

Identification of in vitro and in vivo human metabolites of the new psychoactive substance nitracaine by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

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Abstract The purpose of this work was to investigate the in vitro metabolism of nitracaine, a new psychoactive substance, using human liver microsome incubations, to evaluate the cytochrome P450 (CYP) enzyme isoforms responsible for the phase-I metabolism and to compare the information from the in vitro experiments with data resulting from an authentic user's urine sample. Accurate mass spectra of metabolites were obtained using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) and were used in the structural identification of metabolites. Two major and three minor phase-I metabolites were identified from the in vitro experiments. The observed phase-I metabolites were formed through *N*-deethylation, *N,N*-deethylation, *N*-hydroxylation, and de-esterification, with CYP2B6 and CYP2C19 being the main enzymes catalyzing their formation. One glucuronidated product was identified in the phase-II metabolism experiments. All of these metabolites are reported for the first time in this study except the *N*-deethylation product. All the in vitro metabolites except the minor *N,N*-

deethylation product were also present in the human urine sample, thus demonstrating the reliability of the in vitro experiments in the prediction of the in vivo metabolism of nitracaine. In addition to the metabolites, three transformation products (*p*-nitrobenzoic acid, *p*-aminobenzoic acid, and 3-(diethylamino)-2,2-dimethylpropan-1-ol) were identified, as well as several glucuronides and glutamine derived of them.

Keywords Nitracaine · High-resolution mass spectrometry · In vitro metabolism · Human liver microsomes · New psychoactive substance

Introduction

Nitracaine is a structural analogue of dimethocaine with a *p*-nitro group instead of an amino group (see Electronic Supplementary Material (ESM) Fig. S1). Both substances are local anesthetics with stimulant properties through their dopamine reuptake inhibition and have been shown to display cocaine-like effects [1]. Nitracaine is reported to have emerged as a new psychoactive substance (NPS) in December 2013 [2] and has been suggested in user forums to possess stimulant activity [2, 3]. There is only scarce scientifically reliable information on nitracaine in the literature [4–7], especially on its metabolic fate [2]. This is in contrast to what is known about its analogue dimethocaine [4, 6, 8] which has been extensively studied both in vitro and in vivo, with several metabolites identified in mice urine [8].

In a study conducted by Power et al. (2014), nitracaine was found to generate a desethyl metabolite after in vitro incubations using human liver microsomes [2]. The main and only desethyl metabolite observed in their experiments was characterized using liquid chromatography high-resolution mass

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spectrometry (LC-HRMS). However, in a different *in vitro* study involving dimethocaine, several phase-I and phase-II metabolites were identified [8]. The two studies observed a similar phase-I reaction—deethylation—yet, additional phase-I and phase-II products were identified for dimethocaine. Nevertheless, in both studies, no comparison of the *in vitro*-generated metabolite(s) with the metabolites formed *in vivo* by humans has been pursued.

The NPS drug scene is very dynamic with new compounds substituting controlled drugs. Developing methods for the analysis of these new compounds is a costly challenge due to their rapid transience on the drug scene. However, with the advancements in analytical procedures using HRMS techniques in combination with sophisticated data-processing workflows [9], the detection of NPS biomarkers has improved [9–11]. Reporting of identified metabolites, their accurate mass and product ion profiles, as has been done in several studies [12, 13], can be of relevance in forensic in clinical environments particularly when HRMS is applied to monitor NPS use.

Up to date, no results have been published on the metabolites detected in biofluids (urine or blood) from a nitracaine user. However, this can be of great relevance since the selection of the most suitable biomarker, which can be a metabolite(s) or the parent compound, to track the use of nitracaine is vital in forensic and clinical toxicology. Furthermore, the previous *in vitro* study for nitracaine yielded less results compared to that of dimethocaine [8], indicating the need for further experiments.

The aims of this work were firstly to conduct *in vitro* metabolism experiments using human liver microsomes to generate and identify phase-I and phase-II metabolites of nitracaine. Elucidation of the metabolites was performed using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). Secondly, the main human cytochrome P450 (CYP450) enzyme isoforms responsible for the formation of the metabolites were determined. Thirdly, the *in vitro*-generated metabolic profile of nitracaine was compared to the *in vivo* profile found in an authentic urine sample of a user. Lastly, a metabolic pathway for nitracaine was proposed together with recommendations on which biomarker to target in forensic and clinical situations to confirm the use of nitracaine.

Material and methods

Chemicals and reagents

Nitracaine HCl was obtained from LGC standards (Teddington, Middlesex, UK) at a concentration of 1 mg/mL (as free base) in acetonitrile. The internal standard, theophylline, was obtained as powder (anhydrous, purity > 99 %) from

Sigma-Aldrich (Diegem, Belgium). Pooled human liver microsomes (HLMs, mix gender, $n=200$) were purchased from Tebu-Bio (Boechout, Belgium). Pooled human liver cytosol (HLCYT, mix gender, $n=50$), chemical standards for 2,6-uridinediphosphate glucuronic acid (UDPGA), alamethicin (neat, purity > 99 %), adenosine 3'-phosphate 5'-phosphosulfate (PAPS; neat, purity > 60 %) lithium salt hydrate, 4-nitrophenol (4-NP), 4-nitrophenolglucuronide (4-NP-Gluc; neat, purity > 99 %), 4-nitrophenolsulfate (4-NP-Sulf; neat, purity > 99 %), and NADPH (neat, purity > 99 %) were purchased from Sigma-Aldrich. Baculovirus-insect cell microsomes containing expressed human recombinant CYP enzyme (rCYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1, or 3A4) co-expressed with human CYP oxidoreductase and human cytochrome b5 were purchased from BD Biosciences (Erembodegem, Belgium) and Tebu-Bio. Ultrapure water was prepared using a Purelab flex water system by Elga (Tienen, Belgium). Methanol and formic acid were purchased from Merck. All organic solvents were HPLC grade.

In vitro metabolism assays

Metabolism experiments were carried out following previously reported conditions for other NPS [11, 14]. Phase-I metabolites were generated using a reaction mixture (1 mL, final volume) consisting of 100-mM TRIS buffer (pH adjusted to 7.4 at 37 °C), HLM (0.5 mg/mL, final concentration), and nitracaine (10 μ M, final concentration). The percentage of organic solvent was kept < 1 % to minimize its inhibitory effect towards CYP catalytic activity. This mixture was pre-incubated for 5 min in a shaking water bath at 37 °C, and the reaction was initiated by the addition of 10 μ L of NADPH solution (1 mM, final concentration). After reaction times of 1 and 3 h, the reaction was stopped with 250 μ L of ice-cold acetonitrile containing 1 % formic acid and 5.0 μ g/mL of theophylline (used as internal standard) was added to each sample, which was then vortex-mixed for 30 s and centrifuged at 8000 rpm for 5 min. The supernatant was concentrated to near dryness under nitrogen at 60 °C and reconstituted with 200 μ L of 10 % acetonitrile in ultrapure water.

Generation of phase-II metabolites was investigated in two steps. First, phase-I metabolites of nitracaine were produced by incubating nitracaine with HLMs and NADPH as described above. The reaction was quenched by putting the samples on ice for 5 min and centrifuged at 8000 rpm for 5 min. Then, 940 μ L of the supernatant were transferred to a new tube which contained a fresh aliquot of pooled HLMs or pooled HLCYT (0.5 mg/mL, final concentration) for the samples investigating uridine diphosphate glucuronosyltransferase (UGT) or sulfotransferase (SULT) enzyme-mediated metabolism, respectively. For incubations with UGT enzymes, 10 μ L of alamethicin dissolved in dimethyl sulfoxide (10 μ g/mL, final concentration) was added to the reaction

mixture to increase membrane porosity and therefore facilitate the diffusion of the substrate to the membrane bound UGTs. The reaction was initiated by addition of UDPGA or PAPS cofactors (1 mM, final concentration) to activate UGTs and SULTs, respectively. The samples were incubated for 3 h and prepared as described above.

Positive and negative control samples for each family of enzymes were incubated in parallel under the same conditions described above. In the positive control samples for UGT and SULT activity, 4-nitrophenol (10 μ M, final concentration) was selected as the substrate and the formation of 4-nitrophenolglucuronide and 4-nitrophenolsulfate, respectively, was monitored [15, 16]. For each family of enzymes, negative control samples were prepared as described above but omitting the enzymes, substrate, or the cofactor in the reaction mixture in order to ensure that no false-positive metabolite formation occurred.

The role of individual human CYP isoenzymes in the formation of the metabolites detected incubating nitracaine with HLMS was investigated using a panel of human recombinant CYPs (rCYPs), including human rCYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4. Reaction mixtures were prepared as described above for the phase-I experiments, but using one human rCYP (20 pmol/mL, final concentration) per sample instead of HLMS. The reaction time was 1 h. Enzyme negative control samples were prepared omitting the human rCYP.

LC-QTOF-MS analytical method

Metabolite identification was performed based on liquid chromatography coupled to mass spectrometry. The apparatus consisted of a 1290 Infinity LC system (Agilent Technologies, Wilmington, DE, USA) connected to a 6530 Accurate-Mass QTOF-MS (Agilent Technologies, Wilmington, DE, USA) with a heated electrospray ionization source (JetStream ESI).

Chromatographic separation as previously applied by Kinyua et al. [9] was performed on a Phenomenex Biphenyl column (100 mm \times 2.1 mm, 2.6 μ m) fitted to a SecurityGuard ULTRA Holder for UHPLC columns (2.1–4.6 mm) and maintained at 32 °C. The mobile phase consisted of ultrapure water (A) and of acetonitrile/ultrapure water (80/20, v/v) (B) both with 0.04 % of formic acid, with the following gradient: 0–2 min, 2 % B; 18 min, 40 % B; 25–29 min, 90 % B; and 29.5–33 min, 2 % B. The flow rate and the injection volume were set at 0.4 mL/min and 5 μ L, respectively.

The QTOF-MS instrument was operated in the 2 GHz (extended dynamic range) mode, which provides a Full Width at Half Maximum (FWHM) resolution of approximately 4700 at m/z 118.0862 and 10,000 at m/z 922.0098. Both polarity ESI modes were used under the following specific conditions: gas temperature, 325 °C; gas flow, 8 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 325 °C; and sheath gas flow,

8 L/min. Capillary and fragmentor voltages were set to 3500 and 100 V, respectively. A calibration solution (Agilent Technologies) was continuously sprayed in the source of the QTOF-MS system during sample analysis. The ions selected for (re)calibrating the mass axis, ensuring the accuracy of mass assignments throughout the chromatographic run, were m/z 121.0508 and 922.0097 for positive mode and m/z 112.9856 and 966.0007 for negative mode. The QTOF-MS device was acquiring from m/z 50 to 1000 in MS mode and from m/z 40 to 500 in data-dependent acquisition mode (auto-MS/MS) using three different collision energy values (15, 35, and 40 eV) for the fragmentation of the parent ions. The maximum number of precursor ions per MS cycle was set to three with minimal abundance of 1000 counts. In addition, precursor ions were excluded after every three spectra and released after 0.6 min. For some metabolites, additional injections in targeted MS/MS were necessary in order to obtain proper MS/MS fragmentation data.

Data processing and analysis

Metabolite identification was based on its accurate masses and isotopic abundances obtained in the MS mode, as well as on the MS/MS fragmentation pattern and the accurate masses of the resulting products ions. The open-source software MZmine 2.12 (<http://mzmine.github.io/>) [17] was used to assist in an automated detection of metabolites. This software compares several sets of samples (experiments and controls) by peak picking/deconvolution/alignment algorithms, comparing simultaneously thousands of MS spectra to identify differentially expressed features. In this way, those peaks that did not present a significant increase in abundance after incubations were discarded. After that, the relevant m/z values detected by this software were extracted using MassHunter Workstation software (Agilent Technologies) with a mass window of 10 ppm around the ionized precursor ion to confirm or discard their identity. Structures of the proposed metabolites were drawn using the software ChemBioDraw Ultra 14.0 (PerkinElmer Inc.). LogP were calculated with the software ChemBio3D Ultra 14.0 (PerkinElmer Inc.).

In silico predictions were also obtained using a specific program Meteor Nexus (v1.5, Lhasa Limited, Leeds, UK). This software produces a list of potential metabolites and their structures after selecting the substrate, the families of enzymes, and the species of interest. The likelihood of metabolite formation is also indicated as probable, plausible, or equivocal.

Investigation of nitracaine metabolites in a human urine sample

A urine sample from a 30-year old female nitracaine user was collected by the medical staff at hospital admission due to adverse effects after using nitracaine for two consecutive days.

A 50- μ L aliquot of urine was diluted with 150 μ L of acetonitrile and vortexed for 30 s. The sample was then centrifuged at 10,000 rpm for 2 min, and the supernatant was then transferred into an HPLC vial for analysis.

Results and discussion

Identification of in vitro phase-I metabolites of nitracaine

In order to identify all potential phase-I metabolites generated under our experimental conditions, the Total Ion Current (TIC) chromatograms acquired at two different reaction times (1 and 3 h) were compared with those obtained for the three different negative controls (without substrate, without enzymes, or without co-factor) using the MZmine software. Figure 1 shows the five phase-I metabolites detected in positive mode. The response of each metabolite is expressed as the ratio between the peak area of the metabolite to the peak area of the internal standard. Two major metabolites (M1 and M4) were observed corresponding to the ions at m/z 132.1386 and 281.1503, while the other three metabolites (M3 with m/z 253.1190 and the isomers M2a and M2b at m/z 176.1642 and 176.1645, respectively) were only minor. However, it should be remarked that, although useful for discussion purposes, a higher abundance does not always guarantee a higher concentration of the metabolites as different ESI ionization efficiencies may be expected for different chemical structures. This implies that comparison of abundances can lead to false interpretations. None of these metabolites were found in the negative controls suggesting that they were formed through the involvement of CYP enzymes. Extracted Ion Current (EIC) chromatograms of the identified phase-I metabolites are shown in Fig. 2.

The metabolites could be detected in positive ionization mode due to the presence of an amino group in their structures (Table 1). Their tentative structures were postulated by

interpretation of the product ions generated from auto-MS/MS experiments (see ESM Fig. S2).

The interpretation of the fragmentation pattern of the parent compound usually provides a basis for the structural elucidation of its metabolites because of common fragments. Nitracaine was eluted at 14.06 min and showed a $[M+H]^+$ ion at m/z 309.1806, which fragmented yielding fragment ions at m/z 263.1922, m/z 236.0959, m/z 168.0312, m/z 150.0184, m/z 142.1617, m/z 120.0203, m/z 104.0259, m/z 92.0258, m/z 86.0966, m/z 69.0701, and m/z 58.0670 (Table 1). The ion at m/z 263.1922 corresponded to the loss of the nitro group, whereas the ion at m/z 236.0959 resulted from the loss of the *N,N*-diethylamine moiety. Loss of the ester group from the ion at m/z 236.0959 and the additional loss of a molecule of water led to the ions at m/z 168.0295 and 150.0184, respectively. The product ion at m/z 142.1617 corresponded to the ester moiety of the parent compound, whereas the ion at m/z 120.0203 matched with the radical of the benzoic acid and an additional loss of a hydroxyl group resulted in m/z 104.0259. The product ions at m/z 86.0966 and 58.0670 corresponded to alkyl amine moieties with the molecular formula $[C_5H_{12}N]^+$ and $[C_3H_8N]^+$, respectively, whereas the ion at m/z 69.0701 could be linked to $[C_5H_9]^+$ (ESM Fig. S2). The product ions at m/z 236, 150, 142, and 86 were previously described by Power et al. [2].

M1 (m/z 132.1386), eluting at 1.26 min, presented the same product ions at m/z 69.0704 and 58.0646 as the parent compound. In addition, the ion at m/z 114.1257 was observed which corresponded with a loss of water from the precursor ion. The compound matched with the molecular formula $C_7H_{17}NO$ which corresponded to the loss of the *p*-nitrobenzaldehyde moiety and an ethyl group from nitracaine.

Two metabolites (M2A and M2B) with the same exact mass (m/z 176.1645) were observed at 2.59 and 3.09 min. Both metabolites corresponded to the molecular formula $C_9H_{21}NO_2$. The product ions at m/z 86.0968 and m/z 58.0654, already described in the nitracaine, were observed for M2B. Although the position of the hydroxyl could not be confirmed, it might be located on the nitrogen atom to explain the higher polarity of M1. If so, logP calculated for M2A by ChemBio3D Ultra 14.0 is 0.70, a bit higher than for M1 (logP 0.58). Unfortunately, M2A was not present at high abundance, and no MS/MS spectrum could be acquired.

An additional compound (3-(diethylamino)-2,2-dimethylpropan-1-ol) which corresponded to the non-hydroxylated compound of M2 (at m/z 160.1697) was also detected. The product ions at m/z 86.0963, m/z 74.0966, m/z 69.0708, and m/z 58.0657 were already described for M1, M2, and nitracaine. In addition, a product ion at m/z 98.0957 was also observed. This ion, with molecular formula $[C_6H_{12}N]^+$, corresponded to the loss of methanol and of the two methyl groups from the precursor ion leading to the formation of a

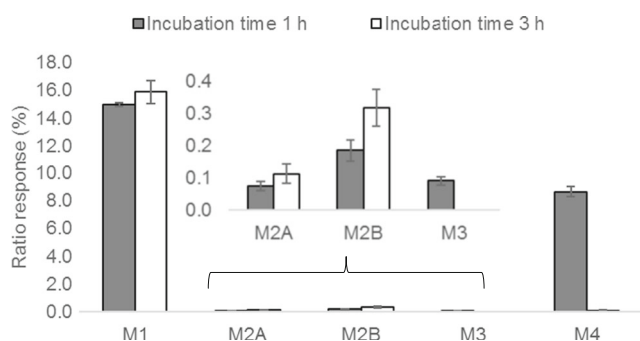
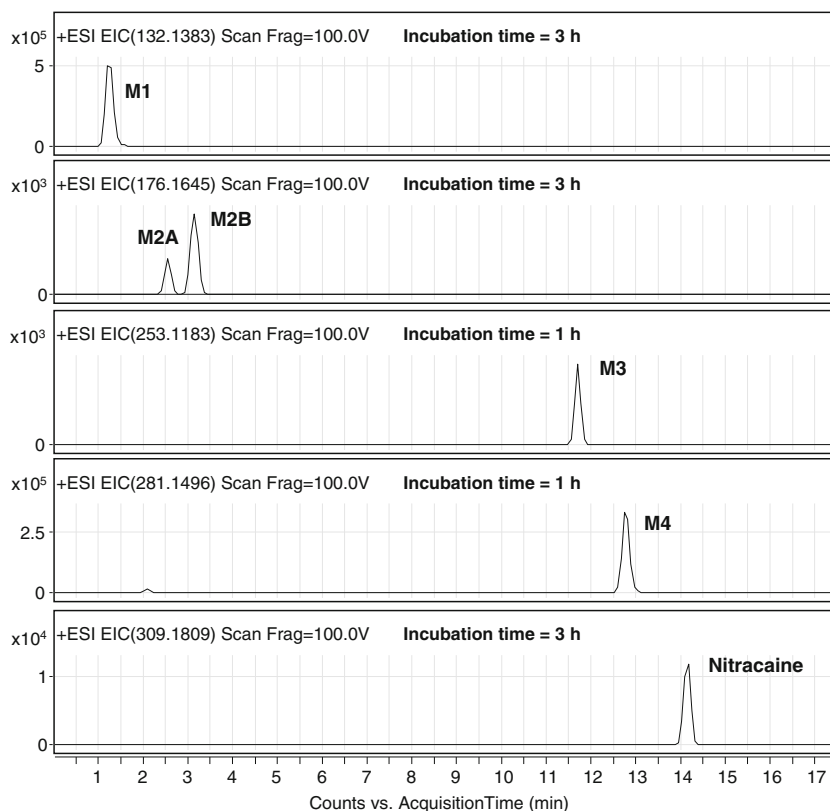


Fig. 1 Ratio response of nitracaine phase-I metabolites formed after 1 and 3 h of incubation with HLM. Ratio response was calculated as the peak area of the compound divided with the area of the internal standard peak and expressed as a percentage

Fig. 2 Extracted ion chromatograms of nitracaine and its phase-I metabolites after incubation with human liver microsomes. Mass window of 10 ppm around the ionized precursor ion



double bond. However, this compound was observed in the negative controls—without enzyme and NADPH with comparable abundances as in the real in vitro incubations. Furthermore, it was also detected when injecting a dilution of the analytical standard. These observations suggest that this compound could be a transformation product of nitracaine but not a human CYP metabolite, since no enzymes were involved in its formation. Subsequently, M1, M2A, and M2B could have been derived from the compound with m/z 160.1697 or directly from nitracaine but the fact that M1, M2A, and M2B were not observed in any negative control (Fig. 1) confirms that these compounds are human CYP metabolites.

M3 (m/z 253.1190), which eluted at 11.71 min, corresponded to the loss of the two ethyl groups of nitracaine (*N,N*-deethylation). The first product ion observed at m/z 236.0929 corresponded to the loss of the amine group. The product ions at m/z 150.0176, m/z 120.0205, m/z 104.0247, m/z 92.0260, m/z 86.0968, m/z 69.0706, and m/z 58.0658 were also observed in the MS/MS spectrum of nitracaine (see above).

M4 (m/z 281.1503), which eluted at 12.75 min, corresponded to the loss of one of the ethyl groups (*N*-deethylation). Its only difference from M3 is the presence of the product ion at m/z 114.1279, which corresponded to the ester moiety from the precursor ion. This metabolite (M4) was the only metabolite found by Power et al. [2] after incubation

of nitracaine with HLMs. The other metabolites described in the present manuscript are detected and reported for the first time.

In negative ionization mode, *p*-nitrobenzoic acid (m/z 166.0147) was observed eluting at 11.64 min. However, it was also detected in the negative controls without enzyme and NADPH suggesting that it is a transformation product of nitracaine and not a CYP metabolite.

Identification of in vitro phase-II metabolites of nitracaine

In the phase-II experiments, one glucuronidated metabolite at m/z 336.2013 was identified. It corresponded to the glucuronidation of the transformation product with m/z 160.1697 ($[M+H]^+$). In the MS/MS spectrum, the characteristic loss of 176 u was observed resulting in the ion at m/z 160.1683 and an additional loss of water led to the ion at m/z 142.1571. No sulphated phase-II metabolites were detected.

In the positive control samples, the glucuronidated (m/z 314.0531, Δm 4.14 ppm) and the sulphated (m/z 217.9776, Δm 5.05 ppm) metabolites of 4-nitrophenol produced by UGTs and SULTs, respectively, were detected in large amounts. This information substantiated the lack of formation of sulphated metabolites of nitracaine under the experimental conditions tested.

Table 1 Retention times (RT), measured accurate m/z , mass measurement errors (Δm , in ppm), tentative formula and product ions for nitracaine, and its metabolites/transformation products after incubation with HLMs

	Precursor ion				Product ions		
	RT (min)	Measured m/z	Δm (ppm)	Tentative formula	Measured m/z [M + H] ⁺	Δm (ppm)	Tentative formula
Nitracaine	14.06	309.1806	−0.97	[C ₁₆ H ₂₅ N ₂ O ₄] ⁺	263.1922	15.96	[C ₁₆ H ₂₅ NO ₂] ⁺
					236.0959	17.79	[C ₁₂ H ₁₄ NO ₄] ⁺
					168.0312	12.50	[C ₇ H ₆ NO ₄] ⁺
					150.0184	−1.33	[C ₇ H ₄ NO ₃] ⁺
					142.1617	18.99	[C ₉ H ₂₀ N] ⁺
					120.0203	−2.50	[C ₇ H ₄ O ₂] ⁺
					104.0259	1.92	[C ₇ H ₄ O] ⁺
					92.0258	1.09	[C ₆ H ₄ O] ⁺
					86.0966	2.32	[C ₅ H ₁₂ N] ⁺
					69.0701	2.90	[C ₅ H ₉] ⁺
M1	1.26	132.1386	2.27	[C ₇ H ₁₈ NO] ⁺	58.0670	32.72	[C ₃ H ₈ N] ⁺
					114.1257	−17.52	[C ₇ H ₁₆ N] ⁺
					69.0704	7.24	[C ₅ H ₉] ⁺
M2A	2.59	176.1642	−1.70	[C ₉ H ₂₂ NO ₂] ⁺	58.0646	−8.61	[C ₃ H ₈ N] ⁺
					86.0968	4.65	[C ₃ H ₁₂ N] ⁺
M2B	3.09	176.1645	0.00	[C ₉ H ₂₂ NO ₂] ⁺	72.0454	13.88	[C ₄ H ₁₀ N] ⁺
TP-160	3.53	160.1697	0.62	[C ₉ H ₂₂ NO] ⁺	58.0654	5.17	[C ₃ H ₈ N] ⁺
					98.0957	−7.14	[C ₆ H ₁₂ N] ⁺
					86.0963	−1.16	[C ₅ H ₁₂ N] ⁺
					74.0966	2.70	[C ₄ H ₁₂ N] ⁺
					69.0708	13.03	[C ₅ H ₉] ⁺
M3	11.71	253.1190	2.77	[C ₁₂ H ₁₇ N ₂ O ₄] ⁺	58.0657	10.33	[C ₃ H ₈ N] ⁺
					236.0929	5.08	[C ₁₂ H ₁₄ NO ₄] ⁺
					150.0176	−6.67	[C ₇ H ₄ NO ₃] ⁺
					120.0205	−0.83	[C ₇ H ₄ O ₂] ⁺
					104.0247	−9.61	[C ₇ H ₄ O] ⁺
					92.0260	3.26	[C ₆ H ₄ O] ⁺
					86.0968	4.65	[C ₅ H ₁₂ N] ⁺
M4	12.75	281.1503	2.49	[C ₁₄ H ₂₁ N ₂ O ₄] ⁺	69.0706	10.13	[C ₅ H ₉] ⁺
					236.0937	8.47	[C ₁₂ H ₁₄ NO ₄] ⁺
					150.0183	−2.00	[C ₇ H ₄ NO ₃] ⁺
					114.1279	1.75	[C ₇ H ₁₆ N] ⁺
					92.0259	2.17	[C ₆ H ₄ O] ⁺
					69.0703	5.79	[C ₅ H ₉] ⁺
TP-GLU	4.29	336.2013	−1.19	[C ₁₅ H ₃₀ NO ₇] ⁺	58.0658	12.06	[C ₃ H ₈ N] ⁺
					160.1683	−8.12	[C ₉ H ₂₂ NO] ⁺
					142.1571	−13.37	[C ₉ H ₂₀ N] ⁺
					113.0214	−16.81	[C ₅ H ₅ O ₃] ⁺
<i>p</i> -Nitrobenzoic acid	11.64	166.0147	0.60	[C ₇ H ₄ NO ₄] [−]	74.0967	4.05	[C ₄ H ₁₂ N] ⁺
					122.0248	−10.65	[C ₆ H ₄ NO ₂] [−]
					92.0268	−17.39	[C ₆ H ₄ O] [−]

Human CYP enzymes involved in the metabolism of nitracaine

The seven most abundant human hepatic rCYPs (rCYP1A2, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4) were tested for their ability to catalyze the formation of in vitro metabolites of nitracaine. As shown in Fig. 3, with the exception of M2B, all metabolites identified in the HLM experiments were found in these experiments. This result suggests that M2B is likely formed by other enzymes present in the HLMs. Among the rCYPs tested, rCYP2B6 and rCYP2C19 were the main enzymes catalyzing the formation of M1, M3, and M4, whereas only rCYP2B6 catalyzed the formation of M2A. A minor contribution was observed for rCYP1A2, rCYP3A4, rCYP2C9, and rCYP2D6, whereas rCYP2E1 did not catalyze the formation of any metabolite of nitracaine in detectable amount. To the best of our knowledge, the role of each individual human CYP enzymes has not been studied before for nitracaine.

Proposed metabolic pathway

The proposed in vitro metabolic pathway of nitracaine is presented in Fig. 4. Nitracaine was found to be metabolized mainly by a loss of an ethyl group (M4) and by a fragmentation of the carboxylic moiety of M4 leading to M1. The loss of a second ethyl group from M4 resulted into M3. Nitracaine could also be transformed into M2 after fragmentation of the carboxylic moiety and hydroxylation of the nitrogen atom from the alkyl chain. For each metabolite, the major CYP enzyme(s) catalyzing its formation are indicated. In addition, a phase-II in vitro metabolite catalyzed by UGT was identified. However, the results suggested that this product was not the result of the glucuronidation of a CYP-mediated in vitro metabolite, but the conjugation of a transformation product.

Since nitracaine is the nitro analogue of dimethocaine, it seems appropriate to compare both metabolic pathways. The major metabolic reaction (*N*-deethylation) found for nitracaine

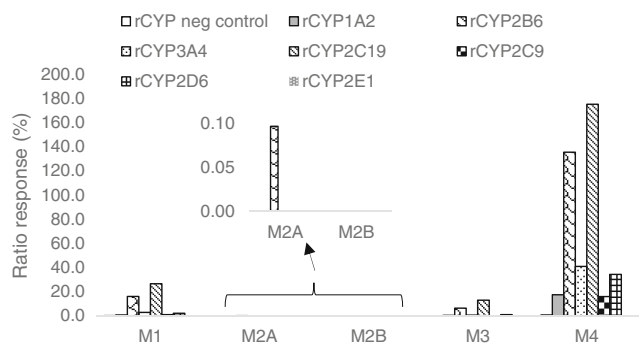


Fig. 3 Ratio response of nitracaine phase-I metabolites formed after incubation of 10 μ M nitracaine (37 $^{\circ}$ C, 1 h) using a panel of seven human recombinant CYP enzymes (20 pmol/mL). Ratio response was calculated as the peak area of the compound corrected with the area of the internal standard peak and expressed as percentage

in this study was also observed for dimethocaine after its incubation with HLMs [18]. However, whereas the hydroxylation at the *p*-aminobenzoic acid moiety was also a major metabolite of dimethocaine [18], hydroxylation of the *p*-nitrobenzoic acid moiety was not detected for nitracaine in the present study. The lack of reactivity observed in the benzene ring of nitracaine may be rationalized by the presence of a nitro group instead of an amine group. The electron-donating properties of an amine group in contrast to the electron-withdrawing effect of the nitro group is a possible explanation for this observation.

In vivo nitracaine metabolites

Both the parent compound and its generated in vitro metabolites were detected in the urine sample from a nitracaine user, except for M3 (Table 2), which was also a minor metabolite in the in vitro experiments. The glucuronidated metabolite (Table 1) was also detected in the urine sample. Besides the two transformation products earlier described, other compounds were tentatively identified in the urine sample but they could not be attributed only to the intake of nitracaine (Table 2). For instance, the ion at *m/z* 138.0551 (in positive mode) or *m/z* 136.0414 (in negative mode) could be tentatively identified as a nitro-reduction of the compound at *m/z* 166.0145 (in negative mode) to an amine group but it also could come from dimethocaine which is structurally similar to the nitracaine. Dimethocaine presents a *p*-nitro group instead of an amino group (see ESM Fig. S1). The last metabolite detected was the conjugation of the compound at *m/z* 166.0145 with glutamine leading to the ion at *m/z* 294.0707. A glucuronidated conjugate of this compound was also detected at *m/z* 336.2032. Another compound at *m/z* 223.0353 was also identified as the loss of the propanamide moiety from 294.0707. Figure 4 shows the proposed metabolic pathway of nitracaine.

Nitracaine was also detected in the urine sample in large amounts, and therefore, the parent drug itself appears to be the key biomarker for screening methods. However, it should be remarked that the excretion pattern of the substance varies with time after intake, and as a result, only metabolites can be detected at certain time point post-intake. This is of importance in clinical and forensic toxicology situations and highlights again the need for information on the metabolic pathway of substances. The in vitro experiments performed in this study indicate that the metabolites M1 and M4 could be considered as better biomarkers for nitracaine use than M2A, M2B, and M3 due to their higher abundance. Nevertheless, though M1 is more intense than M4, its suitability to be a good biomarker is questionable. M1 is present at 1 and 3 h but is not very specific of nitracaine since it could derive also from dimethocaine. Therefore, M4 could be considered as the best biomarker of the intake of nitracaine.

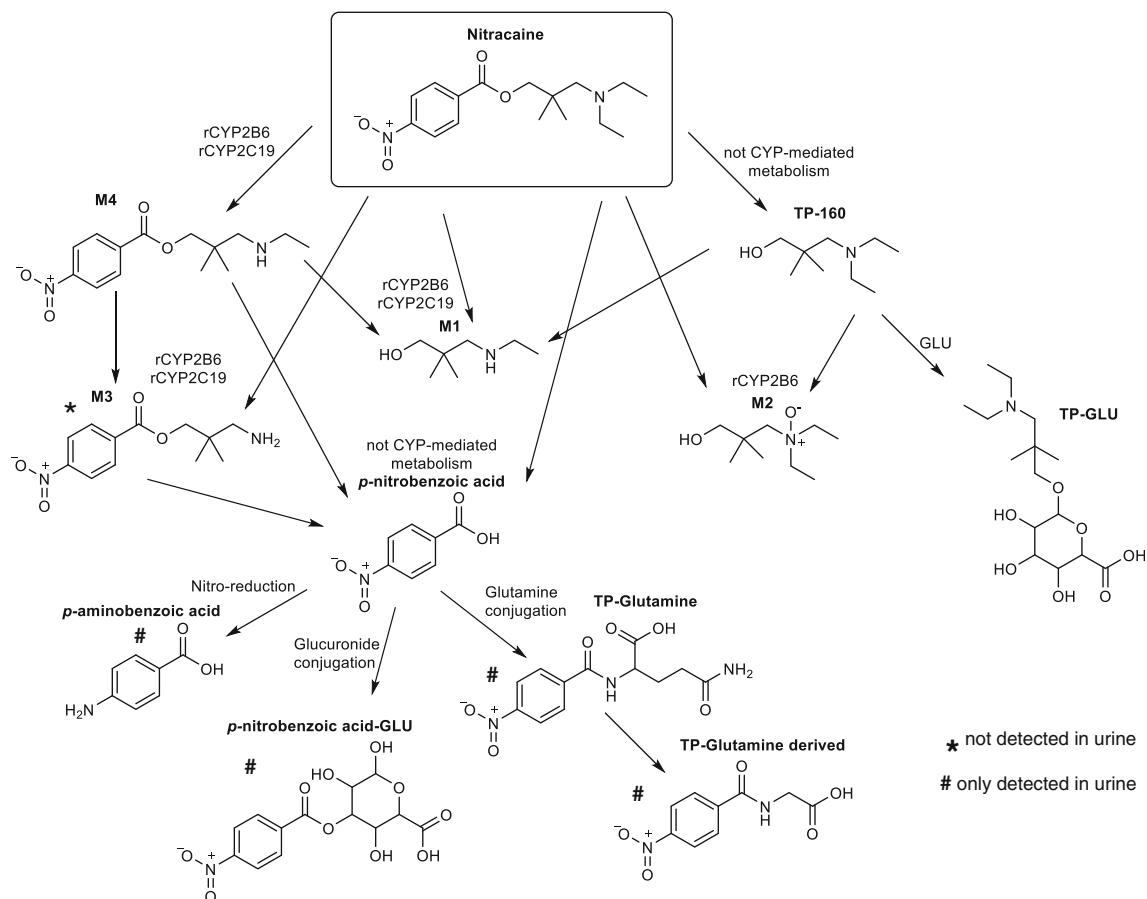


Fig. 4 Proposed metabolic pathway of nitracaine based on in vitro experiments and on information from a urine sample of nitracaine user

Table 2 Retention times (RT), measured accurate m/z , mass measurement errors (Δm , in ppm) and tentative formula for nitracaine, and its metabolites/transformation products in urine from a drug user

	Precursor ion			
	RT (min)	Measured m/z	Δm (ppm)	Tentative formula
Nitracaine	14.09	309.1816	2.26	$[C_{16}H_{25}N_2O_4]^+$
M1	1.26	132.1387	3.03	$[C_7H_{18}NO]^+$
M1 isomer ^a	1.76	132.1382	-0.76	$[C_7H_{18}NO]^+$
M2A	2.59	176.1645	0.00	$[C_9H_{22}NO_2]^+$
M2B	3.09	176.1649	2.27	$[C_9H_{22}NO_2]^+$
M3	—	—	—	—
M4	12.75	281.1499	1.07	$[C_{14}H_{21}N_2O_4]^+$
TP-GLU	4.29	336.2032	4.46	$[C_{15}H_{30}NO_7]^+$
<i>p</i> -Nitrobenzoic acid	11.64	166.0145	-0.60	$[C_7H_4NO_4]^-$
<i>p</i> -Aminobenzoic acid ^a	0.87	138.0551	0.72	$[C_7H_8NO_2]^+$
TP-Glutamine ^a	8.14	294.0707	-5.78	$[C_{12}H_{12}N_3O_6]^-$
TP-Glutamine derived ^a	8.82	223.0353	-3.14	$[C_9H_7N_2O_5]^-$
<i>p</i> -Nitrobenzoic acid-GLU ^a	8.89	342.0460	-2.05	$[C_{13}H_{12}NO_{10}]^-$
<i>p</i> -Nitrobenzoic acid-GLU ^a	9.45	342.0474	2.05	$[C_{13}H_{12}NO_{10}]^-$

^a Only detected in urine

Nexus metabolite prediction

Metabolite prediction with Nexus software definitely speeded up the creation of a list of possible metabolites, and it showed a high confidence in the precursor formulas for the generated metabolites. The software predicted 42 probable and 7 plausible metabolites (see ESM Fig. S3). This list was viewed critically because of its tendency to over predict the amount of metabolites formed, resulting in a large number of false positives. Other studies also observed this over prediction [14, 19]. For this reason, Nexus was useful as a first guidance, but the information obtained from the acquired MS/MS spectra was essential for a correct (and evidence-based) identification of the metabolites.

Conclusions

This study characterized the human in vitro and in vivo metabolism of nitracaine and identified potential biomarkers that can be included in clinical and forensic drug screening methods. The study reveals six in vitro generated metabolites (five phase-I and one phase-II) and the CYP isoenzymes involved in the main metabolic pathway. All metabolites, except the *N*-deethylation are reported for the first time in this study. All the metabolites and transformation products identified in the in vitro experiments were also detected in vivo, except for the *N,N*-deethylation metabolite. On the other hand, six additional metabolites/transformation products were detected solely in the in vivo sample.

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Compliance with ethical standards The human urine sample used in this study was anonymously collected via routine investigations at the Emergency Department of the Universitair Ziekenhuis Brussel for diagnosis purposes, and therefore, it did not involve any ethical issues.

Conflict of interest The authors declare that they have no conflict of interest.

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