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Urinary excretion profile of methiopropamine in mice following intraperitoneal administration: a liquid chromatography-tandem mass spectrometry investigation

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Abstract

We have considered the urinary excretion profile of methiopropamine, a thiophene ring-based structural analog of methamphetamine with similar stimulant effects, with the aim of selecting the most appropriate marker(s) of intake that may be useful in forensic analysis. For this purpose, *in vitro* studies were preliminarily performed on human liver microsomes for tracing the phase I metabolic pathways of methiopropamine, pre-selecting the best candidates as potential target analytes and designing the optimal experimental strategy. *In vivo* studies were then conducted on mice, following the intraperitoneal administration of a 10 mg/kg dose. Urine samples were collected every 3 hours in the first 9 hours and, subsequently, from 24 to 36 hours, stored at - 80 °C until analysis. The measurements were carried out by a targeted procedure based on liquid/liquid extraction followed by liquid chromatography-tandem mass spectrometry analysis. Our results show that in the time interval 0–9 hours after administration, methiopropamine is extensively oxidized mainly to nor-methiopropamine, oxo-methiopropamine, and two hydroxylated (i.e., hydroxy-aryl-methiopropamine and hydroxy-alkyl-methiopropamine). All the phase I metabolites underwent phase II metabolism, with the formation of nor-hydroxy-methiopropamine only in phase II, confirmed by the results obtained following enzymatic hydrolysis with β-glucuronidase and arylsulfatase. In the time interval 24–36 hours after administration, unchanged methiopropamine and nor-methiopropamine were only detected, suggesting that these two markers are those endowed with the highest diagnostic value. The method was validated for these two principal markers, proving to be fit for the purpose of anti-doping, toxicological, and forensic analyses.

Running title: *Excretion profile of methiopropamine in mice*

Keywords: *Methiopropamine; Excretion Profile; Forensic Analysis; In Vitro Metabolism; Novel Psychoactive Substances*

Introduction

Psychoactive substances have a long history of use as doping substances in sports. Stimulant substances were the first to be banned, by the International Association of Athletics Federations, already back in 1928 [1]. The continuous growth of the synthesis of new psychoactive substances (NPSs) has drastically changed the drug scene and consumption among drug users[2-3]. NPSs are also known as 'designer drugs' or 'legal highs', and usually illicitly marketed as 'salt baths', or 'research chemicals', labeled 'not for human use'. NPSs are often produced in amateur or clandestine laboratories to mimic the effects of common illicit drugs, with the aim of circumventing, even if in the short term, the current controlled-substance legislation [4–7]. The pronounced increase in the number and amount of versatile NPSs, with mostly unknown toxicological effects, has also prompted governments to extend and adapt the current legislation for curtailing their production, distribution, and use [8-9]. The main chemical classes of NPSs include psychostimulants, narcotics/hypnotics, synthetic cannabinoids, psychedelic compounds, dissociative compounds, and synthetic opioids [10–12]. Among synthetic stimulants, amphetamines and their derivatives are, perhaps, the most abused compounds, primarily for their stimulant and hallucinogenic properties [13], but also owing to the fact that their detection could be problematic in routine drug tests [14-15]. Although stimulant drugs have been traditionally widely abused for doping purposes, the use of synthetic amphetamine derivatives was not very widespread until the first decade of the 2000s [16]. However, the continuous increase in the synthesis of NPS may become a potential issue also for the anti-doping community.

This study is focused on methiopropamine (MPA; IUPAC name: 1-[thiophen-2-yl]-2-methylaminopropane; other synonyms: methedrene; 2-methiopropamine; Syndrax), a synthetic methamphetamine analog in which the benzene ring is bioisosterically replaced by a thiophene ring [17]. Similar to methamphetamine, the presence of the N-methyl group increases MPA's

lipophilicity, thus enhancing its ability to cross the blood-brain barrier and, consequently, its activity and toxicity[18]. Despite being a relatively 'old' compound (the first synthesis of MPA, described by Blicke and Burckhalter, dates back to 1942 [19]), MPA was newly detected as a recreational drug in Finland in January 2011[17,20,21] . Its diffusion has been confirmed by subsequent alerts in the United Kingdom and supported by the analysis of anonymous urine samples collected from street urinals in London [22]. MPA is readily available by itself or mixed with other substances, in products known as 'Slush Eric'[17], 'Blow' [23], and 'Synthacaine' (a mixture of MPA and 2-aminoindane), at a low cost on various websites [24]. At low doses, MPA is a functional stimulant that creates euphoria, stimulation, and alertness similar to methamphetamine [25,26], further confirming its possible use as a recreational stimulant, as well as a substance of abuse in sport doping.

The most common routes of administering MPA are oral, intranasal, and by inhalation (e.g., smoking). Moreover, the intravenous injection of MPA was also reported in Scotland. In this case MPA was abused as a substitute of methylphenidate derivatives and was under Temporary Class Drug Order (TCDO) [27]; the most commonly reported adverse effects are vasoconstriction, insomnia, nausea and vomiting, skin irritation, increased heart rate, increased sweating, dizziness, decreased energy, difficulty in urinating, and chest pain [28,29]. In a recent study, we have investigated the histological changes in CD-1 male mice following the chronic administration of MPA. Notably, we demonstrated that the mice chronically treated with MPA evidenced myocardial damage and gastrointestinal ischemia, with ischemic-necrotic lesions of variable extent [30]. Acute administration demonstrates that MPA acts as a dopaminergic and noradrenergic stimulating drug. MPA stimulates locomotor activity by increasing heart rate, breath rate and blood pressure [31].

Numerous cases of non-fatal intoxication have been reported in the literature [32-33], with some cases, reporting death owing to the intake of MPA, that was indeed detected in 10

deaths in 2013, 27 deaths in 2014, 23 deaths in 2015, and 2 deaths in 2016 [32]. A case of isolated MPA intake has also been reported [17].

Despite its toxicological properties and growing diffusion, only a few countries have enacted legislation covering the abuse of MPA: Florida and Ohio in the United States, Belarus, China, Denmark, Estonia, Germany, Hungary, Portugal, Slovenia, Sweden, Turkey, and Switzerland. MPA is under a temporary drug classification in the United Kingdom [20, 34]. Previous studies have outlined the phase I and phase II metabolism of MPA in both human and animal models, such as rats [20, 35]. Nonetheless, to the best of our knowledge, no information is yet available on the urinary excretion profile of MPA and its metabolites.

The aim of the present study was to select the most appropriate marker(s) of MPA intake, in order to include them in the routine procedures currently adopted by the WADA accredited anti-doping laboratories to perform doping control tests. For the above reasons, the study was carried out analyzing urine samples from a controlled excretion study in mice after administration of a single dose of MPA.

Experimental

Chemicals

MPA and nor-MPA (also known as thiopropamine), were purchased from Chebios (Rome, Italy); methamphetamine (used as internal standard:ISTD) was purchased from Sigma-Aldrich (Milano, Italy). All the reagents (i.e., formic acid, acetic acid, ammonium formate, ammonium acetate, sodium phosphate, sodium chloride, sodium hydrogen phosphate, potassium carbonate, potassium hydrogen carbonate, acetonitrile, absolute ethanol, methanol, dimethylsulfoxide [DMSO] diethyl ether, ethyl acetate, and *tert*-butyl methyl-ether) were of analytical grade and provided by Sigma-Aldrich (Milano, Italy). Milli-Q (Millipore Italia, Vimodrone, Milano, Italy) ultra-purified water was used in the study; the enzyme mixture β-

glucuronidase/arylsulfatase (from *Helix pomatia*), used for the enzymatic hydrolysis of both the glucorono- and sulfo-conjugates, was purchased from Roche (Monza, Italy).

The enzymatic proteins (human liver microsomes (HLM) from 20 Caucasian male and female donors of different ages), and all the reagents used for the *in vitro* metabolism experiments (sodium phosphate buffer and the NADPH regenerating system consisting of magnesium chloride hexahydrate, $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) were purchased from BD Biosciences (Milano, Italy).

Animals and dose selection

Male ICR (CD-1®) mice, weighing 25–30 g, were purchased from Harlan Italy; (S. Pietro al Natisone, Udine, Italy) with a food diet based on Diet 4RF25 GLP purchased from Mucedola (Settimo Milanese, Milan, Italy). For the *in vivo* studies, MPA was initially dissolved in absolute ethanol (final concentration of 2%) and Tween 80 (2%) and brought to its final volume with saline (0.9% NaCl). The solution made with ethanol, Tween 80, and saline was also used as the vehicle. The drug was administered by intraperitoneal injection at a volume of 4 µl/g. A group of five mice was selected, and single dose of 10 mg/kg MPA was administered to each mouse. The dose was selected based on previous studies in mice [30] and on behavioral and neurological effects reported in human subjects [27]. A drug dose of 10 mg/kg in mouse is equivalent at a human dose of ~0.81 mg/kg (~48 mg of MPA, from common to high dose). The mice were single-housed (1 mouse per cage, with a per-animal floor area of 80 cm^2 and a minimum enclosure height of 12 cm) in a colony room under constant temperature (23–24 °C) and humidity (45-55%).The daylight cycle was artificially maintained (dark between 7 p.m.–7 a.m.). A control group of five mice was also selected for urine blank samples.

Experimental protocols performed in the present study were in accordance with the new European Communities Council Directive of September 2010 (2010/63/EU), a revision of Directive 86/609/EEC, and were approved by the Italian Ministry of Health (license n.

335/2016-PR) and Ethics Committee of the University of Ferrara. Moreover, adequate measures were taken to minimize the number of animals used as well as their pain and discomfort.

Protocol for the *in vitro* studies

All incubation conditions for MPA were optimized (proteins and substrate concentrations, buffer and solvent types, and incubation times), starting from the protocols already published and used by our group [36,37]. Different solvents (methanol, DMSO and acetonitrile), pH values (5.0, 7.4, and 9.0), concentrations of MPA and HLM (0.1, 0.2, 0.5 and 1.0 mg/mL), and incubation times (30 min, 1, 2, 4, 8, 12, and 24 hours) were evaluated with phosphate buffer. The final incubation medium also contained 3.3 mM magnesium chloride, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 0.4 U/mL glucose-6-phosphate dehydrogenase in a total volume of 250 μL. Samples were pre-warmed at 37 °C for 5 min, and the phase I reactions were started with the addition of HLM. After incubation at 37 °C, 250 μL of ice-cold acetonitrile was added to stop the phase I reactions.

The samples were then transferred to an ice bath for the further precipitation of the proteins in the assay medium. The precipitate was subsequently separated from the supernatant by centrifugation at 21.000 *g* (15.000 rpm) at room temperature for 10 min. Each set of assays also included a negative control sample that contained all reaction mixture components excluding the enzymatic proteins to monitor the potential non-enzymatic reactions and a negative control sample that contained all the reaction mixture components a part the substrate, to monitor the potential interferences of the enzymatic system**.** Each incubation was processed in triplicate.

Protocol for the *in vivo* studies

- *Sample collection*

Urine samples were collected every 3 hours in the time interval 0-9 hours and 24-36 hours after the injection of MPA, with the aim of defining the best marker(s) of intake across different periods of time after administration. If not immediately processed and assayed, urine samples were stocked at -80 °C until the analysis. Urine blank samples were also collected within the same hour range and at the same time intervals from the mice control group.

- *Sample pre-treatment*

Sample preparation was based on the procedure optimized by *in vitro* incubation studies. A volume of 200 µL of urine was added with 50 µL of the standard solution of the internal standard methamphetamine (final concentration of 250 ng/mL). The sample was then buffered with 100 µL of a 2 M carbonate buffer (pH 9) and added with 2 mL of *tert*-butyl methyl ether. After 20 min of gentle stirring, the sample was centrifugated at 3000 rpm for 2 min and transferred to an ice bath for 5 min. The organic layer was then collected and evaporated until dry under nitrogen flow at room temperature. The final residue was dissolved in 50 µL of the mobile phase and then analyzed via liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The aqueous layer was stored for later use in phase II metabolism studies.

To the aqueous layer 200 µL of the 2-M acetate buffer (pH 5), 50 µL of the standard solution of methamphetamine and 20 µL of β-glucuronidase/arylsulfatase for the hydrolysis of the glucuronic and sulfate conjugates metabolites, were added and the samples were incubated at 55 °C for 2 hours.

After hydrolysis, to the samples, 300 µL of the carbonate buffer 2 M were added following the same procedure described above after buffer addition.

Instrumental conditions

Samples were analyzed using an Agilent 1200 series HPLC instrument equipped with a SUPELCO C18 column (15 cm x 2.1 mm x 5 µm) coupled with an API4000 QqQ mass

spectrometer (AB Sciex, Monza, Italy) with an ESI source operated in positive ionization mode. Analyses were carried out at a constant flow rate of 250 µL/min using as mobile-phase ultra-purified water, 0.1% formic acid (A), and acetonitrile 0.1% formic acid (B). The selected gradient program started at 2% of B and increased to 30% of B in 10 min, after 4 min up to 40% B, after 3 min up to 60% B in 5 min, and then after 4 min to 100% B. The column was flushed for 4 min at 100% B and finally re-equilibrated at 2 % B for 4 min.

Multiple reaction monitoring (MRM) was used as the acquisition mode for detection, and at least two diagnostic transitions were selected (see Table 1 for the complete list of the diagnostic ion transitions). For the MRM collision-induced dissociation (CID), nitrogen was used as the collision gas at 5.8 mPa, obtained from a dedicated Parker-Balston nitrogen generator system (model 75-A74) with 99.5% gas purity (CPS Analitica Milano, Italy). The mass spectrometric parameters (declustering and needle voltages, gas pressure, source temperature, collision cell exit potential, and collision energy) were optimized by infusing the standard solution of MPA at a concentration of 10 µg/mL. All aspects of instrument control, method setup parameters, sample injection, and sequence operation were controlled by the proprietary software (Analyst® version 1.6.1).

Method Validation

The developed method was validated according to ISO 17025 and WADA-guidelines[38–40]. With this aim, the parameters required for the validation of a qualitative screening procedure for a non-threshold substance were evaluated. No quantitative parameters were estimated as MPA as a stimulant drug prohibited in-competition. The method was validated in terms of selectivity, limit of detection (LOD), recovery, carry over, matrix effect and repeatability of relative retention time (RRT), relative abundances of characteristic ion transitions (RA) and relative abundances of areas (RAA) for MPA itself and nor-MPA, being these analytes available as reference materials in our laboratory. Selectivity was assessed by analyzing blank

mouse urine samples, following the same protocol of positive samples, to verify the presence of compounds that may interfere with the characteristic ion transition included into the acquisition method. In view of the potential application of the method also on human urine, selectivity was also evaluated by analyzing the blank human urine samples from twenty volunteers. For the validation of the other parameters blank human urine samples was next employed. For the determination of LOD, blank urine samples were spiked with MPA and nor-MPA, starting at a concentration of 500 ng/mL with progressive dilution. The LOD was defined as the lowest concentration that shows a signal-to-noise ratio (S/N) ≥ 3, thus indicating the signal-to-noise ratio of the least abundant of the diagnostic ions selected to identify the compounds. Carry-over was studied by analyzing negative urine samples after negative urine samples spiked with the compounds of interest at concentration at least 20 times the LOD.

Recovery was evaluated on eight different urine samples spiked with MPA and nor-MPA at a concentration three times the LOD. The effect of the urine matrix on the ion suppression and ion enhancement was assessed by comparison of the abundances of the signals obtained in 20 negative urine samples spiked with the compounds under investigation with those obtained in water samples containing the compounds of interest at the same concentration.

Repeatability was evaluated using blank urine samples, spiked with a concentration three times the LOD for both MPA and nor-MPA for three parameters RRT, RA, and RAA. Ten samples were analyzed on the first day and the other ten after two days to evaluate the repeatability of analysis with the same operator. The repeatability of RRT was expressed as CV% dispersion for each spiked urine samples. The RA was calculated by dividing the ion trace area of the lowest diagnostic ions (*m/z* 156-58 for MPA and *m/*z 142-125 for Nor-MPA) by the area obtained from the ion trace of the most abundant diagnostic ion (*m/z* 156-97 for MPA and *m/*z 142-97 for Nor-MPA), following WADA guidelines criteria [39]. The repeatability of RA was also expressed as CV%.

Furthermore, the repeatability of RAA (calculated as the ratio between area/ISTD) was evaluated through the one-way ANOVA test. The aim was to estimate the statistical influence of method response in different days of analysis with the estimation of the cumulative variance of the method. With this purpose, the statistical parameters were defined following the UNICHIM guidelines [41], in accordance with ISO 17025 criteria for the estimation of repeatability of a method. The normality of the data distributions was evaluated via Shapiro-Wilk tests (formulas [42]) to conduct statistical tests that require a normal distribution, such as the Dixon test for outliers [43] and homogeneity variance test [44]. More specifically, the variance homogeneity of the two data distributions of MPA and nor-MPA for the two days of analysis was evaluated by two different variance homogeneity tests: 'Cochran's C variance' test and the 'minimum variance test'. These two tests verify the homogeneity of the variance based on the maximum (Cochran's C test) and the minimum value of variance. Variance, confidence intervals, and CVs were also calculated. Variance homogeneity allows the application of a one-way ANOVA test for repeatability [45].

Results and discussion

Instrumental conditions

The instrumental parameters were optimized by infusing the analytes available in our laboratory as reference material, dissolved in the mobile phase, at a concentration of 10 µg/mL. The optimal ionization conditions for MPA ($[M+H]^+$ at *m/z* 156) and nor-MPA ($[M+H]^+$ at *m/z* 142) were obtained by using a curtain gas pressure of 25 psi, a source temperature of 500 °C, an ion source gas 1 (auxiliary gas) pressure of 35 psi, an ion source gas 2 (nebulizer gas) pressure of 40 psi, a declustering voltage of 80 V, and a needle voltage of 5500 V. Different collision energies were evaluated to select the characteristic fragmentation pattern of MPA and nor-MPA. The selected specific product ions are the immonium ion at *m/z* 58,

thiophenylmethylium ion at *m/z* 97, and thiophenylpropylium ion at *m/z* 125; the fragmentation spectra and characteristic *m/z* ion transitions for MPA are reported in Figure 1. Based on the characteristic fragmentation pattern of MPA and nor-MPA and the information obtained from the literature [20], we developed the MRM method with specific ion transitions for each hypothesized metabolite of MPA. Table 1 reports the specifically selected precursor and product ions as $[M+H]^+$ with the respective collision energies.

***In vitro* studies and optimization of the sample preparation protocol**

In vitro metabolism studies were conducted using HLM from a pool of 20 *Caucasian* male and female donors of different ages to minimize the effect of intra-individual variation and present the 'average' enzyme activity. The best results were obtained using methanol as a substrate solvent (the total amount of methanol in the final assay was 1%), substrate concentration of 20 µM, protein concentration of 0.5 mg/mL, phosphate buffer of 0.1 M at pH 7.4, and incubation time of 4 hours at 37 °C. Different extraction solvents (*tert*-butyl methyl-ether, diethyl ether, and ethyl acetate) and pH values (7, 9 and 12) were also comparatively evaluated.

Three phase I metabolites of MPA were identified: nor-MPA (M1), nor-hydroxy-MPA (M2), and hydroxy-alkyl-MPA (M3). The best recoveries (higher than 80%) for nor-MPA and MPA were obtained using sample buffered at pH 12 added with *tert*-butyl methyl ether. However, it was not optimal for the hydroxylated metabolites, which were instead extracted with recovery higher than 80% at pH 7. Being M1 and MPA extracted in very low amounts at pH 7. Samples buffered at pH 9 and next extracted with *tert*-butyl-methyl ether show the best extraction results. MPA and its metabolites were extracted with recoveries higher than 70%. A chromatogram of HLM incubation after extraction at pH 9 is reported in Figure 2.

Validation of the analytical procedure

The following validation parameters were estimated as described in the experimental section: selectivity, limit of detection, recovery, carry over, matrix effect, RTT, RA, and RAA.

The results obtained match both ISO17025 and WADA criteria for a qualitative method of non-threshold substances. In detail, for the estimation of selectivity of the method, blank urine samples collected from twenty volunteers and mice blank urine samples were analyzed using the protocol described in the experimental section. No interferences were identified at the retention times of the MPA and its metabolites. LOD was verified for MPA unchanged and M1, which are the two target compounds excreted during the entire time range (see the following section), available in our laboratory as reference materials. They show similar LOD and S/N values. In details, for MPA (selected ion transition *m/z* 156-58) the LOD was 45 ng/mL and for M1 (selected ion transition *m/z* 142-125) was 40 ng/mL. The LOD values are lower than WADA MRPL for stimulants (100 ng/mL) [40]. In addition to this, the described techniques showed no carryover at the concentrations tested with spiked urine samples. The results show no significant matrix effect (lower than 30%) at the retention times of the analytes. MPA and M1 show recovery near 80% at a concentration of three times the LOD for both compounds. The RRT show good repeatability (CV% lower than 0,5%), which reaches the WADA criteria for a non-isotopic internal standard, where the relative retention time shall not differ by more than 1% from that of the same substance in the spiked urine sample [38]. The repeatability of RA shows CV% lower than 15%. The results of LOD, matrix effect recovery, RRT, and RA with their CV% are reported in Table 2. Data obtained from RAA were than analyzed for the one-way ANOVA test. Data were normally distributed as determined by a Shapiro-Wilk test, and no outliers were found by the Dixon test (data not reported). Based on tabulated value for Cochran's test ($C_{max,a,K,v}$) and calculate C_{max} (ratio between maximum variance and sum of each variance) the condition $C_{max} \leq C_{max,a,K,v}$ was reached. For minimum variance test tabulated value $C_{max,a,K,v}$ and calculated C_{min} (ratio between minimum variance and sum of each variance) reach the criteria $C_{min} \geq C_{min,a,K,v}$.

These two criteria verify the homogeneity of the variances. Results are reported in Table 3. The ANOVA test shows no significant difference in variance over two days analysis of repeatability (F < Ftab) for both substances, the cumulative average variances were calculated. Data obtained are reported in Table 4.

***In vivo* studies after acute administration**

Urine samples collected from male mice in the time interval 0-9 hours and 24-36 hours after administering 10 mg/kg of MPA were analyzed using the analytical protocol set up and optimized for the *in vitro* studies. Blank urine samples were also analyzed to verify the possible presence of matrix interferents. MPA, M1, M3, oxo-MPA (M4), and hydroxy-aryl-MPA (M5) were identified as phase I *in vivo* metabolites, while M2 was identified as a phase II metabolite. All metabolite structures identified *in vitro* and *in vivo* are reported in Figure 3.

The excretion profile of MPA shows a metabolic pathway similar to amphetamine-like drugs, with *N*-demethylated, hydroxylated, and hydroxyl-oxidized metabolites [46]. These data are supported by previous studies on MPA metabolism. In detail, the *N*-demethylated metabolite was confirmed in positive human urine samples by Welter et. al. [20] and by Tyrkkö et.al. [35], while the hydroxy-aryl-MPA was confirmed only in rat urine sample [20] and non-specified hydroxy metabolites were supposed in trace after incubation of MPA with HLM [35]. Furthermore, our result show that the metabolite M4 was found only in mice urine samples in both phase I and phase II. The M4-related results suggests that MPA shows only conversion to oxidized metabolite in mice, but not in humans. This hypothesis is supported by the result reported by Welter et al.[20].

-Phase I and phase II excretion profiles

In the time interval of 0–9 hours after MPA administration, MPA itself and four principal phase I metabolites (M1, M3, M4, M5) were excreted and detectable throughout the entire time period and appeared as soon as 3 hours after MPA administration. The extracted chromatograms of a

representative sample for this time interval are reported in Figures 4 A-B, respectively, for phase I and phase II metabolism. MPA and its phase I metabolites were also excreted in phase II metabolism as glucurono- and sulfo-conjugates. Furthermore, M2 was only excreted in the conjugated form (Figure 4 B). In the time interval of 24–36 hours after the administration, only MPA and M1 were detected. The extracted chromatograms of a representative sample for this range are reported in Figures 5 A-B, respectively, for phase I and phase II metabolism.

To evaluate the excretion profiles of MPA and its metabolites in both phase I and phase II metabolism, the area ratio between the compound and the internal standard were reported *versus* the collection time. Figures 6 A-B report the excretion profile of MPA and its metabolites in the time interval of 0-9 hours and 24-36 hours. The maximum excretion for MPA and its metabolites was in the time interval 3–6 hours after the administration of a single dose of 10-mg/kg MPA, except for M1 (see Figure 6A). M1 shows a maximum excretion value in the first 3 hours from drug administration, whereas hydroxy-alkyl, hydroxy-aryl, and oxidized metabolites show their maximum excretion value in the interval 3–6 hours. The difference between the excretion profiles of M1 and the other MPA metabolites is probably due to the rapid conversion of MPA into M1, followed by the hydroxylation of the structure forming the hydroxy and oxo metabolites.

In the time interval of 24–36 hours after MPA administration, only MPA itself and M1 are still detectable (see again Figure 6 A). The undetectability of the other MPA metabolites is probably due to a low excretion of secondary metabolites after the first hours, or a total excretion in the first 24 hours, owing to their high hydrophilicity, that could have led to their total excretion in the first hours after the intake. It may be worthwhile to highlight that the maximum excretion value of phase I metabolites in the time interval 3–6 hours corresponds to the minimum excretion value of phase II MPA and its metabolites.

The *in vivo* phase II metabolites (Figure 6 B) show maximum excretion values in the first 3 hours for MPA, and all metabolites due to the rapid conversion of phase I metabolites into their conjugate forms with a progressive decrease over the range. All the compounds identified were detected in the time interval 0-9 hours except for M5 that was visible as conjugated metabolite only in the first 3 hours from drug administration. In the time interval of 24–36 hours after the administration of MPA, only MPA and M1 were detectable, confirming the results obtained for phase I metabolites. These observations underline that both MPA and M1 can be selected as the most appropriate markers of MPA intake with high diagnostic value.

With the aim to better understanding the *in vivo* excretion profile, the total excretion profile, as the sum of phase I and phase II, was reported in Figure 7. It is remarkable that MPA and its metabolites show their maximum total excretion after 3 hours with a progressive decrease. The excretion percentages reported in Table 5 were calculated based on the total amount of each metabolite excretion as the sum of their phase I and phase II profile through the entire range of hours of excretion study. In detail, the amount of excretion was estimated by the ratio between the peak areas of each metabolite and the internal standard throughout the hour interval. Subsequently, the sum of the excretion in the two phases (i.e. I and II) over the entire interval of hours was considered as 100% of the excretion of the specific metabolite. The percentage of excretion for each metabolite was calculated from these data for each time interval and reported for phase I excretion.

The results show that MPA, M1, and M4 are excreted mainly in phase I, with percentages ranging from 30 to 100, where 100 represents a total excretion as phase I metabolite. After 24 hours from the intake MPA shows only a small amounts of phase II conjugation while M1 is mainly excreted as phase II.

Conclusion

To the best of our knowledge, this is the first study of MPA urine excretion profiles that defines the markers of intake in a time interval of 0–9 and 24-36 hours after the administration in mice. MPA is extensively metabolized into demethylated, hydroxylated, and oxidized metabolites, with conversion into conjugated phase II metabolites. The maximum excretion levels, calculated as the sum of phase I and phase II excretion profiles, were in the first 3 hours after MPA intake. Despite these results, only MPA and nor-MPA resulted in being detectable over the entire time interval, qualifying as the most appropriate markers of intake, endowed with the highest diagnostic value in a toxicological analysis.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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Table 1. Precursor ions, product ions and collision energies of MPA and its metabolites. The metabolites identified after metabolism studies were reported with their assigned number.

Compound (Metabolite number)	Precursor ion (*m/z*)	Product ions (*m/z*)	Collision energy (eV)
MPA	156	58; 97; 125	35; 30; 25
Nor-MPA (M1)	142	97; 125	30; 25
Nor-hydroxy-MPA (M2)	158	113; 141	30; 25
Hydroxy-Alkyl-MPA (M3)	172	58; 97; 113; 125; 141	35; 35; 30; 30; 25; 25
Oxo-MPA (M4)	170	111; 139	30; 25
Hydroxy-Aryl-MPA (M5)	172	58; 113; 141	35; 30; 25

Table 2. Recovery, LOD, matrix effect, RRT and RA with calculated CV%

Substance	Rec. %	CV %	LOD (ng/mL)	S/N	CV %	Matrix Effect %	CV %	RRT	CV %	RA	CV %
MPA	**84**	7,5	45	3,5	8,2	26	7,3	1,15	0,39	0,24	9,8
Nor-MPA	**80**	7,6	40	3,2	6,7	28	8,9	1,23	0.45	0,19	10,2

Table 3. Statistic value for Homogeneity Variance test where: s^2_{max} and s^2_{max} are respectively maximum and minimum variance of data set, s^2_{tot} = total variance in two days, $C_{max,\alpha,K,v=n-1}$ and $C_{min,\alpha,K,v=n-1}$ are respectively the critical max and min values of *C* critical value at: α= probability, k= number of group, ν= freedom degree.

Variance Homogeneity		**MPA**	**M1**
s^2_{tot}		$1{,}78 \cdot 10^{-4}$	$6{,}29 \cdot 10^{-6}$
s^2_{max}		$1{,}01 \cdot 10^{-4}$	$4{,}09 \cdot 10^{-6}$
s^2_{min}		$7{,}65 \cdot 10^{-5}$	$2{,}20 \cdot 10^{-6}$
C_{max}		0,57	0,65
C_{min}		0,43	0,35
$C_{max,\alpha=0,05,K=2,v=9}$	0,80	$C_{min,\alpha=0,05,K=2,v=9}$	0,20

Table 4. Statistic value for ANOVA test where: SS_E is error Sum of Squares; SS_r is model Sum of Squares; SS_{tot} is Total Sum of Squares;MS_r is model mean squares, MS_E is model error mean squares, F_{exp} is calculated F, $F_{crit,\alpha=0,05,N-K=18,K-1=2}$ is the critical values at: α= probability, k= number of group, N= total freedom degree; S_r^2 is the cumulative average variances.

ANOVA test	MPA	M1
SS_E	$1{,}73 \cdot 10^{-3}$	$5{,}47 \cdot 10^{-5}$
SS_r	$4{,}85 \cdot 10^{-4}$	$9{,}07 \cdot 10^{-6}$
SS_{tot}	$2{,}22 \cdot 10^{-3}$	$6{,}37 \cdot 10^{-5}$
MS_r	$2{,}43 \cdot 10^{-4}$	$9{,}07 \cdot 10^{-6}$
MS_E	$9{,}63 \cdot 10^{-5}$	$3{,}04 \cdot 10^{-6}$
F_{exp}	**2,52**	**2,98**
$F_{crit,\alpha=0,05,N-k=18,k-1=2}$	**4,41**	
S_r^2	$\mathbf{1{,}17 \cdot 10^{-4}}$	$\mathbf{3{,}35 \cdot 10^{-6}}$

Table 5. Percentage excretion of phase I metabolites as calculated from the total amount of excretion per hour for each metabolite.

	Phase I % excretion				
Hours	MPA	M1	M3	M4	M5
0-3	69,67	62,65	1,80	30,97	46,85
3-6	87,08	81,13	5,02	76,51	100,00
6-9	83,27	72,20	3,07	81,49	100,00
24-27	89,71	2,69			
27-30	90,91	1,77			
30-33	93,04	2,46			
33-36	92,02	1,86			

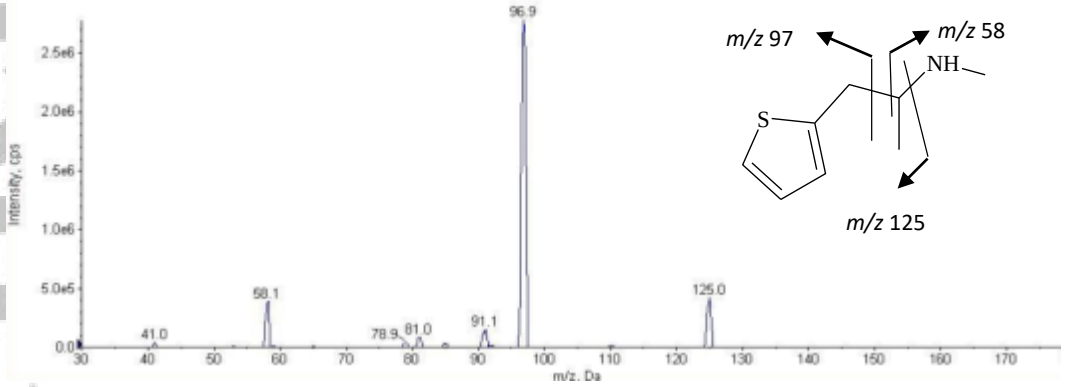

Figure 1. Product ion spectrum of MPA, at a collision energy of 25 eV.

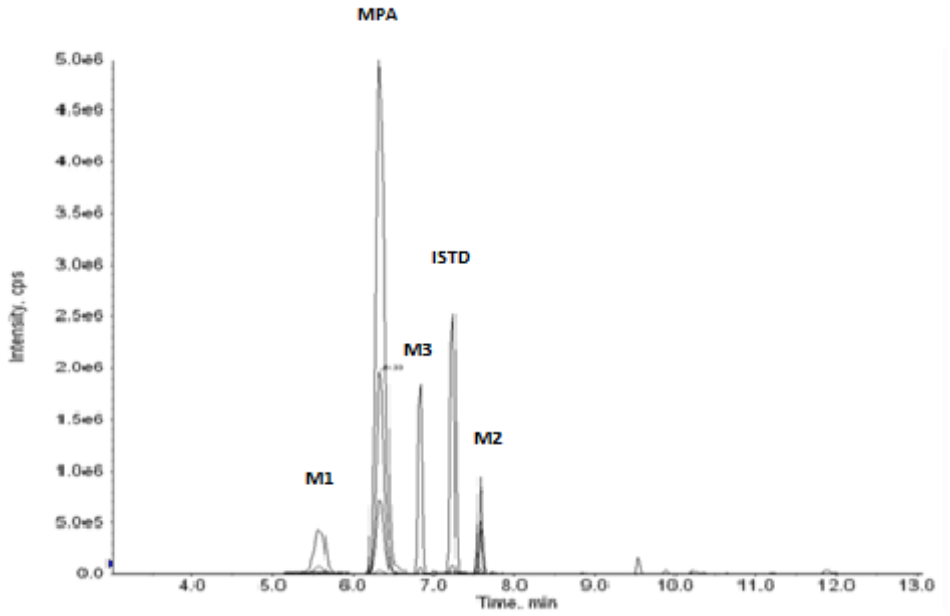

Figure 2. Extracted ion chromatogram of the sample obtained after incubation of MPA with HLM for 4 h at 37 °C.

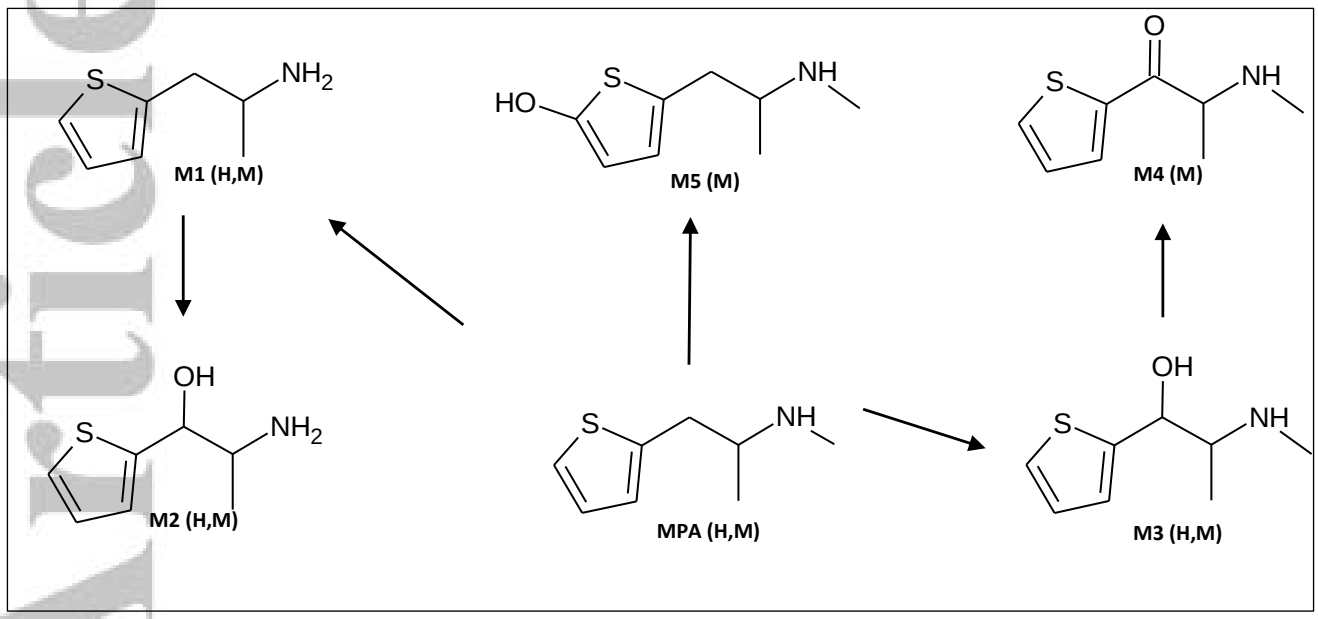

Figure 3. Metabolic profile of MPA in human liver microsomes (H) and in mouse (M). M1. Nor-MPA; M2. Nor-Hydroxy-MPA; M3. Hydroxy-Alkyl-MPA; M4. Oxo-MPA; M5. Hydroxy-Aryl-MPA.

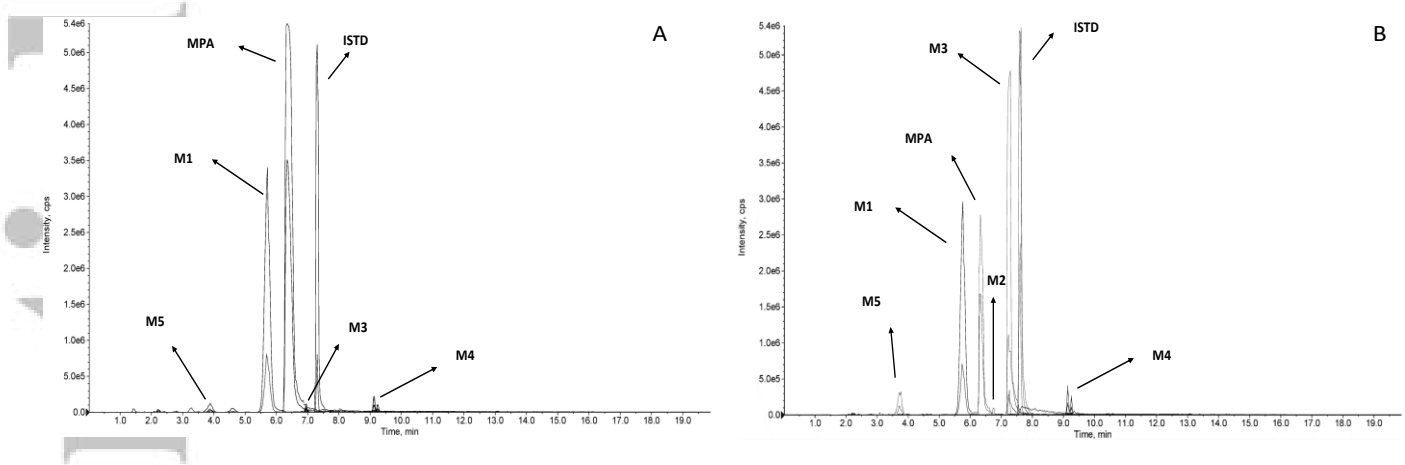

Figure 4. Extracted ion chromatograms of a representative urine sample of *in vivo* studies collected 3 hours after the administration of MPA, respectively of phase I (A) and phase II (B).

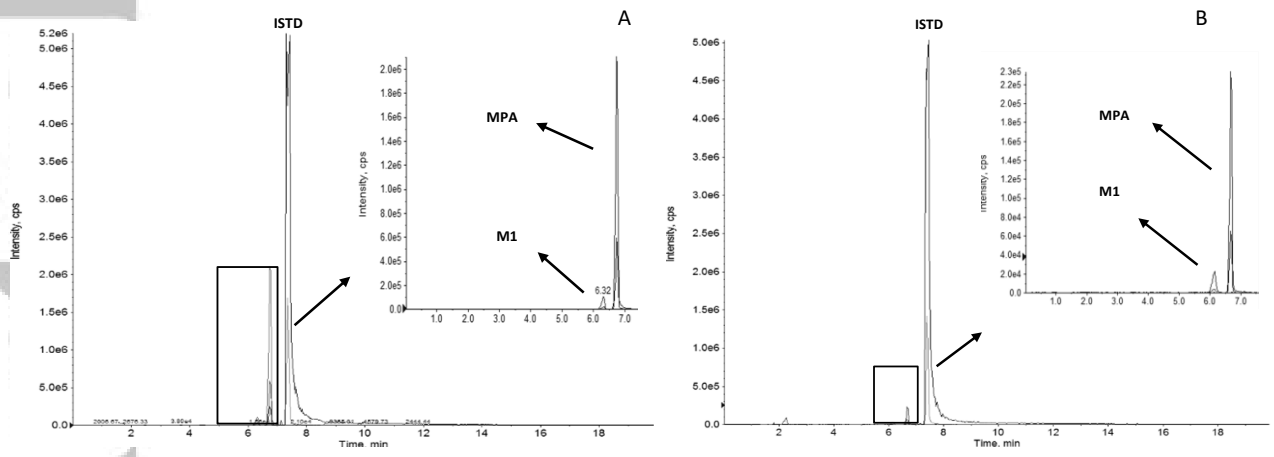

Figure 5. Extracted ion chromatograms of a representative urine sample of *in vivo* studies collected 24 hours after the administration of MPA, respectively of phase I (A) and phase II (B).

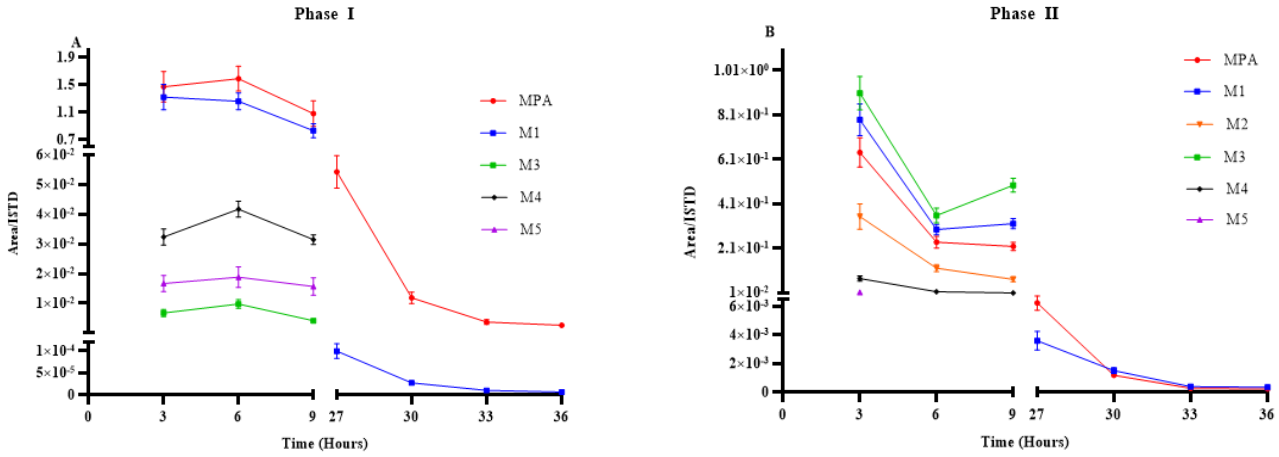

Figure 6. Urine excretion profile, of phase I (A) and phase II (B) metabolites in the time interval 0-9, 24-36 hours after the administration of MPA in mice, shows with average value and SD for each point.

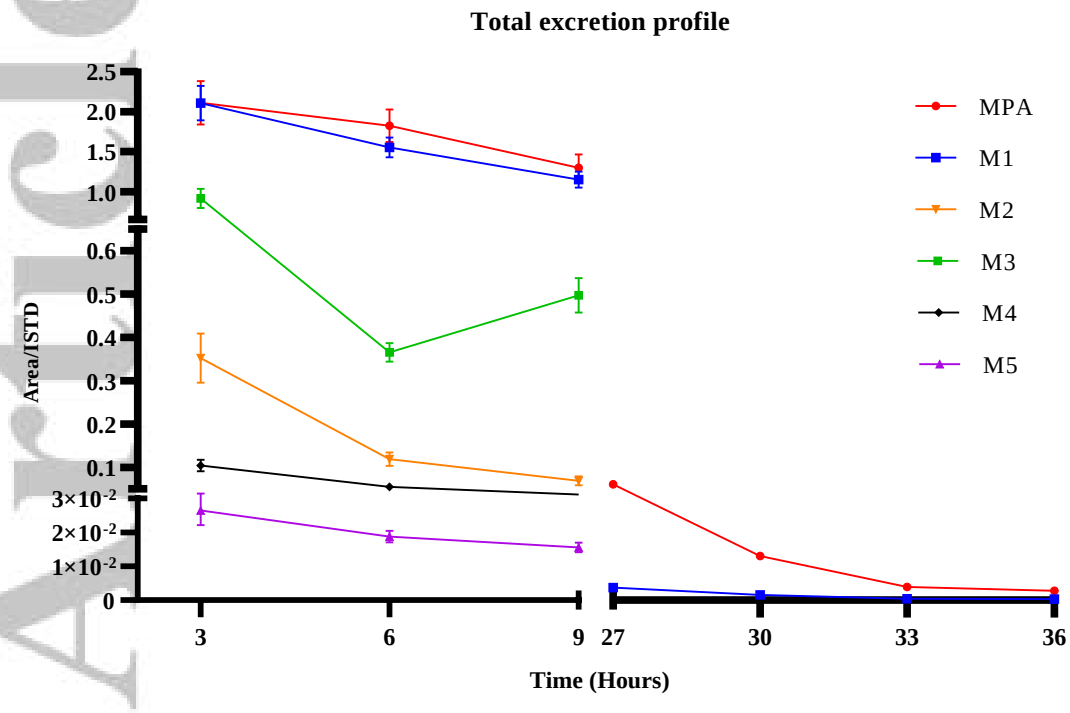

Figure 7. Total urine excretion profile reported as the sum of phase I and phase II excretion in the time interval 0-9, 24-36 h after the administration of MPA in mice, shows with average value and SD for each point.