In vitro Metabolite Profile of Selected Cathinones: identification of Phase I metabolites by Liquid Chromatography High Resolution Mass Spectrometry

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Abstract

The present work was carried out within the scope of the protocol established between the Instituto Superior Técnico (IST), the Faculty of Sciences of the University of Lisbon (FCUL) and the Scientific Police Laboratory of the Judicial Police (LPC-PJ), which encompasses the identification and analysis of New Psychoactive Substances (NPS) in Portugal in multiple matrices.

This work is aimed at identifying the metabolic profiles of four synthetic cathinones: 4'-Methyl-*N*-ethylnorbufedrone (4-MNEB), 4'-Methyl-*N*, *N*-dimethylnorpentedrone (4-MDMP), 4 ' -Methyl-*N*, *N*-dimethylnorbufedrone (4-MDMB) and 4'-Methyl-*N*-ethylnutrentedrone (4-MNEP). Of the cathinones mentioned, only 4-MNEP was reported in Luxembourg in 2014. The 4-MDMB and 4-MNEP cathinones are available from Cayman Chemical. However, similarly with 4-MDMP and 4-MNEB, 4-MDMB have never been reported as NSP. Nonetheless, their structural similarity with other NSP sets their introduction in the NSP market as highly probable.

The metabolites were generated in metabolically competent *in vitro* systems: human liver (HLM), rat liver (RLM), S9 fraction of human liver (S9H) and S9 fraction of rat liver (S9R) microsomes. These incubations were performed in the presence of the cofactor's NADPH (Phase I) and UDPGA (Phase II). The metabolites were subsequently identified by Liquid Chromatography coupled to Tandem High Resolution Mass Spectrometry.

This strategy allowed the firsthand identification of two Phase I metabolites of 4-MNEB, three Phase I metabolites of 4-MDMP and three Phase I metabolites of 4-MDMB, which is expected help the competent authorities to validate the consumption of these cathinones.

Keywords: NPS; microsomes; metabolites; LC-ESI-HRMS; synthetic cathinones.

1. Introduction

In the last 20 years, the abuse drug market has undergone a major change with the appearance and continued introduction of a wide variety of New Psychoactive Substances (NSP). NSP have a dynamic, global, adapted, and diversified market, which is translated into a constant change in

relation to the substances that are available and a rapid distribution, reaching the global market efficiently, thanks to the internet. Thus, it is necessary that measures are taken to mitigate the advance and rapid distribution of these substances.

Synthetic cathinones, the second largest group of NPS reported worldwide (EMCDDA, 2020; UNODC, 2018) are β keto phenethylamines, structural analogues of cathinone, the major psychoactive alkaloid present in the leaves of *Catha edulis*. This class of NPS are known to produce psychostimulant effects similar to methamphetamine, cocaine, or MDMA (ecstasy) by interacting with the plasma membrane transporters of the monoamine neurotransmitters, dopamine, norepinephrine and serotonin, increasing their synaptic cleft concentration (German et al. 2014; Weinstein et al., 2017). Metabolic profiling of cathinones is crucial not only to understand potential adverse effects and link causative agents to toxicities but also to identify unique biomarkers of their intake, that will allow their unequivocal identification in biological matrices. In fact, some cathinones undergo extensive metabolism *in vivo*, (Ellefsen et al., 2016; Uralets et al., 2014) which is translated into low or even negligible urinary levels of the parent cathinone. Therefore, for these cases, the detection of cathinone´s metabolites in biofluids, is the only possible way of proving the consumption of these NPS, in particular several hours after its intake. However, the metabolic profiles of most of the recently reported synthetic cathinones remain unknown.

With the ultimate goal of contributing for a proactive response in tackling the NPS problem, and within the scope of a protocol established between the Forensic Science Laboratory from Portuguese Criminal Police and the Lisbon University (Instituto Superior Técnico and Faculty of Sciences), the present work is aimed at determining the metabolite profile of four selected cathinones (Figure 1): 4'-Methyl-*N*-ethylnorbufedrone (4-MNEB), 4'-Methyl-N, *N*-dimethylnorpentedrone (4-MDMP), 4'-Methyl-*N*,*N*-dimethylnorbufedrone (4-MDMB) and 4'-Methyl-*N*-ethylnutrentedrone (4-MNEP). Of the selected cathinones, only 4-MNEP was reported in Luxembourg in 2014 (EMCDDA, 2015). 4-MDMB is available from Cayman Chemical, however, similarly with 4-MDMP and 4-MNEB, they have never been reported as NPS. Nonetheless, their structural similarity with other NSP sets their introduction in the NSP market as highly probable.

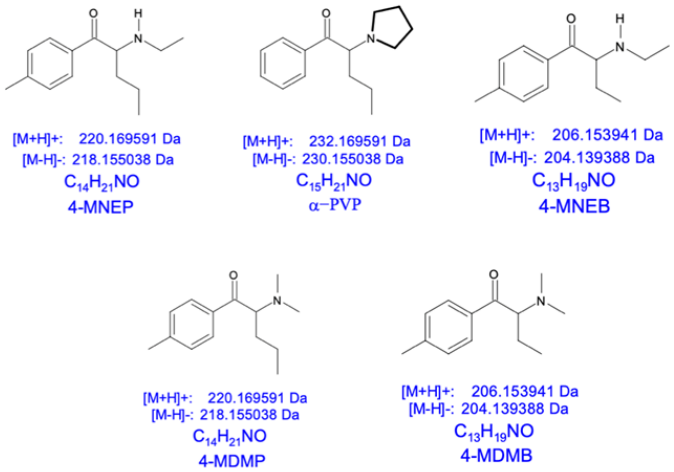


Figure 1 - Structures of the synthetic cathinones used in the current study.

2. Experimental

2.1. Materials and methods

The α-PVP, 4-MNEB, 4-MDMP, 4-MDMB and 4-MNEP standards patterns used in this work were previously synthesized at the Faculty of Sciences of the University of Lisbon (Júlio, S.M.D., 2019). The microsomes of human liver (Pooled), the microsomes of rat liver (Sprague-Dawley), the S9 fraction of human liver (Pooled) and the S9 fraction of rat liver (Sprague-Dawley) are from GIBCO, with a concentration of 20 mg/mL, the "Vivid Regeneration System (x 100)" regeneration system is from Life Technologies. All other commercially available reagents were acquired from Sigma-Aldrich Química, S.A. and used as received.

2.2. Liquid chromatography coupled to tandem High Resolution Mass Spectrometry (LC-ESI-HRMS/MS)

This technique was performed on an UPLC Elute system (Bruker, Bremen, Germany) connected with a quadrupole-time-to-flight hybrid mass spectrometer (QTOF) IMPACT II (Bruker Daltoniks, Bremen) and equipped with an electrospray ionization source (Bruker Daltoniks, Bremen). The samples were separated into Luna® (Phenomenex) 3 µm Polar C18 (2) 100 Å (150 * 2 mm) columns. The flow defined for the mobile phase was 170 µLmin-1 using the following gradient: it starts with 95% 0.1% formic acid in water (eluent A), which is maintained for 1.5 min, followed by a gradient linear from 6.20 min to 100% acetonitrile (eluent B), maintaining these conditions for 1.8 min, a gradient of 0.5 min was followed until the initial conditions, which are maintained for 1 min. The MS spectra were acquired in positive and negative modes with the following parameters: capillary voltage, ± 4.5 kV; nebulizer gas, 4 Bar; drying gas flow, 8 $Lmin^{-1}$, drying gas temperature, 200 ºC. The calibration of each spectrum was performed with an ammonium formate standard that was analyzed by FIA. Data collections were performed in data-dependent mode (DDA, Auto MS) with *m/z* between 50 to 1500 Da. The collected data were processed using the Compass Data Analysis Version 4.4 software and the identification of the compounds was carried out through detailed manual analysis of the full scan and MS/MS spectra.

It was previously built a database with the expected *m/z* values for the Phase I and Phase II metabolites of each of the cathinones selected for this study. This database was built taking into account the various metabolic pathways already described in the literature for this family of compounds (Meyer, *et al.,* 2010*;* Meyer and Maurer, 2010; Strano-Rossi, *et al.*, 2010; Meyer, *et al., 20*12; Negreira, *et al.*, 2015; Zawilska, J.B., 2018). All spectra corresponding to the metabolites were checked manually. The exact mass deviation of the identified cathinone metabolites remained below 5 ppm for the precursor and below 10 ppm for the obtained fragments.

2.3. Generation of Phase I metabolites *in vitro*

Cathinones, at a concentration of 10 µM (1 µL, 5 mM water solution), were incubated with human liver microsomes (HLM) (1 mg/mL), rat liver microsomes (RLM) (1 mg/mL), rat S9 liver fraction

(S9R) (2 mg/mL), human S9 liver fraction (S9H) (2 mg/mL), in the presence of: NADPH (20 mM, 1 µL) and the Vivid Regenaration System 100x (NRS 100x) (2 µL) for a total incubation volume of 200 µL in 50 mM ammonium bicarbonate buffer at pH 7.4. Each incubation was run in duplicate. Control incubations were conducted in the same conditions: 1) using water as a negative control, in the absence of cathinone; 2) in the absence of the NADPH cofactor; and 3) using heat-denatured (90 °C, 15 min) microsomes. Incubation of α-PVP, was run at the same conditions, as a positive control incubation. The mixtures were incubated at 37 °C and 100 µL aliquots were collected following 2 h, 4h and 24h of incubation. Acetonitrile (100 µL) was then added to quench the reactions. Following centrifugation at 10000 g for 15 min at room temperature, the supernatants were collected and analyzed by LC-ESI-HRMS/MS.

2.4. Generation of Phase II metabolites *in vitro*

HLM and RLM (1 mg/mL) or S9R and S9H (2mg/mL) were preincubated for 15 min, in ice, with alamethicin (4 µL, 5 mgmL-1) in 50 mM ammonium bicarbonate buffer at pH 7.4, for a total incubation volume of 200 µL. Following the addition of $MgCl_2$ (400 mM; 1 µL), NRS (4 µL) and cathinone (10 µM final concentration; 1 µL, 5 mM water solution), the resulting solution was incubated for 5 min at 37ºC. NADPH (100 mM, 1 µL) and UDPGA (50 mM, 4 µL) were then added to start the Phase I and II reactions. Incubations were run in duplicate.
Control incubations were conducted in the same conditions: 1) using water as a negative control, in the absence of cathinone; 2) in the absence of the NADPH and UDPGA cofactors; and 3) using heat-denatured (90°C, 15 min) microsomes. Incubation of α-PVP, was run at the same conditions, as a positive control incubation. The mixtures were incubated at 37 °C and a 100 µL aliquot was collected following 2 h of incubation. Acetonitrile (100 µL) was then added to quench the reactions. Following centrifugation at 10000 g for 15 min at room temperature, the supernatants were collected and analyzed by LC-ESI-HRMS/MS.

3. Results and discussion

For the *in vitro* generation of metabolites of the four selected cathinones, we decided to use not only liver microsomes, which were already successfully used for the generation of metabolites that occur *in vivo* for other cathinones, (Meyer *et al*., 2010; Meyer and Maurer, 2010; Strano-Rossi *et al.,* 2010; Meyer *et al.*, 2012; Negreira *et al*., 2015; Zawilska, J.B., 2018), but also human and rat S9 liver fractions (S9H and S9R). This decision was made based on the fact that S9 liver fractions contain not only microsomal metabolizing enzymes but also cytosolic, (Richardson *et al.*, 2016; Wagmann *et al.,* 2019) which might contribute for the formation of a larger number of metabolites. The metabolites generated in this biologically competent systems, in the presence of Phase I and Phase II co factors, NADPH and UDPGA, were subsequently identified by Liquid Chromatography coupled to High Resolution Mass Spectrometry with Electrospray Ionization (LC-ESI-HRMS) was subsequently used to identify the metabolites generated. Towards this goal,

a database was initially built with the *m/z* values of all expected metabolites for each of the cathinones, taking into account the metabolic pathways already known from other structurally similar cathinones. Subsequently, the extracted ion chromatograms (EIC), with a mass window of ± 5 ppm, were performed for searching the protonated or deprotonated molecules of the expected metabolites in the full scan spectra obtained for each aliquot analyzed. For positive identifications in the full scan spectra, the formation of metabolites was subsequently confirmed in the MS/MS spectra where fragments with less than 10 ppm were considered. 885 full scan and MS/MS spectra were analyzed, and only those that comply with the mentioned criteria were considered. α-PVP incubations were used as a positive control, since the metabolic pathways of this cathinone are already widely described in the literature (Sauer *et al*., 2009; Tyrkkö *et al*., 2013; Shima *et al*., 2014; Uralets *et al*.; 2014; Zawilska, J.B., 2018). It should be noted that only Phase I metabolites could be identified under the experimental conditions used for this study. In fact, no Phase II metabolites could be identified upon LC-ESI-HRMS (in the positive and negative ESI modes), of incubations run in the presence of NADPH and UDPGA. The fact that no Phase I metabolites were identified in these incubations suggest the lack of enzymatic activity in these incubations. Nonetheless, for incubations run in the presence of Phase I co-factor NADPH, multiple metabolites of the control cathinone (α-PVP), were obtained (Table 1). In particular the identification of the α-PVP metabolite stemming from ketone reduction (M1 α-PVP), which was the most abundant metabolite found *in vivo* for this cathinone (Tyrkkö *et al*., 2013), attests the viability of the experimental conditions used in the current study for identifying the metabolites that are most likely to be found in vivo. Coherently, multiple metabolites could be positively identified for the selected cathinones.

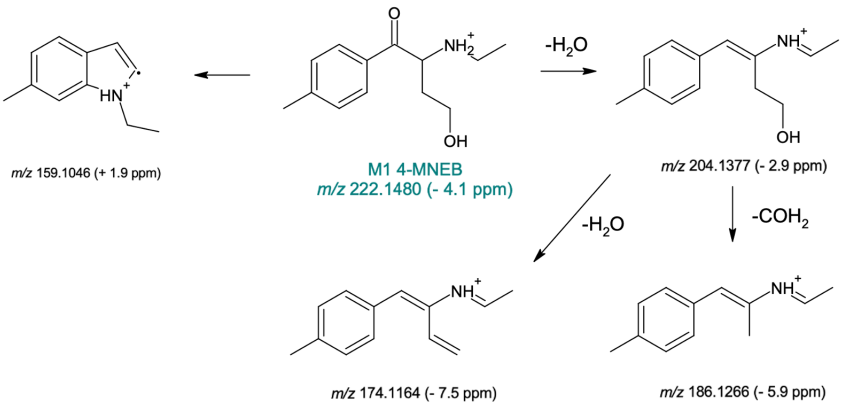
As mentioned previously, a data base containing the *m/z* expected for of all possible metabolites for the 4 selected cathinones was first built. For the identification of metabolites, the extracted ion chromatograms, from all ions corresponding to the expected metabolites for this cathinone, wwere carried out for the multiple aliquots analyzed. When a *m/z* value compatible with the full scan spectrum (with an error of less than 5 ppm) was found, confirmation of the metabolite formation was only achieved upon the identification of plausible fragment ions (with an error less than 10 ppm) in the MS/MS spectrum. For the cathinone 4-MNEB, the formation of metabolites M1-4-MNEB and M2-4-MNEB, were confirmed through the interpretation of the MS/MS spectra. In order to correctly identify the metabolites, it is essential to know the fragmentation mechanisms of the precursor cathinone, in this case 4-MNEB. In the analytical conditions used in the analysis by LC-ESI-HRMS, 4-MNEB has a retention time (t_R) of 7.7 minutes and displays a signal in the full scan spectrum at *m/z* 206.1543 (+ 1.9 ppm), corresponding to the protonated molecule. The base fragment ion in the MS/MS spectrum of the 4-MNEB is observed at *m/z* 188.1434 (0 ppm) and corresponds to the loss of a water molecule from the protonated molecule, which stems from a rearrangement that is characteristic of other cathinones (Vijlder *et al*., 2018). The subsequent loss of the methyl radical results in the formation of the fragment ion a *m/z* 173.1192 (- 4 ppm). The other two ions observed in the MS/MS spectrum at *m/z* 144.0808 (0 ppm) and 159.1046 (+

1.9 ppm result from the cyclization of the molecule resulting in the formation of a indole ring, which has already been reported for other structurally similar cathinones (Pozo *et al.,* 2014).

Table 1 - Retention time and experimental m/z values for the protonated molecule and characteristic fragment ions (shown with the associated error in ppm) for α-PVP and 4-MNEB cathinone and its metabolites identified in incubations with HLM, RLM, S9H and S9R.

Name	t_R (min)	*m/z* exp. $[M+H]^+$ [± error (ppm)]	fragment ions [± error (ppm)]	Identification
α-PVP	6.6	232.1697 (+ 0.4 ppm)	161.0958 (-1.2 ppm) 126.1277 (0 ppm) 189.1150 (+ 1.1 ppm)	HLM,RLM S9H,S9R
M1- α-PVP	6.8	234.1850 (- 0.9 ppm)	216.1737 (- 4.6 ppm) 173. 1188 (-6.4 ppm)	HLM,RLM S9R
M6- α-PVP	12.5	246.1480 (- 3.7 ppm)	161.0948 (-8.1 ppm) 228.1373 (-4.4 ppm)	S9R
M7- α-PVP	6.5	264.1589 (- 1.9 ppm)	246.1495 (+ 2.4 ppm) 228.1370 (-5.7 ppm)	HLM S9R
4-MNEB	7.7	206.1543 (+ 1.9 ppm)	188.1434 (0 ppm) 173.1192 (- 4 ppm) 159.1046 (+ 1.9 ppm) 144.0808 (0 ppm)	HLM, RLM S9H, S9R
M1-4-MNEB	5.1	222.1480 (- 4.1 ppm)	204.1377 (- 2.9 ppm) 186.1266 (- 5.9 ppm) 159.1046 (+ 1.9 ppm) 174.1164 (- 7.5 ppm)	HLM, RLM S9R
M2-4-MNEB	5.3	236.1276 (- 2.1 ppm)	218.1169 (- 3.2 ppm) 200.1079 (+ 4.5 ppm) 174.1271 (- 3.4 ppm)	HLM S9H,S9R

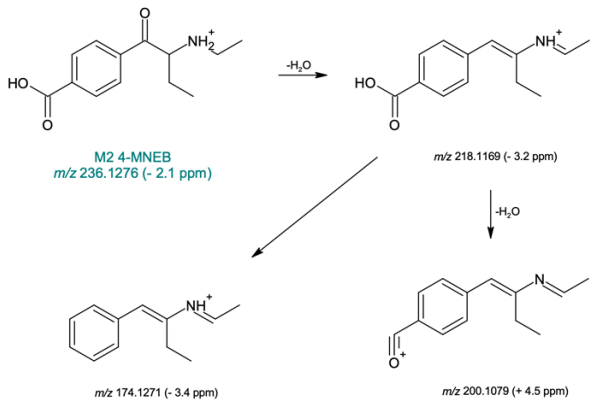
The metabolite M1-4-MNEB elutes at 5.1 minutes and displays a signal at *m/z* 222.1480 (- 4.1 ppm), in the full scan spectrum, which is compatible with the protonated molecule of a metabolite stemming from the hydroxylation of the precursor cathinone (4-MNEB). In the analysis of the MS/MS spectrum of this metabolite are observed, the fragment ions a *m/z* 204.1377 (- 2.9 ppm) and *m/z* 186.1266 (- 5.9 ppm) (Scheme 1), which stem from two consecutive losses of water molecule from the protonated molecule. The base signal of the MS/MS spectrum of this metabolite is identified at *m/z* 174.1164 (- 7.5 ppm) and results from the loss of fomaldehyde from the fragment ion at m/z 204.1377. It should be noted that this fragment ion suggests that hydroxylation occurred in the alkyl chain, since any of the other possible hydroxylation positions (aromatic methyl group or nitrogen substituting group) would result in an identical fragment. This assignment is further reinforced by the identification of the fragment ion at *m/z* 159.1046 (+1.9 ppm), which is only possible if hydroxylation has occurred in the alkyl chain and is in accordance with the fragmentation pattern of other cathinones (Wagmann *et al*., 2019).



Scheme 1 - Proposed fragmentation pattern for the M1-4-MNEB.

M2-4-MNEB elutes at 5.3 minutes and displays a signal in the full scan spectrum at *m/z* 236.1276 (- 2.1 ppm), which is compatible with the protonated molecules of a metabolite stemming from three consecutive metabolic reactions: 1) hydroxylation of the methyl group at the position 4' ´of the aromatic ring; 2) oxidation of benzyl alcohol to formaldehyde; and 3) oxidation of the formaldehyde to carboxylic acid. The MS/ MS spectrum of this metabolite displays three fragment ions: 1) at *m/z* 218.1169 (- 2.1 ppm) the fragment ion that results from the water loss, characteristic of the cathinones (Vijlder *et al*., 2018); 2) at *m/z* 174.1271 (-3.4 ppm), the loss of CO_2 from the previous ion; and 3) *m/z* 200.1079 (+ 4.5 ppm), the loss of water from the first fragment ion.

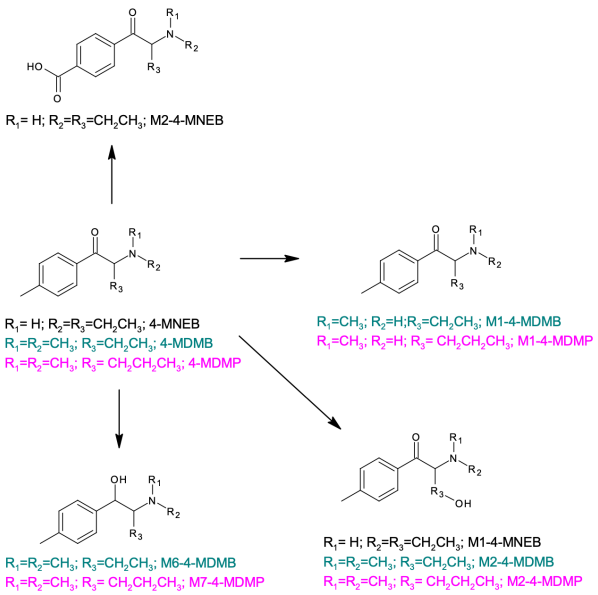
The identification of all metabolites from the other three selected cathinones was performed by a similar procedure. The results are displayed in Table 2. Of note is the fact that for 4-MNEP no metabolites were identified at the experimental conditions used in the current study, albeit Benedicte et al. (2019) have consistently identified two Phase I metabolites in HLM incubations and human urine. A possible explanation for these apparently contradictory results might be related to the low metabolic conversion of this cathinone. In fact, for the identification of metabolites Benedicte et al. (2019) used a cathinone concentration of 100 and 500 µM (opposed to the 10 µM used in the current study) and even at these conditions, these authors mentioned the low metabolic conversion of 4-MNEP.



Scheme 2 - Proposed fragmentation pattern for the M2-4-MNEB.

Table 2 - Retention time and experimental m/z values for the protonated molecule and characteristic fragment ions (shown with the associated error in ppm) for 4-MDMP, 4-MDMB and 4-MNEP and its metabolites identified in incubations with HLM, RLM, S9H and S9R

Name	t_R (min)	*m/z* exp. $[M+H]^+$ [± erro (ppm)]	fragment ions [± error (ppm)]	Identification
4-MDMP	8.3	220.1690 (- 2.7 ppm)	175.1110 (- 4 ppm)	HLM, RLM S9H, S9R
M1-4-MDMP	8.2	206.1536 (- 1.5ppm)	188.1440 (+ 3.2 ppm)	HLM, RLM S9H, S9R
M2-4-MDMP	5.6	222.1484 (- 2.3 ppm)	174.1271 (- 2.3 ppm) 144.0808 (0 ppm) 161.0954 (- 4.3 ppm) 186.1272 (- 2.7 ppm) 204.1388 (+ 2.4 ppm)	HLM S9R
M7-4-MDMP	8.4	222.1857 (+ 2.3 ppm)	204.1756 (+ 4.4 ppm) 161.1216 (+ 9.9 ppm) 175.1125 (+ 4.6 ppm)	HLM S9H
4-MDMB	7.4	206.1537 (- 1 ppm)	161.0956 (- 3.1 ppm) 177.1137 (- 6.2 ppm)	HLM, RLM S9H, S9R
M1-4-MDMB	7.3	192.1385 (+ 1 ppm)	174.1293 (+ 9.2 ppm) 145.0894 (+ 5.5 ppm)	HLM, RLM S9R
M2-4-MDMB	4.7	208.1326 (- 2.9 ppm)	190.1218 (- 4.2 ppm) 172.1114 (- 4.1 ppm) 147.0809 (+ 3.4 ppm) 131.0735 (+ 3.8 ppm) 160.1120 (- 0.6 ppm) 177.1137 (- 6.2 ppm)	HLM S9R
M6-4-MDMB	7.5	208.1692 (- 1.9 ppm)	190.1595 (+ 2.6 ppm)	HLM, RLM S9H
4-MNEP	8.6	220.1696 (0 ppm)	202.1596 (+ 3 ppm) 173.1207 (+ 4.6 ppm) 144.0815 (+ 4.9 ppm) 131.0738 (+ 6.1 ppm) 160.1120 (- 0.6 ppm)	HLM, RLM S9H, S9R



Scheme 3 - General scheme of the metabolites identified in this study in HLM, RLM, S9H and S9R incubations.

Scheme 3 shows the metabolic pathways identified for the cathinones selected for this study. A common metabolic pathway of the two cathinones with a tertiary amine is the *N*-dealkylation and the reduction of the ketonic moiety. The hydroxylation of the alkylic chain is observed for three of the selected cathinones. For 4-MNEB it is also observed the carboxylic acid metabolite resulting from the oxidation of the aromatic methyl substituent.

4. Conclusion

This study was aimed at identifying the metabolites of the cathinones 4-MNEB, 4-MDMP, 4-MDMB and 4-MNEP. Several metabolically competent *in vitro* systems were used to generate these metabolites: RLM, HLM, S9H and S9R. The incubations were performed in the presence of the NADPH cofactor, in the case of Phase I, and UDPGA, in the case of Phase II. The generated metabolites were identified by LC-ESI-HRMS. α-PVP was used as a positive control, and the identification of the most abundant metabolite found *in vivo* for this cathinone attests the viability of the experimental conditions used in the current study for identifying the metabolites that are most likely to be found in vivo. However, the fact that no Phase I or Phase II metabolites were identified in incubations run in the presence of NADPH and UDPGA, suggests the lack of enzymatic activity. Coherently, only Phase I metabolites were identified for the cathinones selected for this study. In in the current work it was possible to identify, for the first time, two Phase I metabolites of 4-MNEB, three Phase I metabolites of 4-MDMP cathinone and three Phase I metabolites of 4-MDMB cathinone. However, no metabolites were identified for 4-MNEP. It is important to note that the *in vitro* systems that enabled the identification of a larger number of metabolites were HLM and S9R. The hydroxylation of the alkylic chain was observed for three of the selected cathinones and a common metabolic pathway of the two cathinones with a tertiary amine is the *N*-dealkylation and the reduction of the ketonic moiety.

The identification of metabolites reported for the first time in this work may play an important role in helping the competent authorities to validate the consumption of these cathinones upon analyzes of biological fluids. This is just the first step for the identification of the metabolites of these cathinones. It would be important for future work to be able to analyze urine and blood samples from people who are under the effect of these substances, in order to validate the presence of these metabolites in these biological samples. Subsequently, once its formation is attested, they could be synthesized for its subsequent toxicity evaluation.

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