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Metabolic study of new psychoactive substance methoxpropamine in mice by UHPLC-QTOF-HRMS

Marta Massano¹  | Enrico Gerace² | Martina Borsari¹ | Matteo Marti^{3,4,5}  |
Micaela Tirri³ | Christina Ververi¹  | Eugenio Alladio¹ | Marco Vincenti^{1,2}  |
Alberto Salomone^{1,2}

¹Department of Chemistry, University of Turin, Turin, Italy

²Centro Regionale Antidoping, Orbassano, TO, Italy

³Department of Translational Medicine, Section of Legal Medicine, LTTA Center, University of Ferrara, Ferrara, Italy

⁴University Center for Studies on Gender Medicine, University of Ferrara, Ferrara, Italy

⁵Collaborative Center for the Italian National Early Warning System, Department of Anti-Drug Policies, Presidency of the Council of Ministers, Rome, Italy

Correspondence

Marta Massano, Department of Chemistry, University of Turin, Turin, Italy.
Email: marta.massano@unito.it

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Abstract

Methoxpropamine (MXPr) is an arylcyclohexylamine dissociative drug structurally similar to 3-methoxyeticyclidine, ketamine, and deschloroketamine, recently appeared in the European illegal market, and was classified within the new psychoactive substances (NPS). Our study investigated the metabolism of MXPr to elucidate the distribution of the parent drug and its metabolites in body fluids and fur of 16 mice. After the intraperitoneal administration of MXPr (1, 3, and 10 mg/kg), urine samples from eight male and eight female mice were collected every hour for six consecutive hours and then at 12- to 24-h intervals. Additionally, plasma samples were collected 24 h after MXPr (1 and 3 mg/kg) administration. Urine and plasma were diluted 1:3 with acetonitrile/methanol (95:5) and directly injected into the UHPLC-QTOF-HRMS system. The phase-I and phase-II metabolites were preliminarily identified by means of the fragmentation patterns and the exact masses of both their precursor and fragment ions. Lastly, the mice fur was analyzed following an extraction procedure specific for the keratin matrix. Desmethyl-MXPr-glucuronide was identified in urine as the main metabolite, detected up to 24 h after administration. The presence of norMXPr in urine, plasma, and fur was also relevant, following a N-dealkylation process of the parent drug. Other metabolites that were identified in fur and plasma included desmethyl-MXPr and dihydro-MXPr. Knowledge of the MXPr metabolites evolution is likely to support their introduction as target compounds in NPS toxicological screening analysis on real samples, both to confirm intake and extend the detection window of the dissociative drug MXPr in the biological matrices.

KEYWORDS

HRMS, metabolites, methoxpropamine, mice, NPS

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1 | INTRODUCTION

The worldwide spread of new psychoactive substances (NPS) in the illicit drug market and their continuous increase in number and type, with the purpose of bypassing the controlled substance legislation, represents an unceasing defy for forensic scientists, clinicians, and enforcement authorities.^{1,2} Nowadays, NPS represent one of most important potential risk factor for public health.³

Unfortunately, a lack of information exists on the metabolic pathway of NPS appearing in the drug scenario, making their identification in biological samples a controversial challenge, especially when the examined samples are limited and the drug is promptly and extensively metabolized.

NPS are often categorized as synthetic cannabinoids, stimulant, depressant (benzodiazepines and opioids), dissociative, 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.⁴

A group of ketamine-like dissociative substances, based on the structure of arylcyclohexamines, represents a newly introduced subclass of NPS.^{5,6} Among these, methoxpropamine (MXPr or 3-MeO-2'-oxo-PCPr) is an arylcyclohexylamine dissociative drug and a homolog of methoxetamine (MXE) that possesses structural similarities with 3-methoxyeticyclidine (3-MeO-PCE), ketamine, and deschloroketamine.⁷ Similarly to its analogs, MXPr acts as a potent antagonist of N-methyl-D-aspartate (NMDA) receptors.⁸ Apparently, MXPr was first synthesized in Denmark in October 2019 and recently entered the chemical market for online sale.⁹ It was then reported as an NPS by the European Monitoring Center for Drugs and Drug Addiction (EMCDDA) in 2020 and was identified for the first time in Italy in the same year.¹⁰

As a recently introduced substance, little is known about MXPr pharmacology, toxicity, and metabolism. Understanding the metabolic fate of the parent drug and identifying the phase I and II products is likely to represent a valid support to develop a targeted analytical method for biological samples, especially blood and urine. For this reason, the present work aimed to study the metabolic profile of MXPr, identify the main metabolites of phase I and II, and observe their pharmacokinetic evolution, in order to single out the most appropriate biomarkers for detecting MXPr abuse.

Quite often, *in vitro* tests and *in vitro* metabolic simulation, for example, with human hepatocytes,^{11,12} human liver microsomes, or with pooled human liver S9 fraction (pHLS9),¹³ are performed to study NPS metabolism.

In our study, we preferred to develop *in vivo* models with male and female mice, possibly simulating the metabolic pathways in humans.¹⁴ After administration of MXPr, urine, blood, and fur were collected and then analyzed. Although an untargeted method was used for data acquisition, the approach used for data processing was typical of a targeted metabolomic analysis.¹⁵ 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 MXPr metabolic profile on the basis of similar studies on the analogous MXE.^{16,17} 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.

2 | MATERIALS AND METHODS

2.1 | Reagents and standards

All chemicals, including methanol, formic acid, and acetonitrile, were purchased from Sigma-Aldrich (Milan, Italy). Ultra-pure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore, Bedford, MA, USA). For UHPLC-QTOF-HRMS quantitative analysis, ketamine-d4 was purchased from LGC Promochem (Milan, Italy) (purity >99%, concentration 1 mg/mL). Methoxpropamine was kindly provided by the Italian National Institute of Health (methanolic solution at a 0.02 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 | Mice study protocol

Overall, 16 ICR (CD-1®) mice (eight male and eight female) weighing 30–35 g were grouped and exposed to a 12:12-h light-dark cycle (light period from 6:30 a.m. to 6:30 p.m.) at a temperature of 20–22°C and humidity of 45–55%. Each cage contained five mice with a floor area per animal of 80 cm² and minimum enclosure height of 12 cm. They were provided *ad libitum* access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in the present study were in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU). Experimental protocols were approved by the Italian Ministry of Health (license n. 335/2016-PR) and by the Animal Welfare Body of the University of Ferrara. According to the ARRIVE guidelines, all possible efforts were made to minimize the number of animals used, to minimize the animals' pain and discomfort.

MXPr was dissolved in Tween 80 (2%) and ethanol (5%), brought to the final volume with saline (0.9% NaCl). Mice were treated by intraperitoneal (i.p.) injection (volume of 4 µl/g) in subsequent days and divided into two groups (4 males and 4 females for each group). Group 1 (G1) were injected with all three doses of MXPr (1, 3, and 10 mg/kg; i.p.), while group 2 (G2) were injected only with dosage of 1 mg/kg. After the first injection of MXPr (1 mg/kg; i.p.) all mice (G1 and G2) were located in metabolic cages (Ugo Basile SRL, Gemonio [VA], Italy) for urine samples collection that was carried out individually for each animal at a specific time point (protocol already validated in the laboratory¹⁴). Subsequently, urine samples were collected only from G1 mice group, after treatment on different days at

different dosages (3 and 10 mg/kg; i.p.). After 20 days of wash out, all mice (G1 and G2) were retreated twice with MXPr (1 and 3 mg/kg; i.p.) in consecutive days for blood and fur samples collection.

2.3 | Urine samples collection and preparation

The urine samples were collected before MXPr administration (time 0; blank sample), then at 1, 2, 3, 4, 5, 6, 12, and 24 h after administration. All samples were collected in 1.5-mL tubes and stored at -20°C . Considering the extremely small volumes available, all urine samples were pooled by collection time and sex. The availability of the samples and the summary of the samples' collection is shown in Table S1. The evolution of MXPr and its metabolites over time was monitored, taking note of the differences between male and female metabolic pathways.

No enzymatic hydrolysis of glucuronides and sulphates was performed on phase II metabolites. Briefly, 50 μL of the urine samples were initially added with 2.5 μL of internal standard (ketamine-d4 at final concentration of 50 ng/mL), and then they were diluted 1:3 with a frozen acetonitrile/methanol (95:5) mixture and vigorously stirred for 5 min. After centrifugation for 5 min at 13,000g, 5 μL of the supernatant was directly injected into the UHPLC system. To allow us to quantify the possible presence of MXPr in urine samples, a urinary matrix negative for the substance was fortified at five concentration levels (10, 50, 100, 500, 1000 ng/mL) with the MXPr working solution and ketamine-d4 was used as the internal standard (ISTD). Each calibration point was repeated in triplicate, for the urine matrix, at five concentration levels.

2.4 | Plasma samples collection and preparation

After 20 days of wash out, mice were readministered with two MXPr doses (1–3 mg/kg; i.p.) in consecutive days, and then the plasma samples were collected by submandibular withdrawal technique.¹⁸ In this case, three samples were obtained: Sample MG1 is the plasma pool relative to the male mice treated on day 1, while samples FG1 and FG2 are the plasma pools relative to the female mice treated at 1 mg/kg (day 1) and 3 mg/kg (day 2), respectively. For the plasma pre-treatment, 50 μL of the samples were initially added with 2.5 μL of internal standard (ketamine-d4 at final concentration of 50 ng/mL), and then they were diluted 1:3 with a frozen acetonitrile/methanol (95: 5) mixture, vigorously stirred for 5 min and placed in the cold room at -20°C for 15 min before centrifugation to facilitate the precipitation of plasma proteins. After centrifugation for 5 min at 13,000g, 5 μL of the supernatant was directly injected into the UHPLC system and the concentration of MXPr was determined using a plasma sample of the mice, previously confirmed as negative to MXPr. The calibration curve was obtained in the range of concentration 10–1000 ng/mL.

2.5 | Fur samples collection and preparation

Fur samples were collected 1 month in advance and at the end of the MXPr treatment. All fur samples were treated with a procedure developed on-purpose for the keratin matrix. Fur samples were pooled by sex in order to obtain a sufficient amount of fur for the analysis. About 50 mg of fur was decontaminated by an initial wash with 1-mL dichloromethane followed by a second wash with 1-mL methanol, each one performed under 3 min stirring. The dried fur was pulverized using six steel balls stirring in a Precellys[®] homogenizer. The pulverized samples were extracted by keeping them immersed in 0.5-mL methanol added with 2.5 μL of ISTD (ketamine-d4) at $+55 \pm 5^{\circ}\text{C}$ for 15 h. At the end of the incubation, the tubes were centrifuged and 5 μL of the supernatant were injected into the UHPLC system. Finally, the fur of the mice, previously confirmed as negative to MXPr, was used to build the calibration curve ranged from 10 to 1000 pg/mg, allowing us to quantify the MXPr.

2.6 | Instrumental conditions

UHPLC separation was performed on the SCIEX ExionLC[™] AC system (Sciex, Darmstadt, Germany) using a Phenomenex Kinetex C18 column (100 \times 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 S2). 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 600.0 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 30-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 MXPr 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 MarkerView™ software from Sciex.

3 | RESULTS AND DISCUSSION

The alleged metabolites were investigated based on an extensive review of ketamine and MXE metabolism.^{17,18} MXE was initially taken as reference because of the similarity of their molecular structures, where the N-ethyl group (MXE) has been replaced by a N-*n*-propyl group in MXPr (Figure 1a). Thus, similar metabolic reactions can be assumed, including reduction, hydroxylation, N-depropylation, dehydrogenation, O-desmethylation (Figure 1b), and di-hydrogenation of the cyclohexanone ring, similarly to ketamine. Figure 1c shows the expected phase I metabolites. Afterwards, the conjugation reactions with glucuronic acid (phase II metabolism) were expected and the confirmation of these metabolites' structures was deduced by the high-resolution mass spectra corresponding to new chromatographic peaks appearing in the urine samples collected after MXPr administration. We speculated that the same metabolic pathways, resulting from a single or a combination of the aforementioned biotransformation, would occur for MXPr. In our study, we developed *in vivo* models using male and female mice, possibly simulating human metabolic pathways. The inclusion of both male and female mice aims to consider the sexual dimorphism potentially related to pharmacodynamic and pharmacokinetic mechanisms that typically characterize NPS profiles.¹⁹ In fact, such sex-related differences have already been described in the literature as variability factors influencing the pharmacotoxicological benchmarks of various therapeutic drugs.²⁰ The urine pool from male mice treated with the highest concentration

of MXPr (10 mg/kg) was initially used for the detection of all metabolites, including the less abundant ones. A total of nine metabolites was identified in urine samples, including phase I and glucuronated phase II metabolites; no mono-hydroxylated nor sulfated metabolites were found (Table 1). Information on the presence of MXPr and its metabolites in urine at different time-points is available in Figure S2. Sulfation is a minor metabolic step in rodents while it seems to be more important in humans compared to glucuronidation. Therefore, further experiments will be considered to confirm or exclude the presence of the sulfate conjugates in humans. The candidate metabolites of MXPr 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 (HRMS fragmentation patterns are available in Figures 2 and S1). A chromatogram obtained from the analysis of a pooled urine sample collected 1 h after the 10 mg/kg MXPr administration is presented in Figure 3. Eight relevant chromatographic peaks were recorded, arising from the presence of MXPr plus seven metabolites. Among these, dihydro-MXPr and dihydro-MXPr-Gluc were not detected in the urine samples collected within the initial 3 h from the administration, whereas their presence was observed in the urine samples collected 4–5 h after the administration, suggesting a delayed formation and urinary excretion.

The presence of an intense peak (Figure 3, peak number 2; RT = 1.8 min) allegedly corresponding to desmethylmethoxpropamine-glucuronide (desmethyl-MXPr-Gluc) was observed in both high and low concentration treatments (1 and 10 mg/kg), suggesting that the metabolic pathway that produces it is the preferential one for MXPr excretion. The most abundant

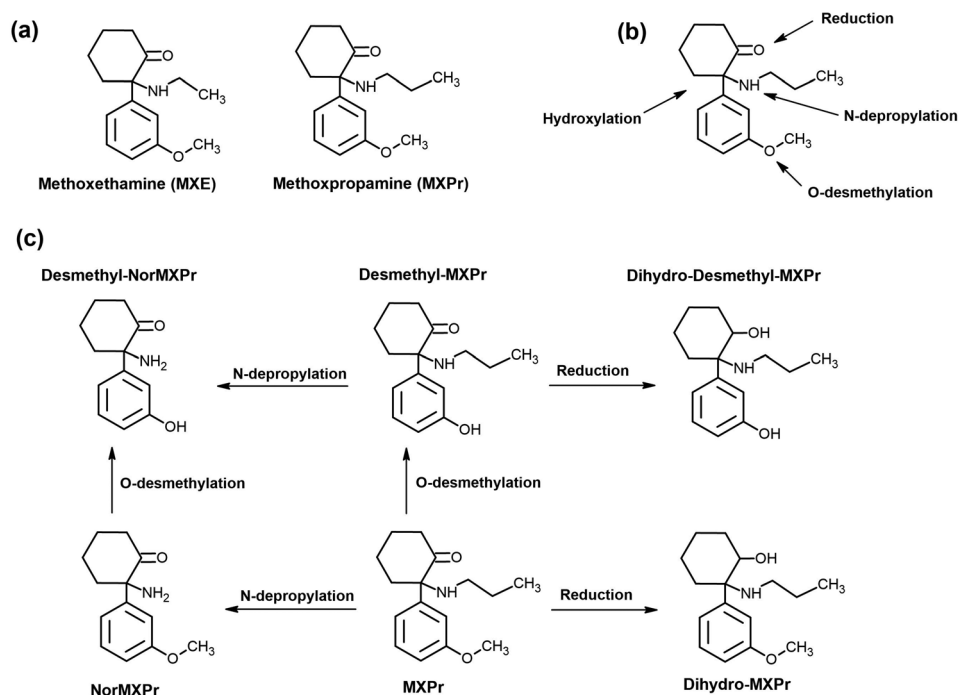
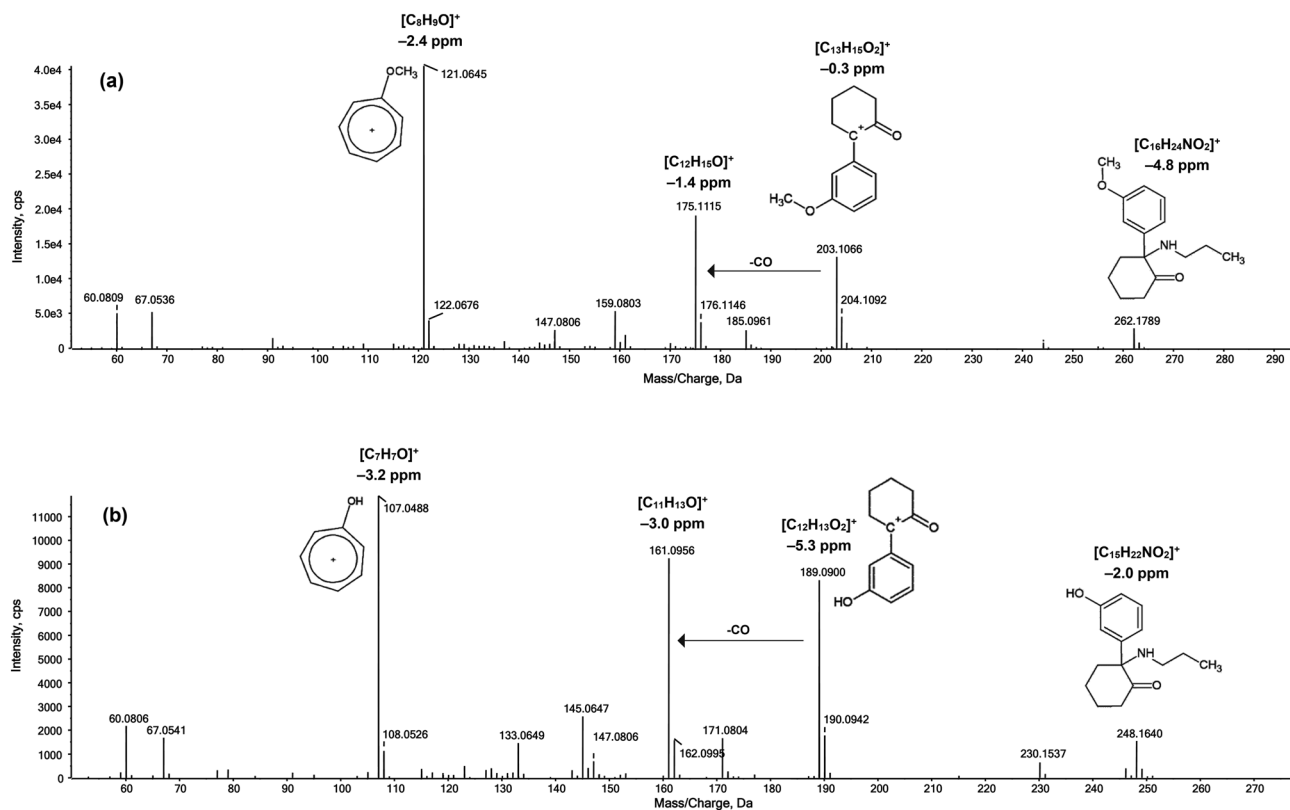


FIGURE 1 (a) Structures of MXE and MXPr, (b) expected metabolic reactions of MXPr, and (c) predicted metabolic pathway of MXPr.

TABLE 1 Name, elemental composition, exact (theoretical) protonated mass, mass error found, and retention time of MXPr and its hypothesized metabolites in UHPLC-QTOF-HRMS.

ID compound	Elemental composition	[M + H] ⁺	Error (ppm)	t _r (min)	Found in mice urine
MXPr	C ₁₆ H ₂₃ NO ₂	262.1802	-2.7	3.05	Yes
Desmethyl-MXPr	C ₁₅ H ₂₁ NO ₂	248.1645	-3.8	2.46	Yes
Dihydro-desmethyl-MXPr	C ₁₅ H ₂₃ NO ₂	250.1802	-0.5	2.64	Yes
Desmethyl-norMXPr	C ₁₂ H ₁₅ NO ₂	206.1176	-0.5	2.00	Yes
NorMXPr	C ₁₃ H ₁₇ NO ₂	220.1332	0.3	2.60	Yes
Dihydro-MXPr	C ₁₆ H ₂₅ NO ₂	264.1958	-2.1	3.25	Yes
Desmethyl-MXPr-Gluc	C ₂₁ H ₂₉ NO ₈	424.1966	-4.3	1.85	Yes
Desmethyl-norMXPr-Gluc	C ₁₈ H ₂₃ NO ₈	382.1496	-0.5	1.42	Yes
Dihydro-MXPr-Gluc	C ₂₂ H ₃₃ NO ₈	440.2279	5.0	3.68	Yes
Dihydro-desmethyl-MXPr-Gluc	C ₂₁ H ₃₁ NO ₈	426.2122	0.7	2.08	Yes
Dihydro-norMXPr	C ₁₃ H ₁₈ NO ₂	221.1410	-	-	No
HydroxyMXPr	C ₁₆ H ₂₃ NO ₃	278.1750	-	-	No
Hydroxy-norMXPr	C ₁₃ H ₁₇ NO ₃	236.1281	-	-	No
Desmethyl-hydroxy-MxPr	C ₁₅ H ₂₁ NO ₃	264.1594	>10	2.00	No
Hydroxy-dihydro-MXPr	C ₁₆ H ₂₅ NO ₃	280.1907	-	-	No
Desmethyl-hydroxy-norMxPr	C ₁₂ H ₁₅ NO ₃	222.1124	>10	1.10	No
Dihydro-hydroxy-norMXPr	C ₁₃ H ₁₈ NO ₂	221.1410	-	-	No
MXPr-Sulf	C ₁₅ H ₂₀ NO ₂ OSO ₃ H	342.1016	-	-	No
Dihydro-MxPr-Sulf	C ₁₅ H ₂₂ NO ₂ OSO ₃ H	344.1173	-	-	No
Desmethyl-MxPr-Sulf	C ₁₄ H ₁₈ NO ₂ OSO ₃ H	328.0860	-	-	No

**FIGURE 2** HRMS fragmentation pattern of MXPr (a) and desmethyl-MXPr (b). The assumed molecular structures of the main fragments, their elemental composition, and the deviation from the exact mass (ppm) are reported.

desmethyl-MXPr-Gluc peak during the excretion profile was observed 1 h after the administration, with an average intensity about 16 times higher than the free phase desmethyl-MXPr, likely representing its phase I precursor.

MXPr was generally excreted as parent drug within the 24 h. Table 2 shows the approximate MXPr concentrations detected in the various urine samples. The identification of the main phase I metabolites is in agreement with the study by Goncalves et al.,²¹ which confirmed the presence of most of the phase II metabolites in urine collected from an MXPr consumer. The MXPr pharmacokinetics profile shows an excretion peak after 1 h, then a progressive decay along 6 to 12 h, even if MXPr is still detectable 24 h after administration of 3 and 10 mg/kg. The same trend is observed for other metabolites, such as norMXPr and desmethyl-MXPr, that exhibit an excretion peak after 1 h followed by a progressive decrease over time, leaving trace concentrations even at 24 h after administration (Figure S2).

Previous studies²¹ identified CYP3A4 as the prevalent enzyme responsible for the N-dealkylation of ketamine, with secondary role played also by CYP2B6 and CYP2C9. In analogy, CYP3A4 is likely to be responsible for the MXPr metabolism to its metabolite norMXPr. Extending the analogy, norketamine is known to be pharmacologically active, making it likely that norMXPr also contributes to the overall MXPr toxicity. It should be noted that the relevant presence of norMXPr in all urine samples makes it the main phase I metabolite, that persists in the urinary excretion even longer than the parent drug MXPr (Figure 4).

Some interesting differences were observed between the metabolites profiles of female and male mice, a phenomenon that could be attributed to sexually dimorphic metabolism,²² involving both phase I and phase II enzymes. Due to the unavailability of female mice urine samples at certain time intervals considered in this study, it is difficult to draw parallel pharmacokinetics curves and observe specific differences in the metabolites profile generated over time. However, it is possible to compare the variation in the levels of MXPr and its main metabolite norMXPr, which definitely exhibit different ratios based on the mouse gender. In particular, the MXPr excretion peak was reached earlier in males than in females, whereas the peak MXPr concentration is significantly higher in female samples for all the administered doses (Figure 5a-c). Accordingly, norMXPr reach higher concentrations in the male urine samples, both evidences suggesting that male mice have a faster metabolism of MXPr than female mice (Figures S4). Nevertheless, the analysis of only one pooled male and one pooled female sample extract is probably not enough to conclude that there are metabolism differences according to gender. Further replicate measurements will be necessary to speculate about possible metabolism differences between males and females.

The analysis of fur and plasma samples did not allow the detection of all target analytes (the metabolites detected in plasma and fur are reported in Table S3). MXPr and only the major phase I metabolites were observed in plasma (average concentration for the parent drug: 1.0 ng/mL) and fur (average concentration for the parent drug: 13 pg/mg). MXPr was eliminated as parent drug in blood before 24 h,

FIGURE 3 Extracted ion chromatogram (XIC) obtained from the urine pool of male mice treated with the highest concentration of MXPr (10 mg/kg) and sampled 1 h after treatment (1. Desmethyl-norMxPr-Gluc, 2. Desmethyl-MxPr-Gluc, 3. Dihydro-desmethyl-MxPr, 4. Desmethyl-norMxPr, 5. Dihydro-Desmethyl-MxPr-Gluc, 6. Desmethyl-MxPr, 7. NorMxPr, 8. MxPr).

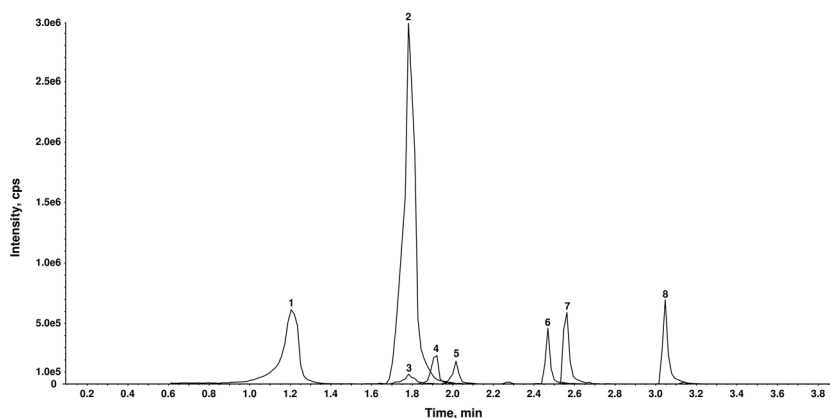


TABLE 2 Urine concentrations of MXPr (expressed in ng/mL) found at the different sampling time after the treatment.

MXPr dosage	Sex	Collection time (h)							
		1	2	3	4	5	6	12	24
1 mg/kg	M	440	141	107	41	18	2	2	n.d.
	F	n/a	572	n/a	210	60	41	10	n.d.
3 mg/kg	M	n/a	629	267	n/a	150	141	58	17
	F	n/a	n/a	n/a	n/a	1050	n/a	339	119
10 mg/kg	M	6730	3070	2870	1820	n/a	588	547	399
	F	n/a	11,300	8580	n/a	n/a	3348	1849	261

Note: n.d.: not detected, n/a: sample not available.

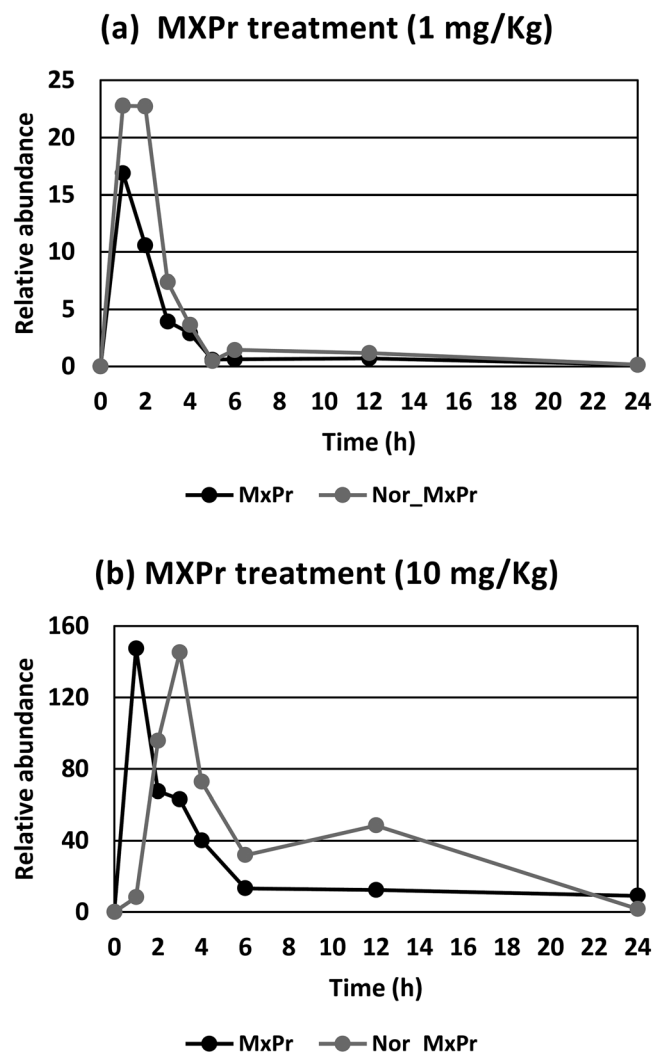


FIGURE 4 MXPr and norMXPr excretion trends at different h for samples: (a) Pooled urine of male mice (1 mg/kg) and (b) pooled urine of male mice (10 mg/kg).

while the presence of the norMXPr and dihydro-MXPr metabolites in the blood sample suggests these metabolites could be long-term drug use indicators. The plasma levels recorded after 10 mg/kg MXPr administration are consistent with those obtained in previous studies involving the administration of 10 mg/kg MXE.²³ Unlike ketamine, subjected to extremely rapid conversion to norketamine, the nor-derivatives of MXE and MXPr were detected in lower concentrations than the parent drugs.²⁴

Currently, very few data are available in the literature concerning the detection of MXPr and metabolites in biological samples. In one study, pooled human liver microsomes (pHLM) assays were performed and analyzed using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF-MS) in order to detect MXPr metabolites. Three metabolites were identified including norMXPr, desmethyl-MXPr, and dihydro-MXPr. Most of the phase II metabolites were confirmed to be present in urine and fur samples collected from mice treated with MXPr.¹⁰

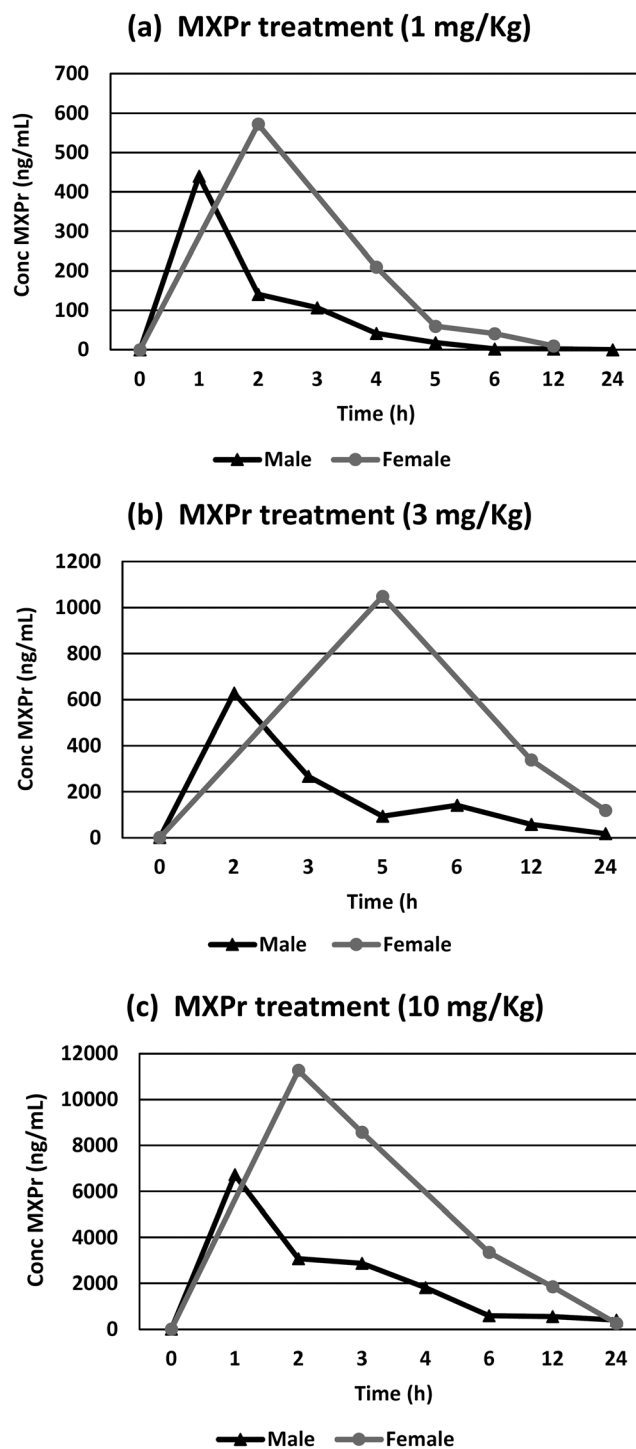


FIGURE 5 Comparison of MXPr concentration in male and female mice urine for the three assays studied, respectively, (a) 1 mg/kg, (b) 3 mg/kg, (c) 10 mg/kg.

A second study refers to the suicide of a drug abuser with past psychiatric disorders; postmortem blood and hair were collected and screened for the presence of xenobiotics by GC-MS and LC-HRMS.¹⁰ The presence of high MXPr concentration was detected in hair (8 ng/mg) and blood (6400 ng/mL) samples, highlighting recent drug consumption before death. The blood sample turned out positive

also for dihydro-MXPr, suggesting the inclusion of this metabolite in the screening procedure devoted to ascertain the detection time of MXPr intake.

4 | CONCLUSIONS

The present study investigates for the first-time the *in vivo* metabolism of MXPr on both male and female mice, in order to identify the main MXPr phase-I and phase-II metabolites and delineate their pharmacokinetic profiles. A significant feature of the resulting data is that a strong sexually dimorphic metabolism of MXPr is observed. In particular, the parent drug undergoes a slower but more extensive metabolism in female mice than in male subjects.

The characterization of the main MXPr metabolites based on LC-HRMS and LC-MS/HRMS allowed the accurate mass determination of their protonated molecular ion and collisionally 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 MXPr and its main metabolites will also allow to develop fit-for-purpose targeted analytical methods useful for the detection of MXPr and its metabolites in biological specimens. In particular, norMXPr, desmethyl-MXPr, and dihydro-MXPr are suggested as target analytes in the toxicological analyses, so as to increase the MXPr detection time after intake and reduce the risk of false-negative results in the forensic cases. This may represent the major strength of this original investigation with particular mention to the translational value of these results. In fact, it is well known that characterizing in different species the metabolic pathways of emerging psychoactive substances provide insight into mechanisms underlying their potential toxicity and data useful for their detection in biological samples. On the other hand, previous data document that some differences due to species-specific influences (i.e., different concentrations of metabolites or enzymatic polymorphism) can be observed relative to metabolism of many drugs.²⁴ Thus, despite the high similarity showed by the CYP3A4 enzyme mainly involved in the metabolism of MxPr and many other psychoactive drugs,²⁴ further studies may be required to speculate on metabolic pathway and related gender-based differences.

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ORCID

Marta Massano  <https://orcid.org/0000-0001-9368-6512>

Matteo Marti  <https://orcid.org/0000-0001-8751-2882>

Christina Ververi  <https://orcid.org/0000-0002-6049-1265>

Marco Vincenti  <https://orcid.org/0000-0002-6275-7194>

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