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Metabolism of *N*-ethylhexedrone and buphedrone: An *in vivo* study in mice using HPLC-MS/MS

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ABSTRACT

N-ethylhexedrone (NEH) and buphedrone (BUPH) are synthetic drugs structurally related to natural cathinone. These synthetic cathinones (SC) are members of the heterogenous family of new psychoactive substances (NPS), which have caused major concern in scientific and forensic communities over the past years, due to their widespread consume. Thus, there is a constant need for monitoring the use of these new substances and gather knowledge on their metabolism and excretion profiles, in order to try to identify markers of NPS consumption.This study aimed at the identification and quantification of NEH, BUPH and selected phase I metabolites using HPLC-MS/MS. NEH, BUPH and some related metabolites were synthesized in-house and quantified in 24 h mice urine, following single dose administration of each drug (64 mg kg^{-1}, *i.p.*). NEH and BUPH were quantified in mice urine at 58.3 ± 14.4 and 146.2 ± 14.9 μg mL^{-1}, respectively. Similar metabolic pathways were observed for both drugs. Among the metabolites studied, the most excreted ones derived from *N*-dealkylation of either NEH or BUPH (at around 80 μg mL^{-1} of urine). Other metabolites resulting from ketone reduction and ketone reduction combined with *N*-dealkylation or 4-aryl hydroxylation (detected for the first time in non-ring substituted SC) were also identified and quantified. Urine samples were screened using liquid chromatography-high resolution mass spectrometry and various phase II metabolites, including *N*-acetylated, glucuronides and dicarboxylic acid conjugates were tentatively identified, some of them for the first time. This work is a contribution to the identification of metabolites from SC that can become potential markers to estimate drug consumption.

1. Introduction

N-ethylhexedrone (NEH) and buphedrone (BUPH) are synthetic drugs derived from cathinone, a natural psychoactive alkaloid isolated from khat plant (*Catha edulis*), that is structurally similar to amphetamine [1–3]. Often labelled as "legal highs", "bath salts", "plant food" or "research chemicals", synthetic cathinones (cathinone derivatives, SC) are readily accessible at low cost via internet, in head shops or through drug dealers, owing their popularity to psychoactive properties similar to amphetamine and to other common illicit drugs, such as cocaine and 3,4-methylenedioxymethamphetamine (MDMA) [4–6]. Together with synthetic cannabinoids, opioids, benzodiazepines and other stimulants, SC are included in a group of compounds denominated as "new psychoactive substances" (NPS) [4,7]. More than 670 NPS have been identified in Europe over the past decade, including a total of 130 SC [4]. The constant entrance of new, or newly synthesized cathinones into the recreational drug market, is enabled by the multitude of possible substitutions to the core skeleton of cathinones. Therefore, addition of substituents to the α-carbon, to the *N*-terminus and/or to the aromatic ring of cathinones may result in new molecules that are not detected in routine drug screening [8–10].

It has been proposed that SC can be grouped into four families according to the *N*-alkyl and aromatic ring substituents [11]: *N*-alkylated cathinones with or without aromatic ring substituents, such as NEH and BUPH; 3′,4′-methylenedioxy-*N*-alkylated cathinones; *N*-pyrrolidine cathinones with or without aromatic ring substituents and 3′,4′-methylenedioxy-*N*-pyrrolidine cathinones. Hence, depending on their chemi-

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cal structure, SC may undergo different preferred metabolic pathways [12,13]. Nonetheless, independent or combined *N*-dealkylation of the primary amine and β-keto reduction to the corresponding alcohol appears to be common phase I metabolic pathways among the four families of SC [12,14–16]. Additionally, hydroxylation of the benzene ring, a major pathway for amphetamines, has also been observed in rat urine following SC administration [13,17]. Phase I hydroxylated SC metabolites may also undergo phase II metabolism, being excreted in urine as glucuronides or sulfates [17–19] and also conjugated with dicarboxylic acids [20,21]. Even though it has been suggested that SC are usually consumed in such doses that allow for the detection of their unchanged form in urine [3,5,13,22], additional information on drug metabolism and on metabolite/drug ratios could be useful to provide estimates of time of drug consumption in addition to the confirmation of drug intake. In a study comprising the analysis of human urine to uncover the metabolic profiles of a range of SC, parent drugs corresponding to *N*-alkylated cathinones with or without aromatic ring substituents, were either absent or less abundant than metabolites [12]. This could be explained by the amount consumed and time of consumption, which were unknown to the authors. In fact, urinary recovery of mephedrone, one of the cathinones considered in that study [12], after 24–48 h post drug administration was practically null, while some metabolites were still being excreted [23]. Furthermore, since the use of SC has been associated with several cases of acute and fatal intoxications, it is imperative that these new substances be subject to controlled pharmacological studies, including evaluation of metabolic and excretion profiles, not only to enable intake confirmation through target biomarkers, but also to link adverse effects to the responsible compound, which can be the drug itself or a metabolite [8,24,25].

Cheap and fast screening methods of drugs of abuse, such as immunoassays, have been used in clinical and forensic studies [26]. However, immunoassays usually only cover a certain number of drugs or drug classes, implying limitations in the identification of new compounds, and are prone to generating false positive or false negative results [27,28]. Thus, in recently reviewed literature regarding analytical methods for the identification and quantification of synthetic cathinones in biological matrices, gas or liquid chromatography coupled to mass spectrometry (GC–MS or HPLC-MS, respectively) have been the most commonly mentioned methodologies and urine the most analyzed matrix [29]. Achievement of high specificity and sensitivity is possible, in tandem MS methods (e.g. HPLC-MS/MS), when 2 or 3 ion transitions per compound are recorded in multiple reaction monitoring (MRM) mode, allowing for more than 40 synthetic cathinones, as well as other drugs, and some metabolites to be correctly identified in urine at the same time [28,30–32].

This study contributes to uncover the metabolism of NEH and BUPH through the identification and quantification of excreted metabolites in urine following *in vivo* studies in mice. These two cathinones were synthesized in-house, as well as metabolites, selected among those expected from literature [12,13,20] and predicted *in silico*. The parent drugs were used for the *in vivo* studies. Following a single dose administration of NEH or BUPH to two groups of mice, 24 h urine was collected and analyzed by HPLC-MS/MS, using optimized and validated methods. HRMS was also used to screen for other phase I and phase II metabolites.

2. Materials and methods

2.1. Chemicals and materials

N-ethylhexedrone (**NEH**) 2-(ethylamino)-1-phenyl-1-hexanone hydrochloride, buphedrone (**BUPH**) 2-(methylamino)-1-phenylbutan-1-one hydrochloride, and corresponding metabolites 2-(ethylamino)-1-phenylhexan-1-ol hydrochloride (**H1**), 2-amino-1-phenylhexan-1-one (**H2**), 2-amino-1-phenylhexan-1-ol hydrochloride (**H3**), 4-(2-(ethylamino)-1-hydroxyhexyl)phenol hydrochloride (**H4**), 4-(2-amino-1-hydroxyhexyl)phenol hydrochloride (**H5**), 2-(methylamino)-1-phenylbutan-1-ol hydrochloride (**B1**), 2-amino-1-phenylbutan-1-one hydrochloride (**B2**), 2-amino-1-phenylbutan-1-ol hydrochloride (**B3**) and 4-(1-hydroxy-2-(methylamino)butyl)phenol hydrochloride (**B4**) were synthesized in-house (Supplementary Data). All compounds were purified to ≥95% purity as determined by HPLC-MS. Chemical structures are depicted in Figs. 1 and 2.

HPLC grade acetonitrile and methanol and formic acid 99–100% p.a. were obtained from Chem-Lab NV (Zedelgem, Belgium). Milli-Q water (18.2 MΩ cm^{-1} resistivity) was obtained from a Millipore-Direct Q3 UV system (Millipore®, USA). PVDF membranes (0.22 μm) were purchased from Merck Millipore (Ireland).

2.2. Preparation of stock, working and control standard solutions

Individual stock standard solutions (S_s) at 1000 μg mL^{-1} were prepared by dissolving each compound in methanol. Individual standard solutions (S_i) of each compound at 100 μg mL^{-1} were prepared from S_s and diluted in acetonitrile.

Two sets of working standard solutions (S_w), one corresponding to NEH and metabolites (H1, H2, H3, H4 and H5), and the other to BUPH and metabolites (B1, B2, B3 and B4), were prepared through the dilution of appropriate volumes of S_s in 50 μL of centrifuged and filtered urine (from control mice, *vide* Sections 2.6.2 and 2.6.3), 200 μL of Milli-Q water and 250 μL of acetonitrile, at a ratio of 1:4:5 (urine:H_2O:ACN, V/V), to a final concentration range of 0.001–20 μg mL^{-1}. Following vortexing and centrifuging at 12,300*g*, for 5 min, supernatants were collected, filtered and analyzed by HPLC-MS/MS. Control standard solutions (QC solutions) were prepared in the same manner as S_w at LOQ of each analyte (lower quality control standard solution, LQC), and for all compounds at a concentration of 12 μg mL^{-1} (higher quality control standard solution, HQC).

2.3. Liquid chromatography tandem mass spectrometry method development

HPLC-MS/MS analysis was performed on a Waters Alliance HPLC system (Waters® 2695 separation module, Ireland) consisting on a system of quaternary pumps, degasser, autosampler and a column furnace. Analyses were carried out on an XBridge BEH C18 XP (50 × 2.1 mm, 2.5 μm) column in an oven at 35 °C. The injection volume was 10 μL. The mobile phase consisted of a mixture of formic acid (0.5% v/v in Milli-Q water) (eluent A) and acetonitrile (eluent B). Method development consisted on varying the percentage of organic solvent so that optimal peak shape and adequate resolution of compounds were achieved. For the analysis of NEH and its metabolites, the mobile phase was held isocratically at 18% B for 10 min, followed by 10 min at 95% B, returning to 18% B in 1 min, and held isocratically at 18% B for stabilization. Total run time was 25 min at a flow rate of 0.3 mL min^{-1}. The elution program used for the analysis of BUPH and its metabolites started with 1% B, increasing to 15% B in 1.5 min, then to 45% B in 5.5 min, and to 95% B in 0.5 min, held isocratically at 95% B for 9.5 min, decreasing to 1% B in 1 min, and finally held isocratically at 1% B for 7 min (adapted from [23,33]). Total run time was 25 min at a flow rate of 0.3 mL min^{-1}.

The mass spectrometer used was a MicroMass Quattromicro® API (Waters®, Ireland). Mass spectrometry was performed using an electrospray ion source in positive ionization mode (ESI^+) operating at 120 °C. High purity nitrogen was used as drying and nebulizing gas, and ultrahigh purity argon was used as collision gas. S_i were infused into the mass spectrometer and different cone voltages (10–60 V) and collision energies (5–30 eV) were tested, aiming to optimize the most adequate conditions for the detection of the parent ions and corre-

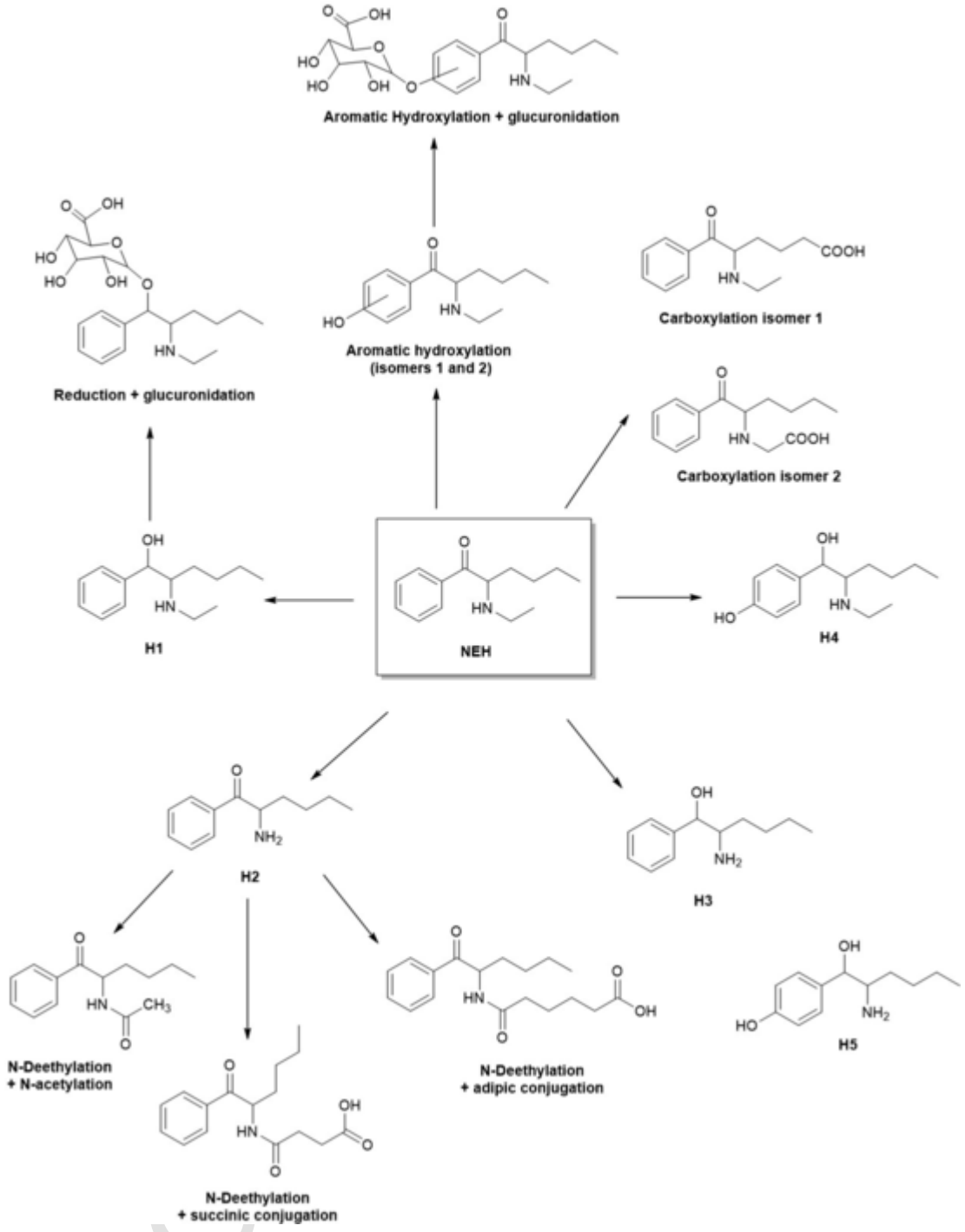

Fig. 1. Chemical structures of NEH and related metabolites; H1, H2, H3 and H4 were synthesized in house and quantified in 24 h mice urine. The remaining metabolites were putatively identified by HRMS.

sponding fragmentation pattern of each compound. The two product ions with the highest signal were selected as the monitored transitions for quantification (MRM1) and confirmation (MRM2) purposes, in order to achieve high selectivity and sensitivity. Additionally, data were acquired in full scan mode, in the *m/z* range 50–1000. For data acquisition and processing MassLynx® 4.1 software (Waters, Ireland) was employed.

2.4. UPLC- Orbitrap HRMS assays

Mice urines were also analyzed on a Q Exactive Focus (Thermo Scientific) coupled to a Dionex Ultimate 3000 UHPLC, using Xcalibur software v.4.0.27.19 (Thermo Scientific). Chromatographic separation was performed using a Waters XBridge C18 (2.1x150 mm, 3.5 µm particle size) column in an oven at 30 °C. The mobile phase consisted of a mixture of formic acid (0.1% v/v in Milli-Q water) (eluent A) and acetonitrile with 0.1% (v/v) formic acid (eluent B). The elution pro-

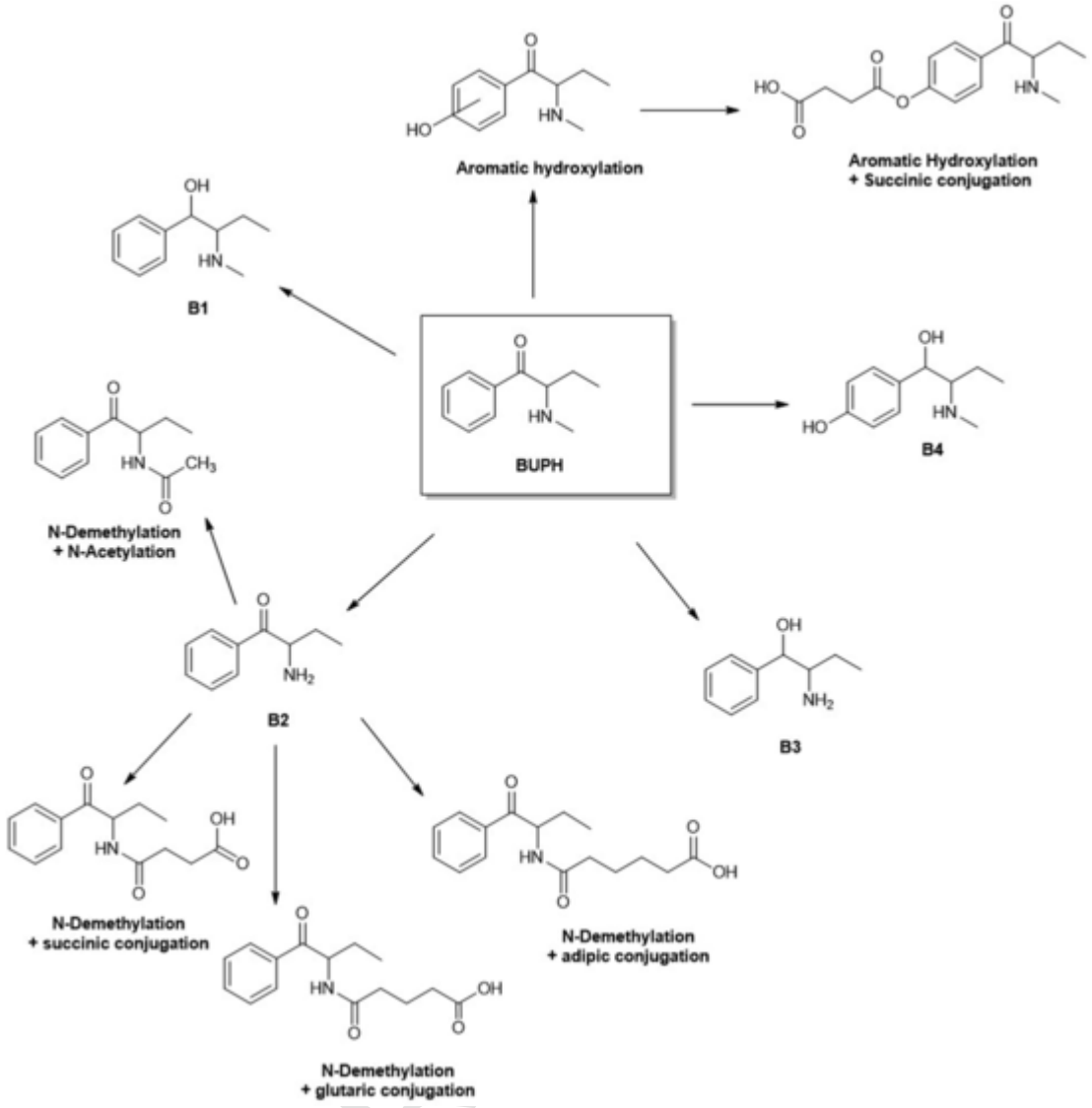

Fig. 2. Chemical structures of BUPH and related metabolites; B1, B2, B3 and B4 were synthesized in house and quantified in 24 h mice urine. The remaining metabolites were putatively identified by HRMS.

gram started with 1% B, increasing to 99% B in 13 min, held isocratically at 99% B for 2 min, decreasing to 1% B in 1 min, and held isocratically at 1% B for 4 min. Total run time was 20 min at a flow rate of 0.4 mL min^{-1}. MS method consisted of several cycles of Full MS scans (R = 70,000; Scan range = 75–1125 *m/z*) followed by 3 ddMS2 scans (R = 17,500; 20, 40, 60 NCE) in positive mode. External calibration was performed using LTQ ESI Positive Ion Calibration Solution (Thermo Scientific) and internal calibration using Lock mass (112.98550 *m/z*). The raw MS and MS/MS data were analyzed using Compound Discoverer software v3.1 (Thermo Scientific).

2.5. Method validation

HPLC-MS/MS method validation was done through the analysis of S_w, evaluating the performance of the methods in terms of specificity, linearity, limits of detection and quantification, precision, accuracy and carry-over. Matrix effect and stability were not determined since all calibration curves were prepared in urine from control mice, S_s S_w were freshly prepared at the time of analysis and samples were always stored at −80 °C until analyses were performed. Detailed information on the method validation process can be found in Supplementary Data.

2.6. In vivo study for metabolites identification

2.6.1. Animals

Male CD-1 mice, from Charles River (France), weighing 37.0 ± 1.5 g, were fed with standard laboratory chow (4RF21 LPG, Mucedola Srl, Milan, Italy) and drinking water *ad libitum* and kept at an average temperature of 21 ± 1 °C with 12-hour light/dark cycle. Animal experiments were carried out in accordance with the relevant European Community and National rules on the protection of animals used for experimental and other scientific purposes (Directive 2010/63/EU; Decreto-Lei 113/2013) and were approved by the animal Ethics Committee of the Faculty of Pharmacy, Universidade de Lisboa and by the Portuguese National Authority (Directorate-General of Food and Veterinary Medicine).

2.6.2. Animal exposure to NEH and BUPH

NEH and BUPH were dissolved in sterile 0.9% saline for single administrations (i.p., 64 mg kg^{-1}, 10 mL kg^{-1}). 24 h urine of each treated animal with NEH, BUPH or saline (control) was obtained by housing the animals in a metabolic cage (Techniplast S.p.A., IT) for 24 h. Ani-

mals (n = 3 mice/group) were randomly assigned to each studied group. Urine samples were collected and stored at −80 °C until further analyses.

2.6.3. Sample preparation

Just before analysis urine samples were centrifuged at 12,300*g*, for 5 min. Supernatants were collected and filtered through 0.22 μm PVDF membranes. Filtered urine from control animals was used to prepare S_w mentioned in Section 2.2 and blanks. Filtered urine (50 μL) from control (blanks) or cathinone-administered animals was diluted in water and acetonitrile at a ratio of 1:4:5 (urine:H_2O:ACN, v:v:v). After vortexing and centrifuging at 12,300*g*, for 5 min, supernatants were collected and analyzed by HPLC-MS/MS.

2.7. Data treatment

Signal ratios between MRM1 and MRM2 transitions (MRM1/MRM2, ion signal ratio) were determined for each standard drug or metabolite. Ion signal ratios and retention times of the different compounds studied, were used to identify the drugs and/or metabolites in urine samples from mice administered with NEH or BUPH. In order to confirm compound identification, maximum acceptable deviations from the ion ratios and retention times determined for synthesized standards were 20% and 5%, respectively. Confirmed compounds were quantified using external calibration, with matrix-matched standards (S_w), due to the absence of labelled internal standards. In vivo results were obtained from 3 independent experiments and were expressed as mean concentration ± SEM (μg mL^{-1}), considering the 10-fold dilution performed during sample preparation. The logP values for BUPH and NEH were estimated using Molinspiration software, https://www.molinspiration.com/cgi-bin/properties.

3. Results and discussion

To the best of our knowledge, this is the first controlled *in vivo* study in mice concerning the metabolism of NEH and BUPH. The lack of commercially available standards of SC or metabolites may at times constitute an impairment to the development of such studies [5,29]. Thus, for this work, NEH, BUPH and some selected metabolites (Figs. 1 and 2) were synthesized in-house. Considering the metabolic pathways known to occur for cathinones [12,13,20] and metabolite prediction *in silico*, through the ADMET Predictor® software (SimulationsPlus, Lancaster, CA, USA), metabolites derived from *β*-keto reduction (H1 and B1), *N*-dealkylation (H2 and B2), *N*-dealkylation and *β*-keto reduction (H3 and B3), *β*-keto reduction and 4-aryl hydroxylation (H4 and B4) of NEH and BUPH were synthesized. Metabolite H5 resulting from the combination of the three main pathways, ketone reduction, N-dealkylation and 4-aryl hydroxylation was also obtained. The structure elucidation of all the compounds was based on their ^{1}H- and ^{13}C NMR and ESI-HRMS data (Supplementary Data). Thereafter, mice were administered with a single drug dose of 64 mg kg^{-1} (*i.p*). Urine (24 h) collected from those animals was analyzed through HPLC-MS/MS for the identification and quantification of parent drugs and respective metabolites.

An HPLC-MS/MS method was implemented according to Al-Saffar *et al.* [33], but it was optimized and validated aiming at the quantification of NEH and BUPH, as well as their respective metabolites. Optimization of cone voltage and collision energies was performed for each substance through direct infusion of S_i into the mass spectrometer. Optimized values are presented in Table 1. The two most intense product ions were selected in order to define MRM1 (quantification transition) and MRM2 (confirmation transition), allowing for a sensitive and selective quantification monitored by MRM mode. Chromatographic separation was achieved for all tested compounds.

Table 1
Optimized MS/MS parameters for NEH, BUPH, and corresponding metabolites. $[M+H]^+$: precursor ion; MRM1: quantification transition; C.E.: collision energy; MRM2: confirmation transition.

Compound	$[M+H]^+$ *m/z*	Cone Voltage/V	Product ions (*m/z*)	MRM1 (C.E./eV)	MRM2 (C.E./eV)
NEH	220	12	91, 118, 119, 146, 175, 202	220 > 202 (15)	220 > 175 (15)
H1	222	12	91, 117, 147, 204	222 > 204 (15)	222 > 147 (15)
H2	192	12	91, 105, 118, 119, 174, 175	192 > 118 (12)	192 > 91 (12)
H3	194	12	91, 117, 176	194 > 176 (10)	194 > 117 (10)
H4	238	12	107, 133, 163, 220	238 > 220 (11)	238 > 163 (15)
H5	210	12	107, 133, 175, 192	210 > 192 (7)	210 > 175 (12)
BUPH	178	12	91, 119, 131, 132, 145, 147, 160	178 > 160 (15)	178 > 132 (15)
B1	180	12	91, 107, 131, 133, 162	180 > 162 (12)	180 > 133 (15)
B2	164	12	91, 117, 118, 119, 146, 147	164 > 118 (12)	164 > 91 (12)
B3	166	12	91, 106, 131, 148	166 > 148 (10)	166 > 131 (10)
B4	196	12	107, 147, 149, 178	196 > 178 (9)	196 > 147 (12)

Chromatograms of urine samples collected from mice administered with NEH or BUPH were acquired in full scan (*m/z* 50–1000) and in MRM mode (Figs. 3 and 4). This allowed for a preliminary screening, searching for parent drugs and expected metabolites. As metabolite B4, derived from *β*-keto reduction and 4-aryl hydroxylation of BUPH, was not detected in these experiments, method validation was performed considering NEH, H1, H2, H3, H4, H5, and BUPH, B1, B2 and B3 (Tables 2 and 3).

Chromatograms of blank urine samples were checked for interfering peaks at the expected retention times (RT) of each compound. When monitoring the quantification transition for H3, an interfering peak was found near the corresponding RT. However, this interference was negligible at concentrations higher than 0.05 μg mL^{-1} in 24 h urine and thus that transition was not excluded from data treatment.

A summary of method validation parameters is presented in Table 2. For all compounds, acceptable linearity was achieved with determination coefficients, r^2, higher than 0.99. The back calculated concentrations of the calibration standards were within ±20% of the nominal value at the LOQ and within ±15% at the remaining concentrations. LOD were between 0.001 and 0.01 μg mL^{-1}, and LOQ were between 0.005 and 0.05 μg mL^{-1}. BUPH could be detected at 0.002 μg mL^{-1} and quantified at 0.005 μg mL^{-1}. Similar results were described by Al-Saffar and co-workers [33] – 0.0008 μg mL^{-1} and 0.003 μg mL^{-1} as LOD and LOQ, respectively. In other studies comprising the simultaneous quantification of up to 40 synthetic cathinones and metabolites in urine [5,22], including BUPH and B1, LOD were between 0.00025 and 0.0025 μg mL^{-1}. Although these limits are lower than the values presented here, it should be noted that Concheiro *et al.* [5,22] submitted their samples to a previous concentration step using solid phase extraction to eliminate co-eluting compounds that could produce matrix effect. Nonetheless, in our study it was decided to minimize sam-

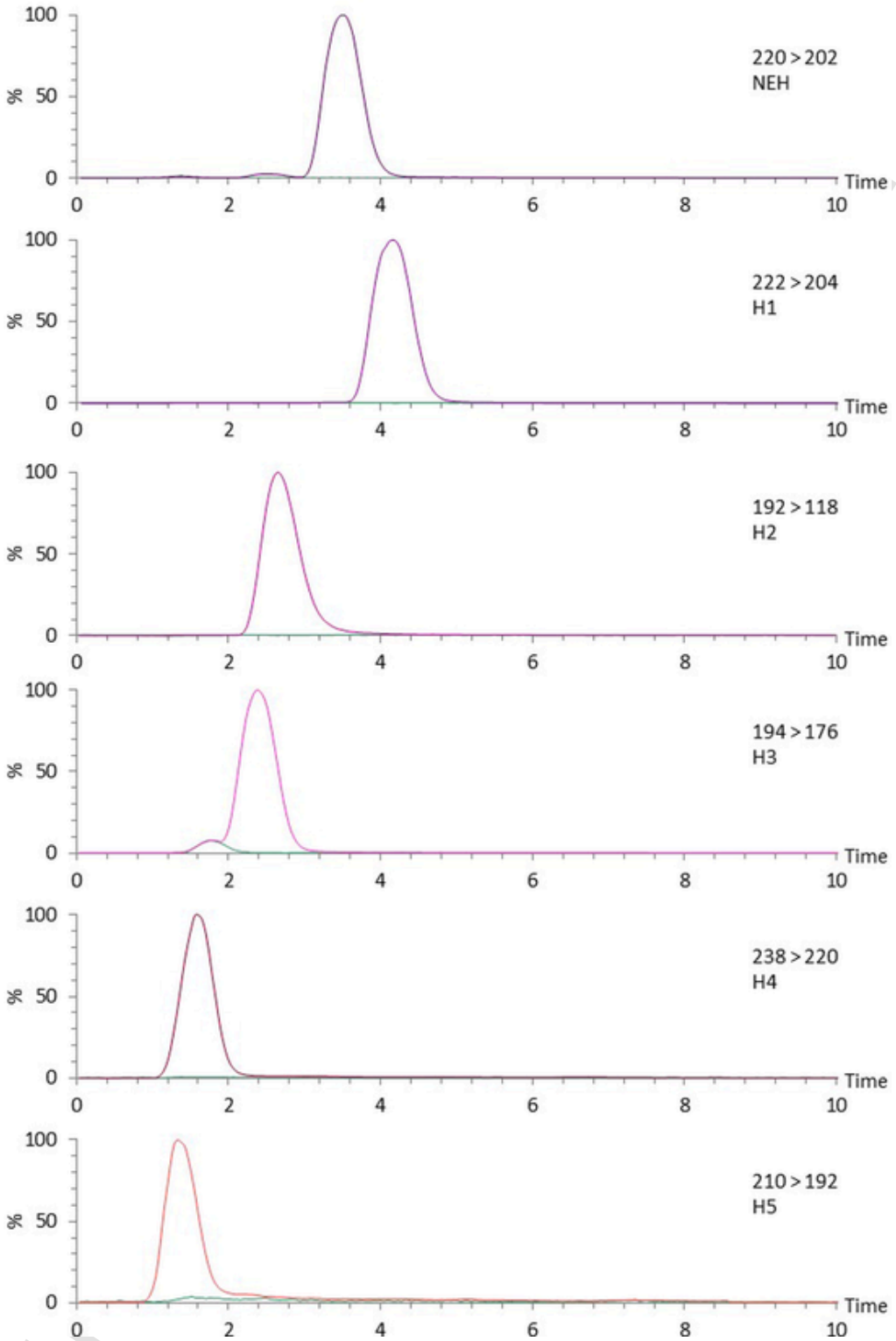

Fig 3. MRM overlay chromatograms of NEH and its metabolites with blank urine matrix (green).

ple preparation opting to reduce possibly interfering salts through a 10-fold dilution of urine in water and acetonitrile, followed by centrifugation [23,34]. No other studies were found presenting the limits of detection and quantification of NEH, its metabolites or the remaining metabolites of BUPH.

Due to limited volume of blank mice urine available, only two concentration levels were considered for precision and accuracy evaluation. Both intra- and interday precision values were considered satisfactory when below 15% (for QC solutions at 12 μg mL^{-1}) or 20% (for QC solutions at the LOQ), which was found to occur in all cases (Table 3). Accuracy values were between 85–115% for HQC solutions and 80–120% for QC solutions at the LOQ (Table 3). No carry-over effect was observed after analysis of HQC solution (Supplementary Data).

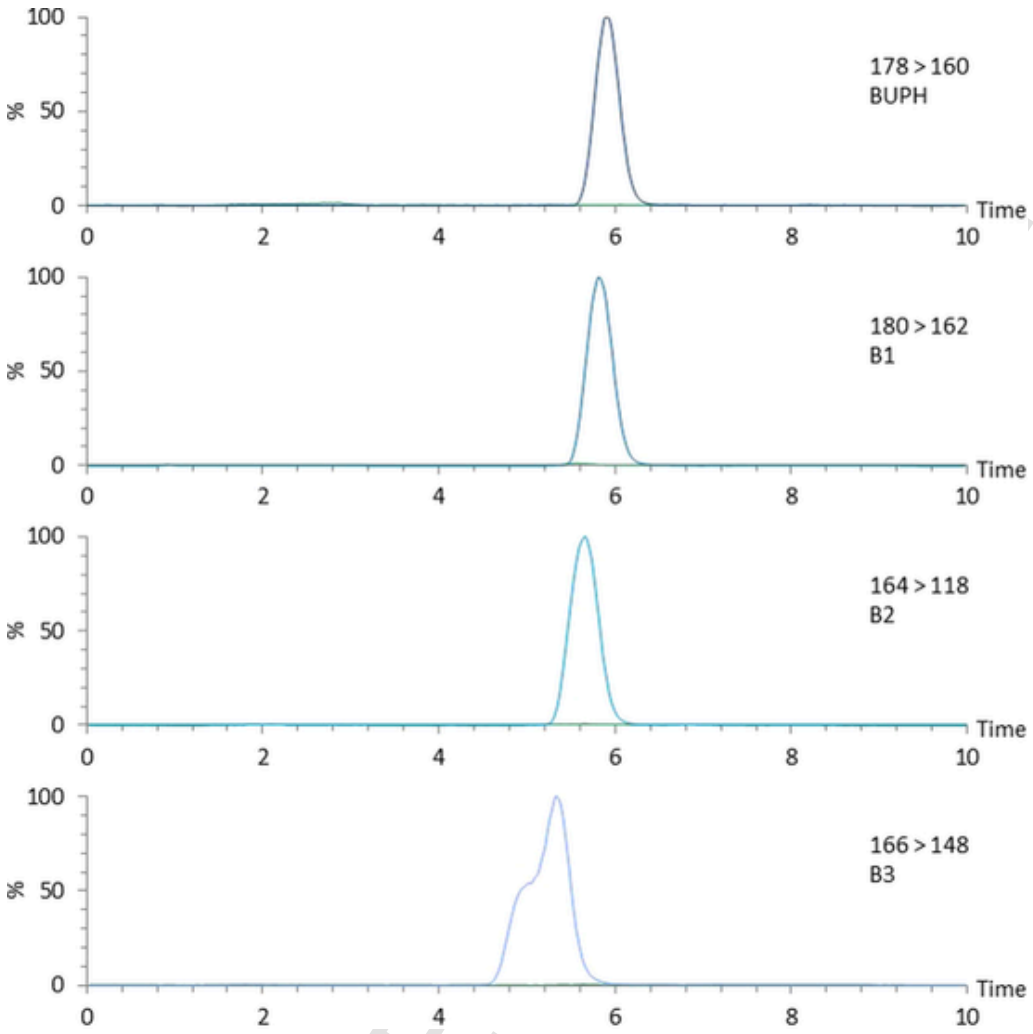

Fig 4. MRM overlay chromatograms of BUPH and its metabolites with blank urine matrix (green).

Table 2
Summary of results for method validation, including limit of detection (LOD), limit of quantification (LOQ), linearity range and calibration curve parameters, slope, intercept and determination coefficient (r^2).

Compound	LOD/μg mL $^{-1}$	LOQ/μg mL $^{-1}$	Concentration range/μg mL $^{-1}$	Linearity/μg mL $^{-1}$		
				Slope	Intercept	r^2
NEH	0.002	0.005	1–15	278,777	164,983	0.9936
H1	0.001	0.005	0.05–1	974,049	4890.3	0.9994
H2	0.002	0.005	1–15	100,366	66,468	0.9989
H3	0.01	0.05	2–15	496,575	−196094	0.9971
H4	0.001	0.005	0.05–1.5	322,908	2411.6	0.9998
H5	0.01	0.05	0.05–1.5	41,573	408.33	0.9984
BUPH	0.002	0.005	6–20	9303	377,119	0.9931
B1	0.002	0.005	0.005–1	555,621	6244.5	0.9999
B2	0.002	0.005	6–20	54,389	256,608	0.9911
B3	0.002	0.005	3–20	35,873	440,012	0.9917

Following optimization and validation, these methods were applied to the analysis of urine samples from mice single-administered with NEH or BUPH, at 64 mg kg^{-1} (*i.p.*). Identification of drugs and metabolites was confirmed through the comparison of RT and ion ratios of each standard compound (S_w) with values obtained for these parameters in urine samples from exposed mice (Table 4). RT and ion ratio deviations obtained for NEH were 1.62% and 0.52%, respectively. For BUPH, RT and ion ratio deviations were −0.90% and 0.44%, respectively.

Quantification results and relative urine distribution for detected compounds are shown in Fig. 5 and Table 5. Data show that during the 24 h that followed drug administration, NEH and BUPH were excreted at concentrations of 58.3 ± 14.4 μg mL^{-1}

Table 3
Intra- and interday precision and accuracy. LQC: 0.005 μg mL $^{-1}$ for NEH, H1, H2, H4, BUPH, B1, B2 and B3; 0.05 μg mL $^{-1}$ for H3 and H5. HQC: 12 μg mL $^{-1}$.

Compound	Intraday				Interday			
	Precision/%RSD		Accuracy/%		Precision/%RSD		Accuracy/%	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
NEH	8.84	2.20	82.87	110.42	2.76	6.78	86.09	100.12
H1	9.12	1.45	115.87	109.23	1.31	5.35	114.35	101.35
H2	5.88	1.92	80.36	109.27	5.42	8.55	82.17	101.37
H3	13.83	2.70	108.58	110.35	6.20	6.00	103.21	101.81
H4	14.86	2.97	112.21	114.47	4.40	6.24	106.47	105.45
H5	13.85	3.48	105.48	109.35	4.11	5.52	99.74	101.70
BUPH	6.95	3.66	120.00	107.27	12.87	4.88	103.25	100.78
B1	7.65	3.83	115.29	94.06	8.68	4.79	102.80	100.16
B2	19.74	3.98	113.91	109.39	6.97	6.47	107.46	100.24
B3	11.43	2.91	84.02	104.74	12.79	3.43	101.52	99.93

Table 4
HPLC-MS/MS parameters of NEH, BUPH and corresponding metabolites (H1, H2, H3, H4, H5, and B1, B2 and B3, respectively) obtained with standard solutions (standards) and in urine samples from mice single-administered with NEH or BUPH (samples). RT and MRM1/MRM2 deviations were calculated comparing data obtained for samples and standard solutions. RT: retention time; SD: standard deviation (%); MRM1/MRM2 ion ratio.

Compound	RT ± SD/min in standards	RT ± SD/min in samples	RT deviation/%	MRM1/MRM2 ± SD/min in standards	MRM1/MRM2 ± SD/min in samples	MRM1/MRM2 deviation/%
NEH	3.52 ± 0.08	3.58 ± 0.16	1.62	4.98 ± 0.02	5.01 ± 0.02	0.52
H1	4.18 ± 0.10	4.23 ± 0.19	1.19	21.85 ± 0.34	17.69 ± 1.25	−19.02
H2	2.82 ± 0.09	2.77 ± 0.10	−1.65	1.51 ± 0.01	1.70 ± 0.01	12.80
H3	2.45 ± 0.04	2.46 ± 0.13	0.43	59.63 ± 1.36	57.06 ± 3.40	−4.30
H4	1.65 ± 0.04	1.71 ± 0.08	3.85	17.88 ± 0.43	15.39 ± 2.49	−13.90
H5	1.30 ± 0.07	1.26 ± 0.04	−3.48	78.40 ± 8.00	0.59 ± 0.05	−99.25
BUPH	5.99 ± 0.04	5.93 ± 0.13	−0.90	1.47 ± 0.01	1.47 ± 0.004	0.44
B1	5.89 ± 0.04	5.96 ± 0.12	1.13	8.16 ± 0.06	7.20 ± 0.327	−11.77
B2	5.72 ± 0.04	5.70 ± 0.11	−0.44	1.47 ± 0.01	1.46 ± 0.01	−0.80
B3	5.40 ± 0.04	5.45 ± 0.13	0.89	23.49 ± 0.16	24.50 ± 0.31	4.30

and 146.2 ± 14.9 μg mL^{-1}, respectively. A small amount of NEH was excreted in urine (2.3%), during the 0–24 h period, while that of BUPH accounted for 10.6% of the administered dose to male mice.

The difference between these concentration values may be related to the extent at which each drug is distributed and metabolized, due to the structural differences between the two drugs. The length of the α-alkyl chain may lead to increased lipophilicity [35], and this is in line with the estimated partition coefficient of NEH (logP = 3.55) that has a longer side chain than BUPH (logP = 2.11). While comparing the plasma pharmacokinetic profiles of methylone, butylone and pentylone, which are 3,4-methylenedioxy cathinone derivatives with increasing α-alkyl chain length, it has been demonstrated that pentylone displayed the highest maximum plasma concentration and the longest elimination half-life ($t_{1/2}$) [35,36]. Thus, having both groups of mice received the same dose of each drug, the lower urine concentration observed for NEH, following the same time-course post dosing, may be explained by a longer elimination $t_{1/2}$ than BUPH.

Two major metabolic pathways have been ascribed to *N*-alkyl cathinone derivatives as NEH and BUPH: reduction of the ketone group at the β-carbon of the side chain, forming hydroxylated metabolites, and *N*-dealkylation [12–14,17,31]. In this work, during the 24 h following exposure to NEH or BUPH, the most abundant phase I metabolite in mice urine, among those studied, was the *N*-dealkylated derivative with intact β-keto group. H2 and B2 were respectively detected at 81.8 ± 14.7 μg mL^{-1} and 80.3 ± 11.4 μg mL^{-1} in urine, representing 3.1% and 6.0% of the administered dose (Fig. 5 and Table 5). It has been suggested that detection of β-keto-*N*-dealkylated cathinone derivatives in human urine likely indicates recent ingestion [12]. Further studies including urine collection at shorter and longer periods of time, could help to prove this hypothesis and these metabolites could be used as markers of time of ingestion.

N-dealkylated alcohols, H3 and B3, generated through ketone reduction combined with *N*-dealkylation, were the second most excreted metabolites (19.4 ± 3.8 μg mL^{-1} and 59.6 ± 4.7 μg mL^{-1}, respectively) (Fig. 5 and Table 5). Metabolite B3 was almost as abundant as B2 in mice urine, representing 4.5% of the administered dose, while H3 occurred as a minor metabolite (<3% of the administered dose). Furthermore, urinary excretion of β-keto reduced *N*-alkylated compounds, H1 and B1, was also quantified in our study but as minor metabolites (<0.5% of the administered dose). Concentrations were 2.25 ± 0.69 μg mL^{-1} for H1 and 5.65 ± 0.48 μg mL^{-1} for B1 (Fig. 5 and Table 5). Despite being a major pathway for amphetamines, 4-hydroxylation of the benzene ring is not a commonly observed pathway in the metabolism of non-ring substituted synthetic cathinones [13]. Nonetheless, H4, the metabolite resulting from ketone reduction followed by 4-hydroxylation of NEH, was detected at 1.23 ± 0.13 μg mL^{-1} in the 24 h urine from mice exposed to NEH, accounting for 0.06% of the administered dose (Fig. 5 and Table 5). This aryl-hydroxylated metabolite were also detected in preliminary *in vitro* metabolism studies using mice microsomes (data not shown). Interestingly, this is the first time, to our knowledge, that 4-hydroxylation is observed for non-ring substituted SC. The analogous BUPH metabolite, B4, was not detected in the analyzed samples. Although a peak was found at the retention time corresponding to H5, the ion ratio observed (0.59) was not in accordance with the expected value for

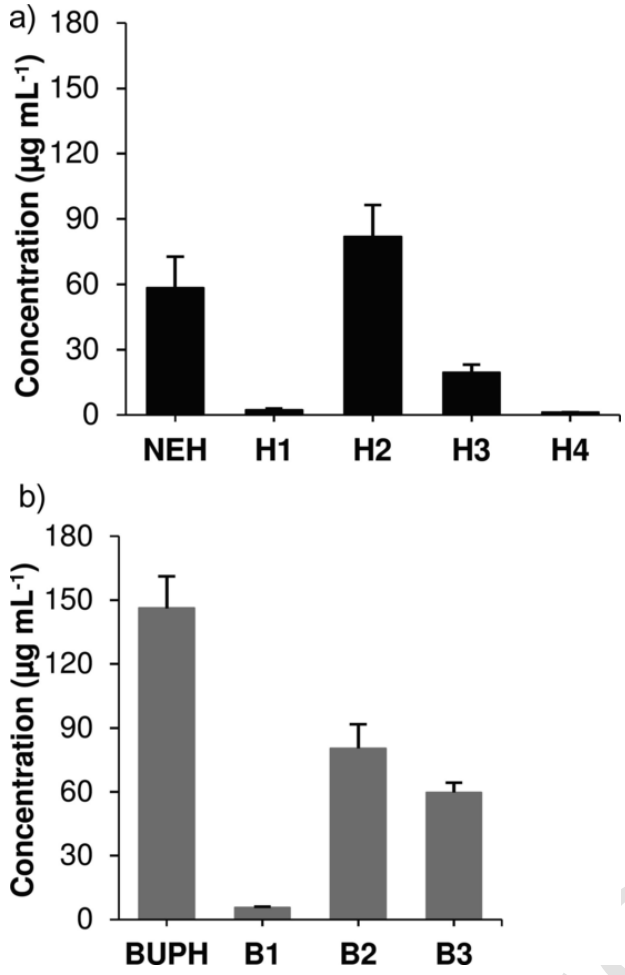

Fig. 5. Quantification of NEH, BUPH, and corresponding metabolites (H1, H2, H3, H4, and B1, B2 and B3, respectively) in 24 h urine samples from mice single-administered with (**a)** NEH or (**b)** BUPH. Results are presented as mean concentration ± SEM (µg mL^{-1}).

Table 5
Quantification of NEH, BUPH, and corresponding metabolites (H1, H2, H3, H4, and B1, B2 and B3, respectively), and their relative distribution in mice urine samples pooled over 0–24 h after a single-administration with NEH or BUPH (64 mg/Kg, *i.p.*).

Compound	Concentration in urine (µg mL^{-1})	% of administered dose
NEH	58.33 ± 14.36	2.31 ± 0.47
H1	2.25 ± 0.69	0.09 ± 0.03
H2	81.80 ± 14.68	3.11 ± 0.25
H3	19.37 ± 3.80	0.80 ± 0.05
H4	1.23 ± 0.13	0.06 ± 0.02
Total	–	6.4 ± 0.7
BUPH	146.25 ± 14.93	10.59 ± 1.34
B1	5.65 ± 0.48	0.41 ± 0.02
B2	80.33 ± 11.37	6.02 ± 2.34
B3	59.59 ± 4.66	4.50 ± 1.27
Total	–	21.5 ± 2.2

this compound (78.40) (Table 4), and thus the combination of the three pathways, ketone reduction, *N*-dealkylation and 4-aryl hydroxylation, was not confirmed to occur for NEH, in mice.

Considering the low excretion rate of the parent drugs and related metabolites during the 24 h study, it was hypothesized that other phase I and phase II metabolites could also be present. It has been reported that cathinones and hydroxylated metabolites are prone to glucuronidation, as well as conjugation with dicarboxylic acids such as succinic, glutaric and adipic acids [17–21]. Therefore, urine samples were also analyzed by UPLC coupled to an Exactive Orbitrap HRMS system, aiming at screening for other metabolites. Based on the structure of NEH and BUPH a list of phase I and phase II metabolites was planned (Supplementary Data, Tables S1 and S2). Extracted ion chromatograms were used for searching the (de)protonated molecule of the predictable molecule on full scan mode, and compared with blank urine to exclude false positives. In addition to the phase I metabolites previously quantified by HPLC-MS/MS, 9 metabolites of NEH and 6 from BUPH were tentatively identified (Tables 6 and 7). The proposed metabolic pathways are depicted in Figs. 1 and 2.

Phase I metabolic reactions included *N*-demethylation, reduction of the keto function, hydroxylation of the aromatic ring and combinations of these reactions. Metabolites resulting from the aliphatic oxidation of the terminal carbon atom of alkyl chains of NEH were also identified. These carboxylated metabolites have been recently identified in human urine by Wagmann et al. [20]. Phase II metabolic reactions included *N*-acetylation (for both NEH and BUPH), glucuronidation (2 metabolites found for NEH) and further conjugation of the *N*-dealkyl metabolites with succinic, glutaric or adipic acids (2 metabolites identified for NEH and 3 for BUPH). *N*-acetylated derivatives and/or amidic dicarboxylic conjugates of mephedrone [21,37], 4-choroethcathinone [20] and methylone [38] have already been detected urines from rats or Humans. It has been suggested that dicarboxylic acid conjugation could be an alternative pathway to *N*-acetylation, since until now, these metabolites have not been detected simultaneously in biological samples [20,38]. However, to the best of our knowledge, this is the first report of *N*-acetyl, succinyl, glutaryl and adipoyl metabolites of NEH and BUPH in mice urine, being also the first time that they were simultaneously identified. Interestingly, a metabolite resulting from the *O*-succinyl conjugation of BUPH hydroxylated metabolite, and not yet described, was also putatively identified in this screening. No conjugated metabolites of dicarboxylic acids and hydroxylated NEH were identified.

4. Conclusions

In this work, NEH, BUPH and corresponding metabolites were quantified for the first time in 24 h urine samples from mice exposed to both drugs, using a HPLC-MS/MS method. Identification was confirmed by comparison with standards synthetized in-house. Metabolites quantified in this study suggest that both drugs follow the same metabolic pathways as other *N*-alkylated cathinones, with or without aromatic ring substituents. The most excreted metabolites were the *β*-keto-*N*-dealkylated compounds, which have been proposed as urine biomarkers of recent human exposure. Additionally, 4-aryl hydroxylated metabolites were detected for the first time in non-ring substituted SC. It was also observed that both NEH and BUPH were eliminated in mice urine in their unchanged form. However, NEH and corresponding *β*-keto reduced metabolite (H1) and *N*-dealkylated alcohol (H3) were excreted at a lower extent than BUPH and its *β*-keto reduced metabolite (B1) and *N*-dealkylated alcohol (B3).

Using liquid chromatography-high resolution mass spectrometry various phase II metabolites, including *N*-acetylated, glucuronides and dicarboxylic acid conjugates, were tentatively identified in mice urines. The simultaneous presence of N-acetyl, succinyl, glutaryl and adipoyl metabolites is reported for the first time.

Adding information on metabolic and excretion profiles allows the identification of potential metabolites that can be present in biological samples or wastewater and can be used as NPS biomarkers to assess the extent and pattern of consumption in specific groups or in the general population. All the research done in order to understand the metabolism and mechanisms of action of SC is important due to the in-

Table 6
Retention time, elemental formula, experimental and theoretical *m/z*, and error obtained for NEH and metabolites quantified (H1, H2, H3 and H4) and putatively identified in mice urine.

Compound ID	Rt (min)	Elemental formula	Experimental *m/z*	Theoretical *m/z*	Error (ppm)
NEH	7.89	$C_{14}H_{21}ON$	219.16231	219.16231	0
Aromatic hydroxylation isomer 1	6.63	$C_{14}H_{21}O_2N$	235.15714	235.15723	−0.38
Aromatic hydroxylation isomer 2	7.10	$C_{14}H_{21}O_2N$	235.15712	235.15723	−0.47
Carboxylation isomer 1	6.53	$C_{14}H_{19}O_3N$	249.13646	249.13649	−0.12
Carboxylation isomer 2	7.73	$C_{14}H_{19}O_3N$	249.13637	249.13649	−0.48
Reduction + glucuronidation	7.50	$C_{20}H_{31}O_7N$	397.20955	397.21005	−1.26
Aromatic Hydroxylation + glucuronidation	5.94	$C_{20}H_{29}O_8N$	411.18959	411.18932	0.66
N-Deethylation + N-acetylation	8.62	$C_{14}H_{19}O_2N$	233.14159	233.14158	0.04
N-Deethylation + succinic conjugation	9.68	$C_{16}H_{21}O_4N$	291.14696	291.14706	−0.34
N-Deethylation + adipic conjugation	12.06	$C_{18}H_{25}O_4N$	319.17836	319.17836	0

Table 7
Retention time, elemental formula, experimental and theoretical *m/z* and error obtained for BUPH metabolites putatively identified in mice urine.

Compound ID	Rt (min)	Elemental formula	Experimental *m/z*	Theoretical *m/z*	Error (ppm)
BUPH (parent compound)	6.48	$C_{11}H_{15}ON$	177.11517	177.11536	−1.07
Aromatic Hydroxylation	5.77	$C_{11}H_{15}O_2N$	193.11028	193.11028	0
N-Demethylation + N-acetylation	7.47	$C_{12}H_{15}NO_2$	205.11027	205.11029	−0.1
Hydroxylation + succinic conjugation	7.99	$C_{15}H_{19}O_5N$	293.12634	293.12632	0.07
N-Demethylation + succinic conjugation	8.32	$C_{14}H_{17}O_4N$	263.11565	263.11576	−0.42
N-Demethylation + glutaric conjugation	8.49	$C_{15}H_{19}O_4N$	277.13142	277.13141	0.04
N-Demethylation + adipic conjugation	10.42	$C_{16}H_{21}O_4N$	291.14696	291.14706	−0.34

creased consumption of this type of products and the associated health risk concerns.

CRediT authorship contribution statement

Joana Carrola: Methodology, Investigation, Validation, Writing - original draft. **Noélia Duarte:** Supervision, Writing - review & editing. **Pedro Florindo:** Investigation. **Sara Henriques:** Investigation. **Gustavo da Silva:** Investigation. **Lubertus Bijlsma:** Investigation. **Rui Moreira:** Supervision. **Catarina Correia:** Investigation. **Maria de Jesus Perry:** . **Álvaro Lopes:** Conceptualization, Project administration, Funding acquisition. **Cristina de Mello-Sampayo:** Investigation, Writing - review & editing. **Maria do Rosário Bronze:** Supervision, Writing - review & editing.

CRediT authorship contribution statement

Joana Carrola: Methodology, Investigation, Validation, Writing - original draft. **Noélia Duarte:** Supervision, Writing - review & editing. **Pedro Florindo:** Investigation. **Sara Henriques:** Investigation. **Gustavo da Silva:** Investigation. **Lubertus Bijlsma:** Investigation. **Rui Moreira:** Supervision. **Catarina Correia:** Investigation. **Álvaro Lopes:** Conceptualization, Project administration, Funding acquisition. **Cristina de Mello-Sampayo:** Investigation, Writing - review & editing. **Maria do Rosário Bronze:** Supervision, 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.

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Appendix A. Supplementary material

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