly interesting are the trends in Fig. 3, which show that all metabolites increase in abundance after 2 h after intake, while NEP decreases; this phenomenon is not observed in the case of the 40 mg. It can therefore be assumed that an increase of 10 mg in the dose taken may cause a slowdown in metabolic processes or a saturation of the NEP metabolic pathway.

Concerning NEH, the trends of variation in the abundances of the compound and its metabolites can be observed in Fig. S6 (30 mg dose) and Fig. S7 (40 mg dose). For both the doses, only the increase in the signal intensity of the metabolite hydroxy-NEH, 2 h after sample administration, can be appreciated; whereas for the other metabolites, no particular trend is observed between the two-time ranges. The inclusion of both male and female volunteers aims to consider the sexual dimorphism potentially related to pharmacodynamic and pharmacokinetic mechanisms that typically characterize NPS profiles [27]. In fact, such sex-related differences have already been described in the literature as variability factors influencing the pharmaco-toxicological benchmarks of various therapeutic drugs [28]. The study of this phenomenon for NEP and NEH compounds can be observed in Figs. S4, S5 and S8, S9. However, only the hydroxy-NEP metabolite appears to be affected by a sex-related effect, showing a significant increase in women 2 h after consuming the substance (Fig. S4).

3.2. Metabolism of 4-CMC

As expected, the metabolic profile of this cathinone (Fig. 4) was very similar to the one observed for 4-chloroethoxyquinone (4-CEC) [29]. Three Phase I metabolites were identified (Table 2): 1) at *m/z* 200.0836, hydroxy-4-CMC, stemming from reduction of the 4-CMC keto group; 2) at *m/z* 184.0523, nor-4-CMC, the metabolite formed by N-demethylation, which subsequently affords hydroxy-nor-4-CMC upon reduction of α-keto group, observed 3) at *m/z* 186.0680. Similar to NEP and NEH, the phase II metabolite 4-CMC-glucoronate was identified and no sulphated metabolites were found. Candidate metabolites were identified from the chromatographic profile of the full-scan analysis by checking the exact mass of the corresponding protonated molecular ion, and the elemental composition of the relevant fragment ions was checked in the MS/HRMS spectra to confirm the identification of the provisional metabolite. An

Table 3
Concentration (ng/mL) of NEH, NEP and 4-CMC in urine sample.

NEH								
Volunteer	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
Time (h)	**Urine (ng/mL)**							
0-2	1630	403	290	484	309	no sample	15	887
2-4	2030	222	325	406	383	202	11	87
NEP								
Volunteer	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**
Time (h)	**Urine (ng/mL)**							
0-2	8140	20	no sample	909	159	372	492	361
2-4	1770	347	1160	857	1230	504	483	119
4-CMC								
Volunteer	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**
Time (h)	**Urine (ng/mL)**							
0-2	437	1270	429	156	601	4620	20300	126
2-5	8210	1100	7770	4460	5530	4280	5910	11500

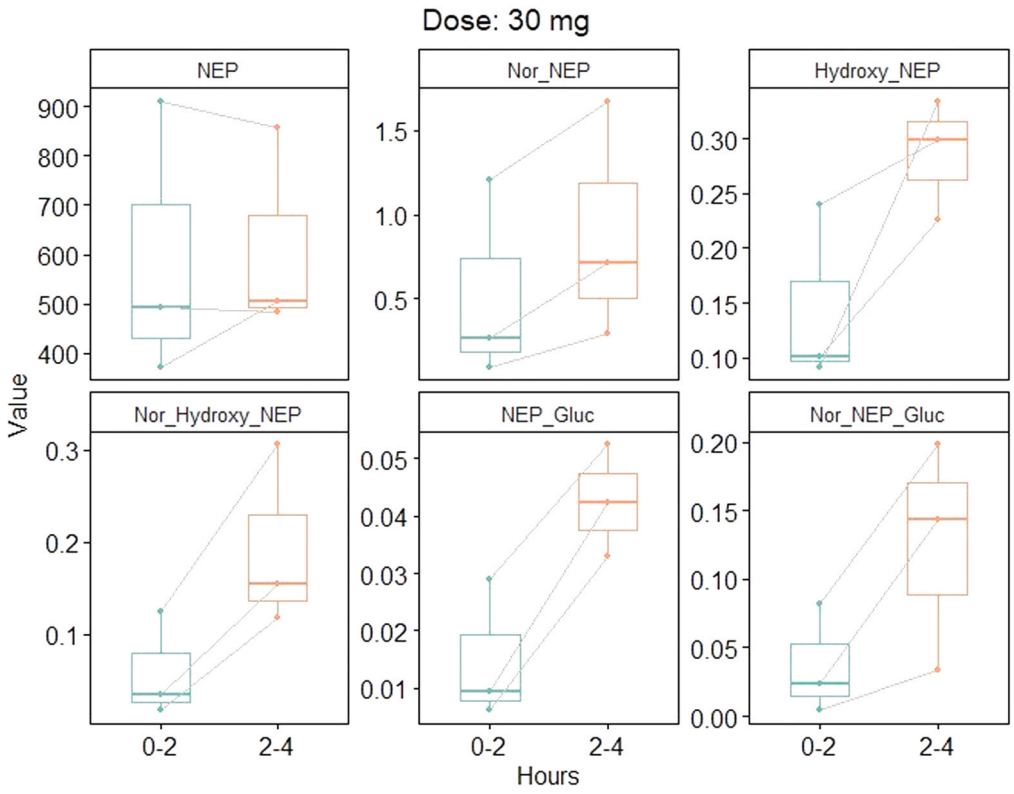

Fig. 3. Boxplot illustrating the ratio between the analyte and the internal standard of NEP and its metabolites at 0–2 and 2–4 h after the administration of 30 mg dose.

example of the MS/MS fragmentation spectrum of hydroxy-4-CMC, identified in group 3, sample 49 (range 2–5 h after consumption of 4-CMC), is shown in Fig. 5.

Compared to NEH and NEP, the formation of metabolites in the urine after intake of 4-CMC shows a different behaviour. In fact, based on $t_{1/2}$ values, 4-CMC is estimated to be a compound with low clearance ($t_{1/2}$ > 60 min) [14] and high half-life time ($t_{1/2}$ =105 min) [21], thus suggesting a lower propensity towards metabolic reaction and thus making 4-CMC abundant in the urine even 5 h after administration (Table 3). Only the hydroxy-4-CMC metabolite was observed in both time ranges, while the others were detected only after 2 h from administration. Furthermore, as can be seen from Fig. 6, the hydroxy-4-CMC has a statistically significant increase in signal strength at dose of 150 mg (p-value = 0.02857), and for male and female volunteers together (p-value = 0.0006216). These results could identify this metabolic pathway as the preferred pathway for this synthetic cathinone.

4. Conclusions

The present study investigates the in vivo metabolism of NEP, NEH and 4-CMC on both male and female volunteers, in order to identify the main phase-I and phase-II metabolites and investigate their variation in the two observed time ranges. A significant feature of the resulting data is that a strong sexually dimorphic metabolism of hydroxy-NEP and hydroxy-NEH is observed. In particular, the hydroxy-metabolite showed a significant increase in women two hours after the consumption of the substances. The characterization of the main NEP, NEH and 4-CMC metabolites based on LC-HRMS and LC-MS/HRMS allowed the accurate mass determination of their protonated molecular ion and collisional activated fragment ions resulting in the reliable definition of their structure and the outlining of the major metabolic routes for the tested substance. The knowledge of the fragmentation pattern for both the parent drugs and their main metabolites will also allow to develop fit-for-purpose targeted analytical methods useful for the detection of them in biological specimens. In particular, hydroxy-NEP, hydroxy-NEH and hydroxy-4-CMC are suggested as target analytes in the toxicological analyses, so as to increase these synthetic cathinones detection time after intake and reduce the risk of false-negative results in the forensic cases. In fact, it is well known that characterizing of different species produced by the metabolic pathways of emerging psychoactive substances provide i) insight into mechanisms underlying their potential toxicity, and ii) useful data for their detection in biological samples. On the other hand, further studies may be required to speculate on metabolic pathway and related gender-based differences at other time points, so that the pharmacokinetics of these three synthetic cathinones can be further investigated.

Author agreement statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

CRediT authorship contribution statement

Pichini Simona: Supervision. **Salomone Alberto:** Visualization, Supervision, Conceptualization. **Massano Marta:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization.

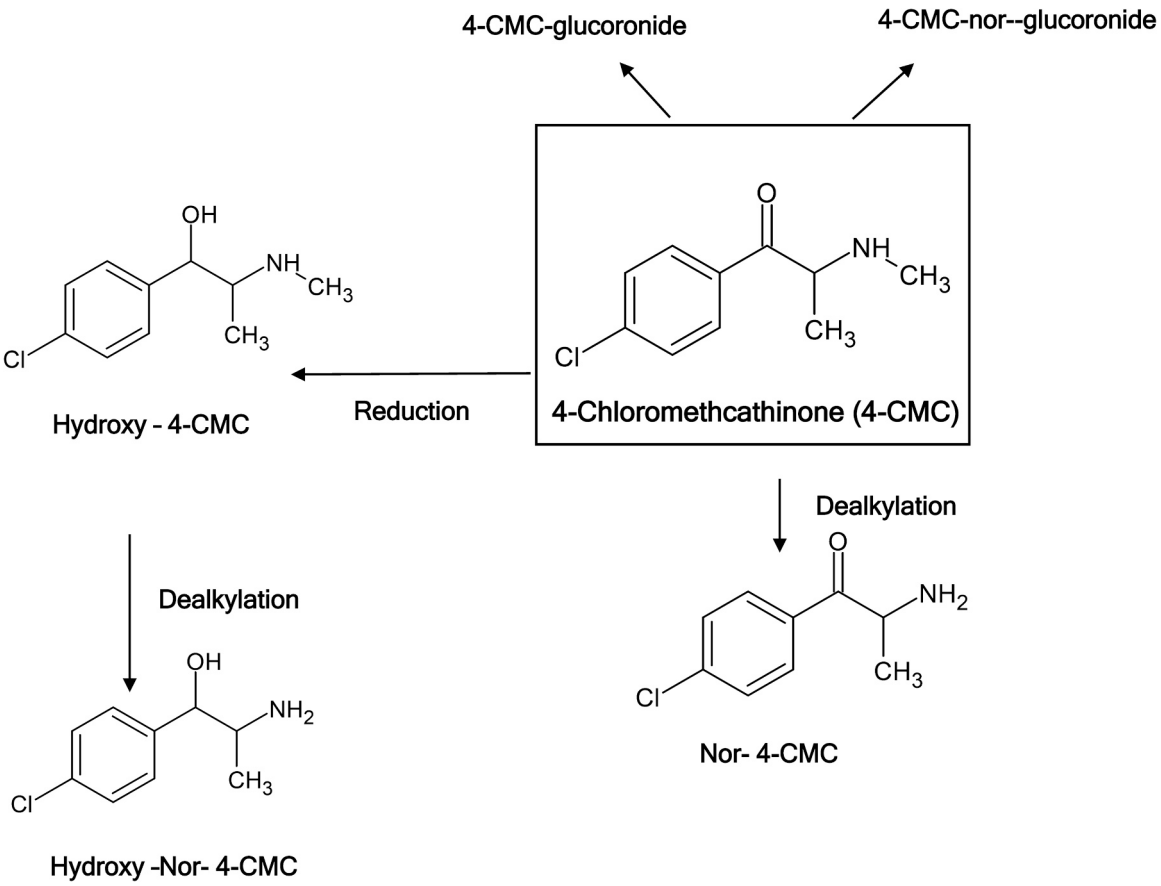

Fig. 4. Expected metabolic reactions of 4-CMC.

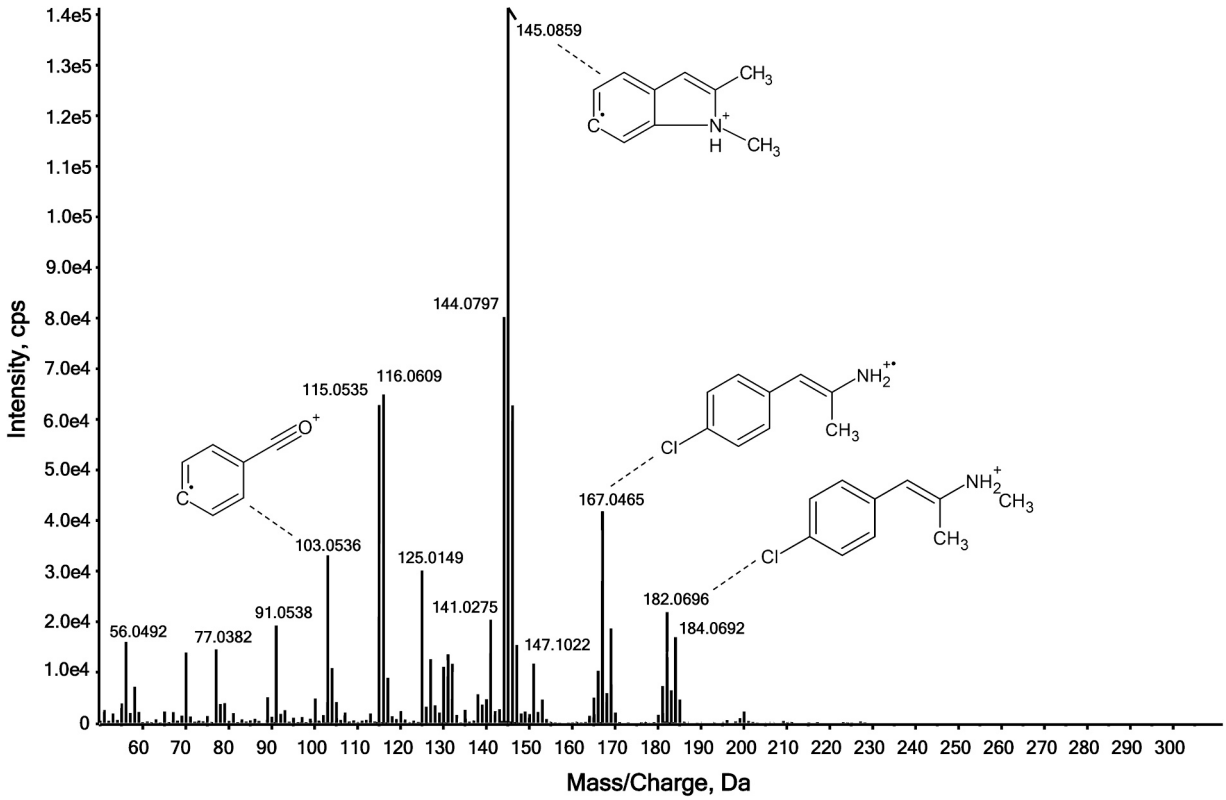

Fig. 5. HRMS fragmentation pattern of hydroxy-4-CMC. Sample 49, 2–5 h after substance consumption.

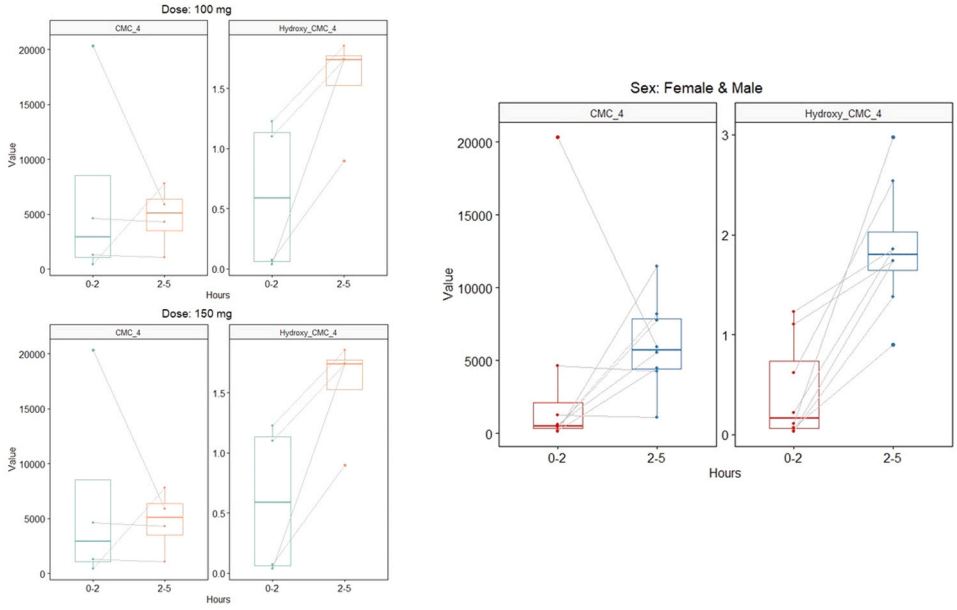

Fig. 6. Boxplots illustrating the ratio between the analyte and the internal standard of 4-CMC and its metabolites at 0–2 and 2–5 h after the administration of 100 and 150 mg doses and for both male and female volunteers.

Farrè Magi: Supervision. **Papaseit Esther:** Supervision. **Nuñez-Montero Melani:** Supervision. **Pérez-Maña Clara:** Supervision. **Hladun Olga Alvaro:** Supervision. **Marchei Emilia:** Supervision. **Ventura Mireia:** Supervision. **Gerace Enrico:** Supervision. **Alladio Eugenio:** Data curation.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2024.115994.

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