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Prince Sellase Gameli

`prince.sellase.gameli@liu.se`

Linköping University

Robert Kronstrand

Linköping University

Henrik Green

Linköping University

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**Metabolism of brorphine, chlorphine, fluorphine and *N*-propionitrile
chlorphine in human hepatocytes and authentic *N*-propionitrile chlorphine
case studies using high-resolution mass spectrometry**

Prince S. Gameli^{1*}, Robert Kronstrand^{1,2}, Henrik Gréen^{1,2,3}

¹Department of Biomedical and Clinical Sciences, Division of Clinical Chemistry and Pharmacology, Linköping University, Linköping, Sweden

²Department of Forensic Genetics and Forensic Toxicology, National Board of Forensic Medicine, Linköping, Sweden

³Department of Biomedical and Clinical Sciences, Science for Life Laboratory, Linköping University, Linköping, Sweden

Corresponding author

Prince S. Gameli

Division of Clinical Chemistry and Pharmacology

Department of Biomedical and Clinical Sciences

Linköping University

SE-581 85, Linköping

Sweden

Email; prince.sellase.gameli@liu.se

Authors' ORCiD

PSG: 0009-0001-3194-325

RK: 0000-0002-4222-9597

HG: 0000-0002-8015-5728

Abstract

Novel synthetic opioids represent an increasingly important sub-group of new psychoactive substances to monitor due to their association with severe and fatal intoxications, especially when combined with alcohol and other drugs. The recent emergence of orphines, which are potent μ -opioid receptor inhibitors, has raised health and safety concerns. As such characterizing orphine analogues' metabolism is essential for reliable identification and interpretation in analytical toxicology. This study aimed at comprehensively characterizing the metabolites of orphine analogues, i.e. bromphine, chlorphine and fluorphine, and *N*-propionitrile chlorphine using pooled human hepatocyte incubations and data acquisition with liquid chromatography tandem high-resolution mass spectrometry (LC-HRMS). Orphines were individually incubated with hepatocytes, alongside controls, and data acquired with LC-HRMS. Blood and urine samples from authentic *N*-propionitrile chlorphine cases were also analyzed. Tentative metabolites were elucidated based on their accurate masses, isotopic distributions and fragmentation patterns. In total, 11 metabolites were detected for bromphine and chlorphine, 12 metabolites for fluorphine, and 13 biotransformational products for *N*-propionitrile chlorphine. Three and eight *N*-propionitrile chlorphine metabolites were found for blood and urine, respectively. The common main site for biotransformation was the benzimidazolone unit, for all orphines. Additionally, *N*-dealkylation, to nor-metabolites, at the piperidine was common for all compounds while *N*-oxidation, also at the piperidine, was observed for fluorphine and *N*-propionitrile chlorphine. Although not compound specific, we recommend incorporating nor-metabolites as consumption markers for orphine sub-classes in analytical toxicology to reduce plausible false negative results, especially in urine screening as orphines can undergo extensive metabolism leaving no traces of the parent.

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26 opioid

1. Introduction

Novel synthetic opioids (NSO), which target μ -opioid receptors (MOR), represent a major class of novel psychoactive substances (NPS) due to their extreme potency and implications in fatal overdose, making them a serious threat to public health (Vandeputte et al. 2022; Shafi et al. 2022). These NSO include fentanyl and its analogues, and non-fentanyl like compounds such as piperazines, cyclohexylbenzamides, 2-benzylbenzimidazolones and benzimidazolones and “atypical” MOR agonists (Zawilska et al. 2023). Interestingly, while the third wave of opioid crisis led to the emergence of fentanyl analogues, the blanket legislations scheduling fentanyl and its analogues may have triggered potent nitazene production (Pereira et al. 2025). It is thus not surprising that recent nitazene regulations, perhaps in part, may have also led to novel orphines’ emergence (The Colombo Plan 2026; UNODC 2026a).

Orphine, the most basic structure of this emerging NSO sub-class, consists of a benzimidazolone core and a phenylethylpiperidine group (UNODC 2026b). The first orphine to emerge on the illicit drug market was brorphine, a bromobenzyl orphine analogue, in 2019 (Marchei et al. 2020). It was subsequently detected in toxicological cases in the USA and Germany in 2020 and 2024, respectively (Vohra et al. 2021; Grafinger et al. 2021). Afterwards, chlorphine was detected in 2024 (UNODC 2026c), and one can speculate that other halogenated analogues including fluorphine and iodorphine are likely to emerge. Studies on these halogenated derivatives suggest that brorphine, chlorphine and iodorphine are highly potent MOR agonists that cause severe respiratory depression in male CD-1 mice (Vandeputte et al. 2024). Notably, *N*-propionitrile chlorphine, also known as cycchlorphine, with a propionitrile substitution at the benzimidazolone (see Figure 1) has gained widespread notoriety due to its implication in a substantial number of non-fatal and fatal intoxication cases (Raffa et al. 2026; Kusko et al.

2026). Preliminary data, on this NSO sub-group show that they are potent human-ether-a-go-go (hERG) inhibitors consistent with other opioids (El Sherbini et al. 2024).

These emerging orphine analogues necessitate continuous monitoring in clinical and forensic toxicology (Ip et al. 2026). To identify reliable diagnostic markers of consumption, useful in analytical toxicology, it is particularly important to map their metabolism pathways (Wagmann et al. 2021). Brorphine, for instance, undergoes extensive metabolism leaving only minor traces of the parent in urine (Grafinger et al. 2021). However, there are limited metabolism data on the other halogenated analogues and whether they have similar metabolism profiles. For *N*-propionitrile chlorphine, pooled human liver microsomes (HLM) data suggests the primary biotransformation includes hydroxylations and *N*-dealkylations particularly at the benzimidazolone resulting in chlorphine (Bassman et al. 2025). If confirmed *in vivo*, this finding may provide useful insights on the toxicological profile of *N*-propionitrile chlorphine and contribute to a better understanding of its toxidrome and implications in intoxication cases.

To obtain metabolism data in a timely manner, especially when authentic intoxication cases are unavailable and concomitant drug use is commonplace, *in vitro* models including HLM, HepRG and human hepatocytes, are useful alternatives (Ooka et al. 2020; Wagmann et al. 2021). Among these metabolism models, hepatocytes simulate both phase I and phase II human metabolism with better accuracy as they provide sufficient metabolic enzymes, cofactors and transporter proteins for xenobiotic biotransformation (Sevior et al. 2012; Li 2000). This experimental setup combined with high-resolution mass spectrometry (HRMS) for data acquisition provides an avenue for analytical toxicologists to obtain diagnostic markers to improve authentic positive case detections (Chen et al. 2007; Weston et al. 2022).

In this study, we employed ultrahigh-performance liquid chromatography (UHPLC) tandem quadrupole time of flight mass spectrometry (QToF-MS) to comprehensively characterize hepatic metabolites of bupropion, chlorphene and fluorphene, and *N*-propionitrile chlorphene. Additionally, blood and urine samples from *N*-propionitrile chlorphene intoxication case were analyzed to identify ideal markers of consumption for analytical toxicology and potentially active biotransformation products that may be of interest to monitor in the drug supply chain.

2. Materials and methods

2.1. Chemicals and reagents

Chemical standards of bupropion (B), chlorphene (C), fluorphene (F) and *N*-propionitrile chlorphene (N) were obtained from Cayman Chemicals (Ann Arbor, MA, USA). Stock solutions of B, C and N were prepared at 1 mg/mL and F was at 0.5 mg/mL in methanol. LC-MS grade methanol, acetonitrile, and formic acid were obtained from Thermo-Fisher Scientific (Gothenburg, Sweden). Positive control stock solution; 5 mg/mL caffeine, 1 mg/mL bupropion, 1 mg/mL diclofenac, 1 mg/mL omeprazole, 1 mg/mL dextromethorphan, 1 mg/mL chlorzoxazone and 1 mg/mL midazolam and internal standards (ISTD); 15 µg/mL D₈-amphetamine, 5 µg/mL D₅-diazepam, 2.5 µg/mL D₃-mianserin and 15 µg/mL D₅-phenobarbital were procured from Thermo-Fisher Scientific (Gothenburg, Sweden). Hepatocytes (LiverPool®) pooled from 20 diverse donors and InVitro HT thawing media was purchased from Bioreclamation IVT (Brussels, Belgium). Other hepatocyte incubation reagents including William's E media, L-glutamate, and HEPES buffer were procured from Thermo-Fisher Scientific (Gothenburg, Sweden). Finden B-One β-glucuronidase was obtained from Kura Biotech (Puerto Varas, Chile). Erythrosin B was procured from Sigma Aldrich (Missouri, USA) and finally, ultra-pure water,

obtained from an in-house MilliQ gradient unit from Millipore (Massachusetts, USA), was used for sample preparation.

2.2. *In vitro* human hepatocyte incubations

Hepatocyte incubation was carried out by making minor modifications, where necessary, to a previous protocol (Watanabe et al. 2017; Lindbom et al. 2025). Briefly, human hepatocytes were thawed, transferred to 48 mL of InVitro Gro HT thawing media, and then centrifuged at 100 ×g for 5 min. Hepatocytes were re-suspended in 50 mL of Williams E media, supplemented with L-glutamine and HEPES (SWME) at 2 and 20 mmol/L, respectively, after discarding the thawing media. After centrifugation at 60 ×g for 5 mins, hepatocyte amount was estimated using erythrosin B exclusion method. The final hepatocyte amount was adjusted to 2×10^6 cells/mL with SWME.

For hepatocyte-drug incubations, 50 µL drug (10 µmol/L) dissolved in SWME, < 0.5% organic solvent, was gently mixed with 50 µL of hepatocytes in a 96-well plate. Hepatocyte incubations with bromophine, chlorophine, fluorophine and *N*-propionitrile chlorophine were performed individually for 0 and 3 hours (h), and the reaction quenched with 100 µL ice-cold acetonitrile with ISTD. Ice cold acetonitrile was added prior to adding hepatocytes and compounds for 0 h incubations. After quenching the reaction, the plates were centrifuged at 2500 ×g for 15 min (4°C), the supernatant transferred to LC-MS vials, and 10 µL was injected for HRMS data acquisitions. Experiments, performed in duplicates, had negative (hepatocytes without drugs), degradation (drugs without hepatocytes) and positive (compounds selectively metabolized by cytochrome P450 isozymes) controls, for 0 and 3 h.

2.3. Authentic blood and urine intoxication cases

Authentic cases that were confirmed as positive for *N*-propionitrile chlorphine at the National Board of Forensic Medicine, Sweden were added for analysis. For blood samples, from four cases, 300 μ L of ice-cold acetonitrile/methanol (90:10) was added to 150 μ L of blood with 5 μ L methanol containing ISTD (250 ng/mL). The mixture was centrifuged at 15,000 $\times g$ for 15 min (4°C). The supernatant was collected and dried under nitrogen at 50 °C. After drying, the extract was reconstituted into 50 μ L of mobile phase A (MPA):mobile phase B (MPB), (50:50).

For urine (five cases), both non-hydrolyzed and hydrolyzed samples were prepared for HRMS data acquisition as follows: 50 μ L of urine was mixed with 50 μ L of milliQ water or β -glucuronidase for non-hydrolyzed or hydrolyzed samples, respectively. Enzymatic hydrolysis was performed at 37 °C for 2 h. Subsequently, 25 μ L of methanol and ISTD in methanol (200 ng/mL) were added to both hydrolyzed and non-hydrolyzed samples. After centrifugation at 15,000 $\times g$ for 15 mins, the supernatant dried under nitrogen at 50 °C. This was then reconstituted into 50 μ L of MPA:MPB (50:50).

Ten μ L of all extracts, i.e. blood, hydrolyzed and non-hydrolyzed urine were injected for data acquisition. Negative blood and urine samples were prepared alongside, under the same experimental conditions to ensure tentative metabolite identification.

2.4. Data acquisition with UHPLC-QToF-MS

UHPLC was performed using Agilent 1290 Infinity (Agilent Technologies, Kista, Sweden) with an Acquity HSS T3 column (150 mm $\times$ 2.1 mm, 1.8 μ m; Waters, Sollentuna, Sweden) and an Acquity VanGuard pre-column (Waters). Mobile phases were made of 0.1% formic acid in water as MPA, and 0.1% formic acid in acetonitrile as MPB. Chromatographic elution was as follows: MPB, initiated with 1% for the first 0.60 min, and increased to 5% within 0.10 min for 10.0

mins. B was then increased to 20% for 8.0 mins and then to 50% for another 3.0 mins. B was sharply raised to 95% at 21.0 min and maintained till 24.0 min, finally the initial starting conditions were restored within 0.10 min. The entire gradient run was 27.0 min at a flow-rate of 0.5 mL/min and column temperature maintained at 60 °C.

HRMS data was acquired using an Agilent 6550 iFunnel QToF-MS from Agilent Technologies with a dual Agilent Jet Stream electrospray ionization source. HRMS acquisition was performed in positive ionization mode with Auto MS/MS settings as follows: full scan MS, m/z 100-900; and MS/MS, m/z 50-950; precursor intensity threshold, 5000 counts; pre-cursor number per cycle, 5; fragmentor voltage, 380 V; collision energy, 30 eV; isolation width, 1.3 m/z ; gas temperature, 150 °C; gas flow, 18 L/min; nebulizer gas pressure, 345 kPa; sheath gas temperature, 375 °C; and sheath gas flow, 11 L/min.

HRMS data was processed with Agilent MassHunter Qualitative Analysis software (B.07.00), using an in-house library of plausible biotransformations. Metabolite identification involved an initial screening using the following parameters; mass error, 15 ppm; absolute peak area threshold, > 20,000 counts; maximum number of matches, 20; and extraction window, 100 ppm. A metabolite is then tentative identified if the mass error of the protonated molecule was < 5 ppm, (unless there was peak saturation of the detector), presence of a consistent isotopic pattern, a product ion spectrum that matched the proposed chemical structure, the retention time was plausible (from 2.0 to 19.0 mins), the peak shape was appropriate and the proposed metabolite was absent in negative controls.

3. Results

3.1. HRMS/MS fragmentation of buporphine, chlorphine, and fluorphine

Brorphine, chlorphine and fluorphine eluted at 13.97, 13.53 and 12.08 mins, respectively, and *N*-propionitrile chlorphine at 14.36 min. Due to peak saturation of brorphine, chlorphine, fluorphine and *N*-propionitrile chlorphine at 10 μ L, hepatocyte incubates were re-injected at 1 μ L to obtain un-interrupted chromatographic peaks of the parent compounds (see Figure 2).

Brorphine had precursor ion at m/z 402.1007, chlorphine at m/z 356.1513 and fluorphine at m/z 340.1806. The MS/MS fragmentation pattern of brorphine, chlorphine and fluorphine consistently had fragment ion (FI) at m/z 218.12 ($C_{12}H_{16}N_3O^+$) consistent with cleavage of the halogenated benzyl ring at the piperidine. Further piperidine contraction resulted from ammonia (NH_3), at m/z 201.10 ($C_{12}H_{13}N_2O^+$), and ethylene (C_2H_4) losses at m/z 173.07 ($C_{10}H_9N_2O^+$). FI at m/z 135.05 corresponding to the benzimidazolone unit was detected with minor intensity. Fragments at m/z 189.98, 139.03, and 123.06, consistent with the bromo-, chloro- and fluoro-benzyl moiety of brorphine, chlorphine and fluorphine, respectively, were the base peaks. Bromide ion loss was observed at m/z 104.05 ($C_8H_8^+$) for brorphine while hydrogen chloride or fluoride ions losses were at m/z 103.05 ($C_8H_7^+$) for chlorphine and fluorphine. Finally, FI at m/z 84.08 ($C_5H_{10}N^+$) corresponding to the piperidine was observed, and a further C_2H_4 loss at m/z 56.04 ($C_3H_6N^+$).

The precursor for *N*-propionitrile chlorphine was detected at m/z 409.1780 with fragmentation pattern similar to chlorphine. Cleavage at the piperidine occurs at m/z 271.15 ($C_{15}H_{19}N_4O^+$) and subsequent NH_3 and C_2H_4 losses at m/z 254.12 ($C_{15}H_{16}N_3O^+$) and 226.09 ($C_{13}H_{12}N_3O^+$), respectively. FI at m/z 188.08 ($C_{13}H_{12}N_3O^+$) corresponds to the benzimidazolone and propionitrile sub-units. Other FIs at 139.03, 103.05, 84.08, and 56.04, previously elaborated, were detected in the MS/MS of *N*-propionitrile chlorphine. The MS/MS spectra and

fragmentation patterns of brorphine, chlorphine, fluorphine and *N*-propionitrile chlorphine are depicted in Figure 3.

3.2. *In vitro* human hepatocyte metabolites

Figure 4 shows the extracted ion chromatographs (EIC) of brorphine (B), chlorphine (C), fluorphine (F) and *N*-propionitrile chlorphine (N) metabolites. In total, 11 metabolites were identified for brorphine and chlorphine, while 12 metabolites were detected for fluorphine. For *N*-propionitrile chlorphine, nine and four phase I and II metabolites, respectively, were found. Proposed biotransformations, retention times, predicted molecular formulas, accurate masses, Δ ppm and peak areas of metabolites in both replicates are reported for brorphine, chlorphine, fluorphine and *N*-propionitrile chlorphine in Table 1, 2, 3, and 4, respectively. The estimated peak area of the parent compounds from 1 μ L injections, ten-fold, are highlighted with * in the table for comparison.

3.2.1. Phase I metabolites

***N*-Dealkylation**

Metabolites B2, C2 and F2 were all detected at the same retention time with precursor ion at m/z 218.1293. The MS/MS spectra of these metabolites consisted of previously described fragments at m/z 201.10, 173.07 and 135.05. Additionally, FI at m/z 84.08, major FI, and 56.04 were detected. Fragment at m/z 55.05 ($C_4H_7^+$), cyanide (CNH_3) loss, likely results from m/z 84.08.

N1 was detected at 5.66 min with accurate mass at m/z 271.1551. The spectrum of N1 had FI at m/z 254.12, an NH_3 loss from N1, and m/z 226.09, consistent with subsequent C_2H_4 loss. FI at m/z 185.07 ($C_{11}H_9N_2O^+$), likely results from combined propionitrile and methyl (CH_3) loss from m/z 254.12. Finally, fragments at m/z 135.05, 84.08, and 56.04 were also identified.

The metabolite N10, with precursor ion at m/z 356.1542, was detected at 13.66 min. N10 had MS/MS fragmentation pattern consistent with that of chlorphine.

Hydroxylation(s)

The metabolites B6, B8 and B11 had precursor ion at m/z 416.0966, and C6, C8 and C11 at m/z 372.1472 and F6, F8 and F11, with m/z at 356.2767, were detected. Notably, the MS/MS of these metabolites had FI at m/z 234.12 ($C_{12}H_{16}N_3O_2^+$), 217.09 ($C_{12}H_{13}N_2O_2^+$), 189.06 ($C_{10}H_9N_2O_2^+$) and 151.05 ($C_7H_7N_2O_2^+$), albeit a minor fragment. The base peaks of these metabolites were detected at m/z 189.98, 139.03, and 123.06, for brorphine, chlorphine and fluorphine metabolites, respectively. Additionally, FI at m/z 103.05, 84.08, 56.04 were found.

N7 and N11 both had precursor ion at m/z 425.1733. Fragments at m/z 287.15 ($C_{15}H_{19}N_4O_2^+$), and 270.12 ($C_{15}H_{16}N_3O_2^+$) were detected. FI at m/z 204.07 ($C_{10}H_{10}N_3O_2^+$) may have resulted from cyclopentyl loss from m/z 270.12. A methyl cyanide loss at m/z 163.04 ($C_8H_7N_2O_2^+$) from m/z 204.07 was observed. Other notably fragments included m/z 139.03, 103.05, 84.08 and 56.04.

N-Oxidation

F12 also had accurate mass at m/z 356.1770, albeit with slightly different MS/MS fragmentation compared to F6, F8 and F11. The MS/MS of F12 also had FI at m/z 234.12 ($C_{12}H_{16}N_3O_2^+$) and m/z 201.10, 188.07 and 135.05 were found. FI at m/z 100.07 is consistent with $C_5H_{10}NO^+$ and subsequent water (H_2O) loss was observed at m/z 82.06 ($C_5H_8N^+$). Additional fragments include FI at m/z 123.05 and 103.05.

Similarly, the metabolite N13, with accurate mass at m/z 425.1736, also had fragments at m/z 287.15 ($C_{15}H_{19}N_4O_2^+$) and minor FI at m/z 188.08. Previously described FIs at m/z 139.03, 103.05, 82.06, which was the base peak, and 56.04 were detected.

***N*-Dealkylation and Hydroxylation**

B1, C1, F1, which were the earliest eluting metabolites, were found at 2.50 min with precursor at m/z 234.1241. We also found FIs at m/z 234.12, 217.09, 189.06 and 151.05.

N4 and N5 also eluted at 11.36 and 11.66 mins, respectively, and had precursor ion at m/z 372.1472. The retention times, precursor ions and MS/MS patterns of N4 and N5 were consistent with C6 and C8, respectively.

***N*-Oxidation and Hydroxylation**

N12 was found at m/z 441.1692 and had MS/MS fragmentation pattern with FI at m/z 303.14 ($C_{15}H_{19}N_4O_3^+$), 139.03, 82.06 (base peak of N12), and 56.04.

De-cyanation to alcohol

The metabolite N9 had accurate mass at m/z 400.1782 and MS/MS with FI at m/z 262.15 ($C_{14}H_{20}N_3O_2^+$) and NH_3 loss at m/z 245.13 ($C_{14}H_{17}N_2O_2^+$), cyclopentyl loss from m/z 245.13 occurred at m/z 179.08 ($C_9H_{11}N_2O_2^+$) and H_2O loss from m/z 179.08 at m/z 161.07 ($C_9H_9N_2O^+$). Other FIs at m/z 139.03, 103.05, 84.08 and 56.04, previously explained, were detected.

3.2.2. Phase II metabolites

***N*-Glucuronidation**

B7, C7 and F7 were respectively detected with precursor ions at m/z 576.1345, 532.1842 and 516.2137. The MS/MS had FI at m/z 394.16 ($C_{18}H_{24}N_3O_7^+$), H_2O loss from m/z 394.16 was found at m/z 376.14 ($C_{18}H_{22}N_3O_6^+$). FI at m/z 260.13 consistent with the formula $C_{14}H_{18}N_3O_2^+$ and m/z 218.12 and 86.09 ($C_5H_{10}N^+$) were also detected.

Hydroxylation and *O*-Glucuronidation

We identified three metabolites each for the halogenated orphines, namely, B3, B4 and B5 with precursor at m/z 592.1291, C3, C4 and C5 at m/z 548.1795 and F3, F4 and F5 at m/z 532.2090.

For bromophine metabolites, FI at m/z 416.09 ($C_{20}H_{23}BrN_3O_2^+$) was detected. Furthermore, the MS/MS fragmentation of all metabolites had FI at m/z 410.15 ($C_{18}H_{24}BrN_3O_8^+$) consistent with loss of the halogenated benzyl moiety. FIs at m/z 372.1459 ($C_{20}H_{23}ClN_3O_2^+$) and 356.1746 ($C_{20}H_{23}FN_3O_2^+$) were observed in the MS/MS of chlorphine and fluorphine metabolites, respectively. Fragments at m/z 234.12, 217.09, 189.06, 151.05, 103.05, 84.08 and 56.04 were detected. As previously elucidated, FI at m/z 182.97, 139.03 and 123.05 were detected for bromophine, chlorphine and fluorphine metabolites, respectively.

For *N*-propionitrile chlorphine metabolites N2, N3 and N6, both with precursor at m/z 601.2091, had MS/MS with FI at m/z 287.15, 270.13 (minor fragment), 204.07, 139.03, 103.05, 84.0804, and 56.04.

Hydroxylations and *O*-Methylation

The metabolite B10, detected at m/z 446.1078, C10 at m/z 402.1578 and F11 at m/z 386.1878 underwent similar biotransformations. Their fragmentation pattern had FIs m/z 264.13 ($C_{13}H_{18}N_3O_3^+$), an NH_3 loss from m/z 264.13 at m/z 247.105 ($C_{13}H_{15}N_2O_3^+$) and C_2H_4 loss from m/z 247.10 at m/z 219.075 ($C_{11}H_{11}N_2O_3^+$). Fragment at m/z 207.07 corresponding to $C_{10}H_{11}N_2O_3^+$

was found. FI at m/z 180.05 likely results from cyclopropene loss (C_3H_4) from m/z 219.075 and fragment at m/z 109.06 was consistent with the formula $C_7H_9O^+$. Other FIs, described previously, m/z 182.97 (brorphine), 139.03 (chlorphine), 123.06 (fluorphine), and common fragments 103.05, 84.08 and 56.04 were present in the MS/MS spectra.

N8 had an accurate mass at m/z 455.1875 and MS/MS with FIs at m/z 317.16 ($C_{16}H_{21}N_4O_3^+$) and others at m/z 271.15, 254.12, 226.09, 188.08, 139.03, 103.05, 84.08 and 56.04.

Hydroxylation and O-Sulfation

B9, C9 and F10, respectively, had precursor ions at m/z 496.0538, 474.0852, and 436.1337. FIs at m/z 314.07 ($C_{12}H_{16}N_3O_6S^+$), 234.12, 218.12, 217.09 were the major fragments found in the MS/MS spectra, among the common FIs for brorphine, chlorphine and fluorphine metabolites.

3.3. *In vivo* N-propionitrile chlorphine phase I and II metabolites

In total, three *N*-propionitrile chlorphine phase I metabolites, namely N1, N7 and N10, were detected across four cases in blood. For non-hydrolyzed urine, four phase I metabolites; N1, N7, N10 and N11, and three phase II metabolites; N2, N3 and N6 were found. After hydrolysis for 2 h, four phase I metabolites, previously detected for non-hydrolyzed urine, and N13 were found. No phase II metabolites were found after enzymatic hydrolysis. Figure 5 illustrates the EIC of *N*-propionitrile chlorphine and metabolites detected in blood, non-hydrolyzed and hydrolyzed urine samples from case #2. The predicted biotransformations, retention times, molecular formulas, accurate masses, mass errors in Δ ppm of *N*-propionitrile chlorphine metabolites detected in authentic cases are reported in Table 5. The MS/MS fragmentation of all metabolites are included in Supplementary Material.

4. Discussion

In this study, we comprehensively characterized the biotransformation of emerging halogenated orphine analogues and *N*-propionitrile chlorphine using pooled human hepatocyte incubations and data acquisition with HRMS. The detection of both phase I and II metabolites in positive controls implied the hepatocyte incubations were viable. Additionally, authentic intoxication cases positive for *N*-propionitrile chlorphine were analyzed.

4.1. Comparing *in vitro* phase I metabolism of brorphine, chlorphine and fluorphine

Brorphine, chlorphine and fluorphine had similar biotransformational pathways (see Figure 6). Phase I reactions included *N*-dealkylation, and *N*-oxidation at the piperidine for fluorphine, hydroxylations at the benzimidazolone, and combinations of *N*-dealkylation and hydroxylation (benzimidazolone) reactions. *N*-Dealkylation, a major metabolite found for brorphine, chlorphine and fluorphine resulted in nor-orphine and was previously detected in authentic human intoxication casework (Krotulski et al. 2021; Grafinger et al. 2021). Interestingly, the nor-metabolite was also detected in degradation controls, albeit at a lower intensity, suggesting hepatocytes mediate minimally in the formation of this metabolite. By comparing the peak areas of the nor-metabolite from the hepatocytes and degradation controls, the nor-metabolite showed a decreasing intensity in the order of fluorphine > chlorphine > brorphine. This suggests a structure activity relationship for nor-orphine, perhaps due to steric hindrance of the enzymes at this site.

Hydroxylation and *N*-oxidation reactions were characterized by a 15.99 Da shift (+O) from the parent (Kostiainen et al. 2003; Anari et al. 2004). Hydroxylations occurred on the benzimidazolone moiety as indicated by the characteristic fragments at *m/z* 234.12, 217.09,

189.06 and 151.05, consistent with 15.99 Da shift from the parents' fragments. These metabolites likely followed hydroxylation at either the para, meta or ortho positions (Grafinger et al. 2021). *N*-Oxidation occurs at the piperidine as shown by FIs at m/z 234.12, 100.07 and 82.06, and was detected for fluorphine. Trace of F12 in degradation controls was identified, however, the peak area of F12 was tenfold higher after incubation with hepatocytes. Although a similar metabolic product, known as *N*-oxide, was found as the most intense metabolite for bromphine in urine (Grafinger et al. 2021), in this study, *N*-oxide was detected at the same intensity for bromphine and ten times higher for chlorphine in degradation controls compared to hepatocyte incubations. As such, this urinary metabolite may be minimally mediated by hepatocytes, if any.

Combination of *N*-dealkylation and hydroxylation was detected as B1, C1 and F1 for bromphine, chlorphine and fluorphine respectively. Hydroxylation occurred at the benzimidazolone as previously established, however, it was not possible to identify whether this hydroxylation occurred at the para, meta or ortho position.

4.2. Comparing *in vitro* phase II metabolism of bromphine, chlorphine and fluorphine

B7, C7 and F7 were shifted by +176.03 Da ($+C_6H_8O_6$) from their respective parents consistent with glucuronidation (Kostiainen et al. 2003). Fragmentation of these metabolites points to this reaction occurring at the benzimidazolone as opposed to the piperidine. B7 was a minor metabolite detected in hepatocyte incubations, perhaps a reason why it was not previously reported in bromphine authentic samples (Krotulski et al. 2021; Grafinger et al. 2021).

B3/C3/F3, B4/C4/F4 and B5/C5/F5 had a mass difference of +192.02 Da from bromphine, chlorphine and fluorphine. These metabolites underwent hydroxylation (+15.99 Da) and further glucuronidation (+176.03 Da) on the benzimidazolone. Based on their retention times, these

metabolites likely follow their respective phase I hydroxylated metabolites B6/C6/F6, B8/C8/F8 and B11/C11/F11.

The metabolites B9, C9 and F9 were consisted with hydroxylation (+15.99 Da) and further phase II sulfation (+79.96 Da) (Yi et al. 2006). Based on their MS/MS pattern as previously elaborated, sulfation reaction followed hydroxylation at the benzimidazolone.

Similarly, B10, C10 and F10 had a mass shift of +46.00 Da from the parent compounds and points to di-hydroxylation (+31.99 Da, +2O) and methylation (+14.01, +CH₃) reaction(s) occurring at the benzimidazolone. Although Grafinger et al. 2021, posited combined reactions at the piperidine and benzimidazolone, fragments at *m/z* 247.10, 207.07 and 84.08, without *m/z* 82.06 resulting from water loss at the piperidine, are consist with di-hydroxylation and a subsequent methylation at the benzimidazolone moiety. However, a hydroxy methyl metabolite was not detected, even after retrospective analysis, which suggests this metabolic transformation (*O*-methyl metabolite) may have occurred at an earlier stage.

4.3. *In vitro* and *in vivo* *N*-propionitrile chlorphine phase I metabolites

N1 similar to B2, C2 and F2 underwent *N*-dealkylation at the piperidine, however, unlike brorphine, chlorphine and fluorphine, N1 did not undergo further hydroxylation. On the other hand, N10 transformed via *N*-dealkylation at the benzimidazolone had accurate mass and fragmentation pattern consisted with the propionitrile loss forming chlorphine. Furthermore, transformation of N1 and N10 seem to be catalyzed by hepatocytes, compared to the *N*-desalkyl (nor-orphine metabolites) metabolite of brorphine, chlorphine and fluorphine, as traces of these metabolites were not found in the degradation controls. N10 was further hydroxylated to form N4 and N5, corresponding to C6 and C8, respectively. In blood and urine samples, N1 was the

most intense metabolite detected at the same or higher intensity compared to *N*-propionitrile chlorphine. Where *N*-propionitrile chlorphine was extensively metabolized (case #5), N1 was found with peak area at 1.5×10^7 , making it an important marker of consumption for propionitrile orphine analogues. N10, on the other hand, was found 100-fold less than the parent in three blood samples and tenfold less than one urine sample. Thus, the overall contribution of this metabolite to the MOR activity of *N*-propionitrile chlorphine, if any, may be minimal. Nonetheless, further experiments may be needed to tentatively confirm this assertion.

Hydroxylation and *N*-oxidation at the benzimidazolone and piperidine, respectively were observed with +15.995 Da mass difference. N7 and N11 from their elucidated MS/MS was consistent with hydroxylation at the benzimidazolone. N13, characterized by FI at m/z 100.07 and 82.06, was transformed via *N*-oxidation. Similar to F12, N13 was ten-fold higher in intensity after incubation of *N*-propionitrile chlorphine with hepatocytes. N12, based on its MS/MS spectra, was consistent with hydroxylation and *N*-oxidation, as opposed to combination of N7 and N11. N12 is thus a combination of N13 and either N7 or N11. In three human urine samples, N7 and N11 were predominant metabolites, however, only N7 was detected in blood. After enzymatic hydrolysis N7 and N11 were detected across four urine samples, consistent with successful hydrolysis of phase II glucuronides.

N9 was likely formed via oxidation on the cyanomethyl of the propionitrile, resulting in a cyanohydrin and subsequent loss of the cyano group. Further reduction of the oxidation then forms the alcohol on the propyl chain (Grogan et al. 1992; Åstrand et al. 2018). Similarly, results were obtained for the metabolism of the cyano-containing synthetic cannabinoid receptor agonists in human hepatocyte incubations and authentic urine samples, albeit subsequent decyanation to carboxylic acid or glucuronidation reactions were not observed for *N*-propionitrile

chlorphine (Åstrand et al. 2018; Norman et al. 2025). This biotransformational pathway potentially leads to the release of cyanide, which is likely detoxified through conversion to thiocyanate to facilitate urinary excretion (Lachowicz et al. 2024).

4.4. *In vitro* and *in vivo* *N*-propionitrile chlorphine phase II metabolites

N2, N3 and N6 both shifted by +192.02 Da were consistent with hydroxylation and glucuronide conjugation. The MS/MS spectra of these metabolites points to the biotransformation occurring at the benzimidazolone. Notably, N6, based on its elution, is likely an N11 phase II glucuronidation metabolite. N2 and N3 had a similar biotransformation as N6. Considering the coelution of N2 and N3, it is likely that *N*-propionitrile chlorphine underwent para, meta and ortho hydroxylations and further glucuronide conjugation. However, under the present analytical conditions, it was not possible to detect the third hydroxylated metabolite. It is plausible that N7 contained both isomers that were not chromatographically resolved. Further experiments with chiral chromatography will be necessary in confirming this assertions. In *in vivo* urine samples, enzymatic hydrolysis did not also yield this third metabolite, which is unsurprising under the present analytical conditions. Nonetheless N3 had markedly increased intensity and improved chromatographic peak shape in urine samples as compared to hepatocyte incubations, confirming it is a distinct metabolite.

Finally, N8 also had the same biotransformation pathway as B10, C10 and F10 with hydroxylations and *O*-methylation occurring at the benzimidazolone unit. Similar to the metabolism of brorphine, chlorphine and fluorphine, the initial hydroxy-methyl metabolite was not detected under this experimental condition. The metabolic fate of *N*-propionitrile chlorphine is depicted in Figure 7.

4.5. Implications for clinical and forensic toxicology testing

As already established, brorphine and other orphine analogues can undergo extensive metabolism thus requiring other markers of consumption (Grafinger et al. 2021). In two urine samples, case#04 and case#05, the intensity of *N*-propionitrile chlorphine was either minimal or completely absent, and thus urinary screening of these parents, depending on the amount consumed and time since exposure, may result in false negative. Given nor-metabolites were prevalent across both *in vitro* and *in vivo* systems and were detected with high chromatographic peak area, we recommend nor-orphine and nor-propionitrile orphine as sub-class biomarkers of consumption of orphine and its analogues. Detecting these nor-metabolites and traces of the parent should be tentative of exposure to orphines. Quantitative measurement can subsequently be performed using highly sensitive, low-resolution instrument such as LC tandem triple quadrupole. For *N*-propionitrile orphine, N7 can be used as blood and urine markers, where hydrolysis of urine is performed and N2 and N6 as urinary markers when enzymatic hydrolysis is not performed. Detecting these metabolites alongside N1 and with or without *N*-propionitrile chlorphine should be indicative of exposure to *N*-propionitrile chlorphine.

Chlorphine formed via *N*-dealkylation of *N*-propionitrile chlorphine was minor in authentic samples, nonetheless their interpretation in clinical and forensic settings, especially in urine samples, needs to be circumspect especially in cases of co-consumption. Additionally, further experiments may be necessary to grasp the nature of the combined activity of chlorphine and other halogenated orphines and their *N*-propionitrile analogues on MOR as well as their potential remedies, such as naloxone treatment in clinical setting.

Furthermore, other major metabolites including nor-metabolites and hydroxylated metabolites may be active as posited by Vandeputte et al. 2024. Although, given that preliminary

data suggests 5,6-dichloro nor-orphine, a likely *N*-dealkyl metabolite of 5,6-dichloro orphine analogues, is inactive, it is a reasonable presumption that these nor-metabolites of bromphine, chlorphine, fluorphine and *N*-propionitrile chlorphine may be inactive as well. Notwithstanding, additional pharmacodynamic experiments will be necessary to confirm this assertion.

5. Conclusion

In total, 11 metabolites were detected for bromphine and chlorphine, 12 for fluorphine and 13 metabolites for *N*-propionitrile chlorphine. The metabolism of the halogenated orphine analogues was substantially similar, whereas *N*-propionitrile chlorphine exhibited a distinct metabolic profile. Fourteen metabolites were detected for *N*-propionitrile chlorphine after LC-HRMS analysis of hepatocytes and authentic blood and urine samples. We recommend nor-metabolites of these novel orphines and their *N*-propionitrile analogues as sub-class consumption markers and should be included, alongside hydroxy and hydroxy-glucuronide metabolites, as urinary screening marker for emerging orphine analogues. Additionally, enzymatic hydrolysis of urine samples should be performed for urine samples suspected of containing *N*-propionitrile chlorphine, where detecting the hydroxylated metabolite N7 is useful indication of consumption, as *N*-propionitrile chlorphine undergo extensive metabolism.

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537

Table Captions

Table 1

Proposed biotransformations, molecular formulas, retention times, accurate molecular masses, mass errors (Δ ppm) and peak areas in both experimental replicates of bromophine metabolites after incubation with human hepatocytes.

Table 2

The metabolic biotransformations of chlorphine metabolites, their molecular formulas, retention times, accurate molecular masses, mass errors (Δ ppm) and peak areas in both experimental replicates after incubation with hepatocytes.

Table 3

Proposed biotransformations, molecular formulas, retention times, accurate molecular masses, mass errors (Δ ppm) and peak areas in both experimental replicates of fluorophine metabolites after incubation with human hepatocytes.

Table 4

Metabolites identified following incubation of *N*-propionitrile chlorphine with human hepatocytes for three hours, their proposed biotransformations, molecular formulas, retention times, accurate molecular masses, mass errors (Δ ppm) and peak areas in both replicates.

Table 5

Proposed metabolic biotransformations, molecular formulas, retention times, accurate molecular masses, and mass errors (Δ ppm) of *N*-propionitrile chlorphine and metabolites found in blood,

non-hydrolyzed and hydrolyzed urine samples. Metabolites with ✓ denotes that they were detected while n.d. means not detected.

Figure Legends

Figure 1

Depiction of the core sub-structure of orphine and emerging analogues in this study.

Figure 2

Chromatographic elution of brorphine, chlorphine, fluorphine and *N*-propionitrile chlorphine after three hour human hepatocytes incubations and UHPLC-QToF-MS analysis using 1 μ L injection volume.

Figure 3

MS/MS fragmentation spectra of brorphine, chlorphine, fluorphine and *N*-propionitrile chlorphine with their predicted fragmentation pattern. FI in [] were either detected with minor intensity or proposed (and not detected).

Figure 4

Extracted ion chromatographs of phase I and II metabolites of (A) brorphine (B) chlorphine (C) chlorphine (D) *N*-propionitrile chlorphine after incubations with pooled human hepatocytes for three hours and 10 μ L injection for UHPLC-QToF-MS analysis.

Figure 5

EIC of *N*-propionitrile chlorphine and its metabolites from case #02 detected in (A) blood (B) non-hydrolyzed urine (C) hydrolyzed urine following 2 h of enzymatic hydrolysis with β -glucuronidase.

Figure 6

Proposed biotransformational pathways of novel halogenated orphine analogues brorphine, chlorphine and fluorphine after *in vitro* metabolism studies. Reactions in [] are likely intermediate steps not detected.

Figure 7

Metabolic fate of *N*-propionitrile chlorphine proposed after *in vitro* human hepatocyte incubations. Biotransformation steps predicted as possible intermediates are in [].

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Author contributions

Conceptualization: PSG, HG; Methodology: PSG; Formal analysis and investigation: PSG;

Writing – original draft: PSG; Writing – review and editing: PSG, RK, HG; Data curation: PSG;

Visualization: PSG; Resources: RK, HG; Funding: HG

Funding Source

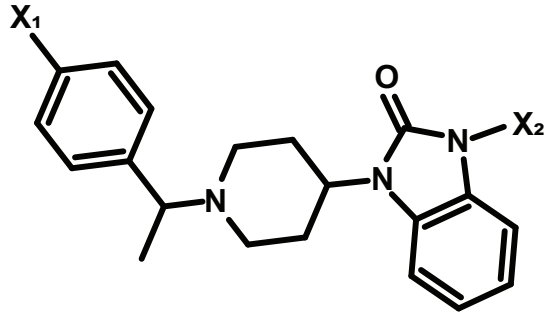
This research was funded by the Eurostars-3 (Eureka Network) Joint Programme by the European Commission [Grant number: E!2297, EUFORiaR (Eurostars-3)].

Data availability

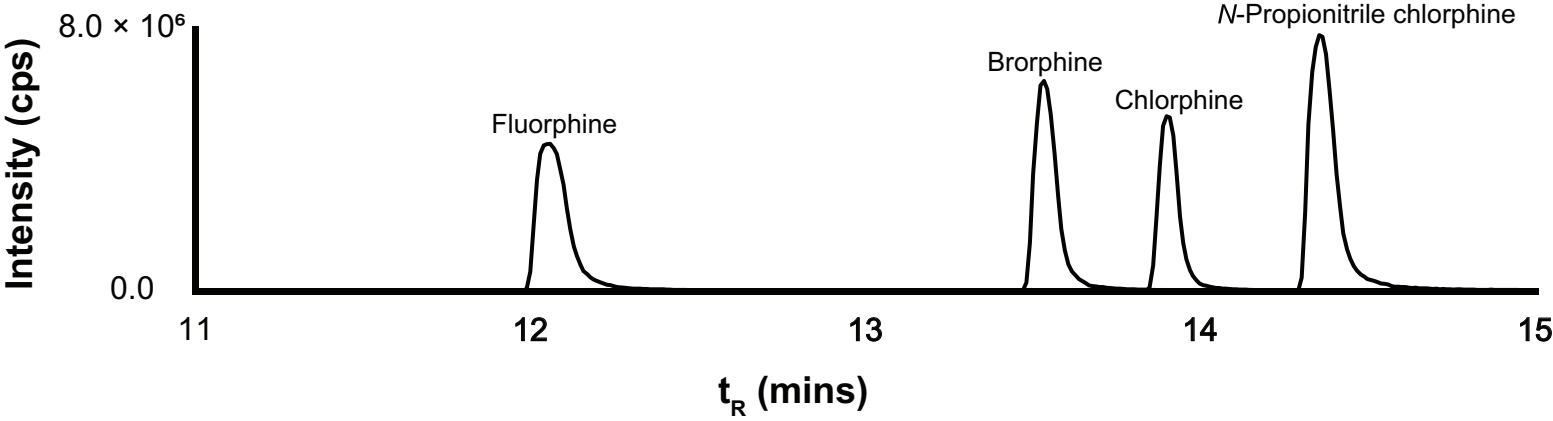
Data in support of the present findings are included in this study or supplementary materials, further inquiries can be directed to the corresponding author.

Declarations

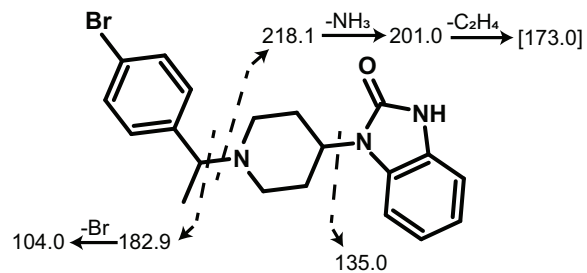
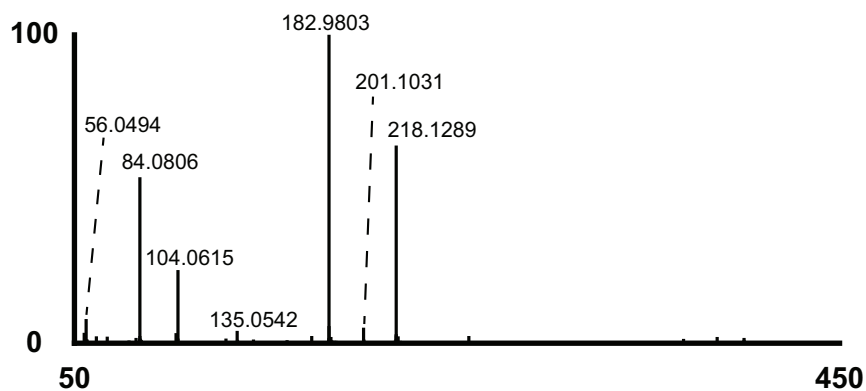
Authors do not have any competing interests to declare.



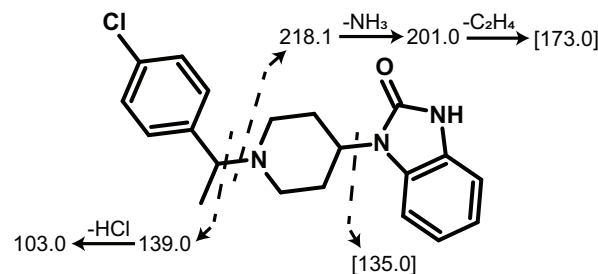
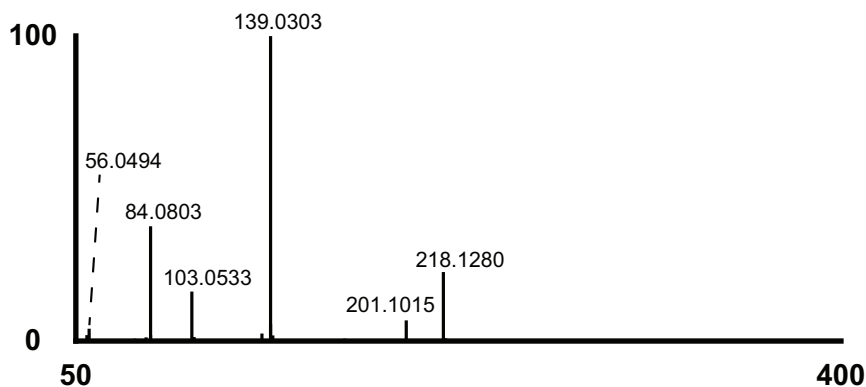
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Chlorphine (C)	Cl	H
Fluorphine (F)	F	H
N-Propionitrile chlorphine (N)	Cl	C ₃ H ₃ N



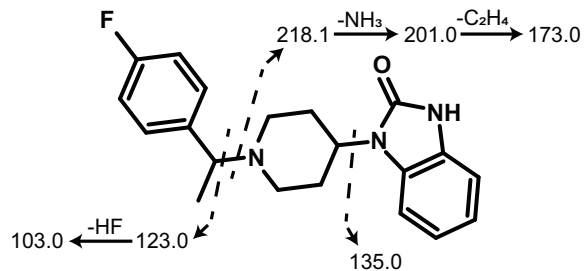
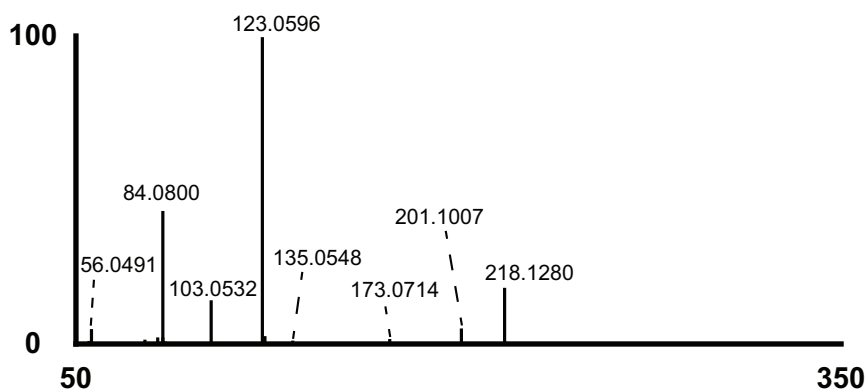
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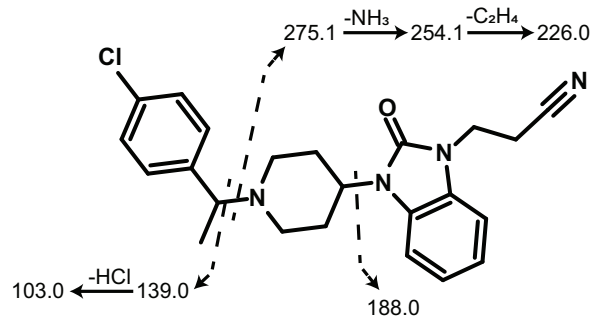
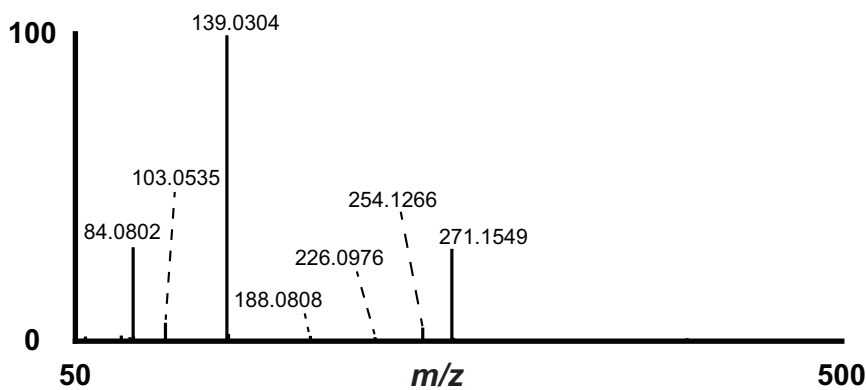
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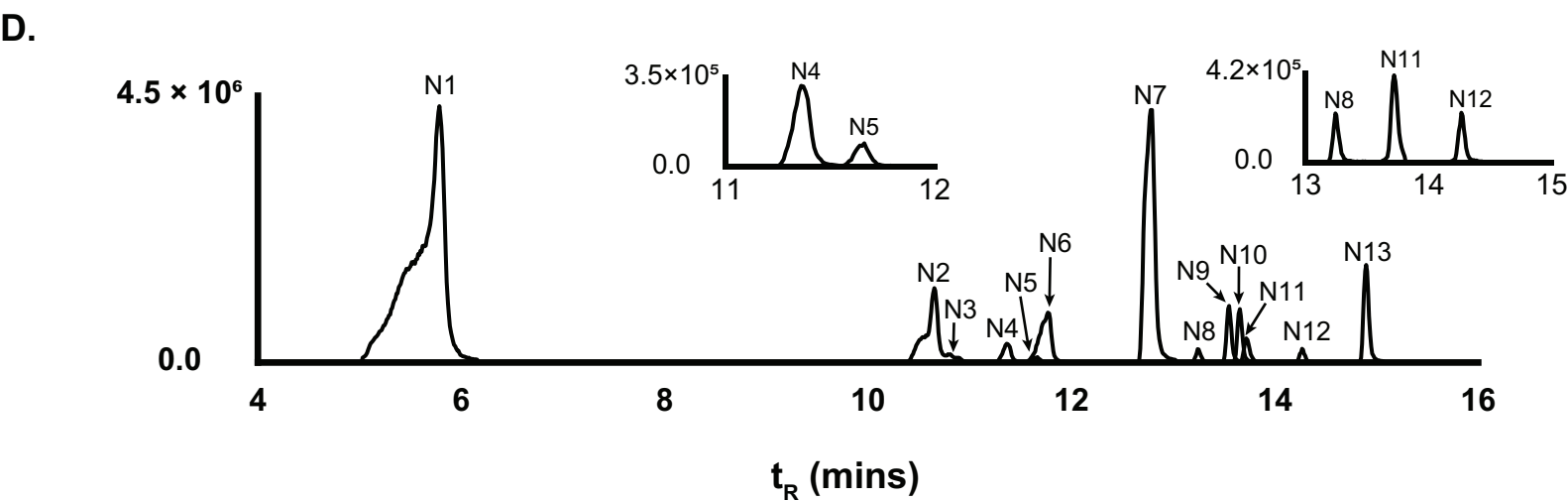
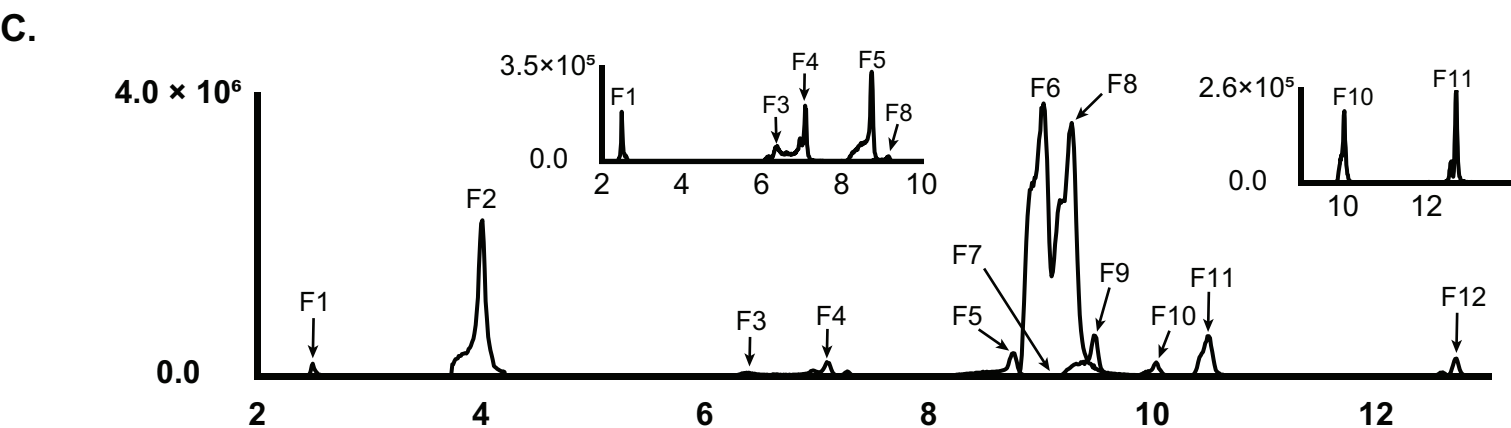
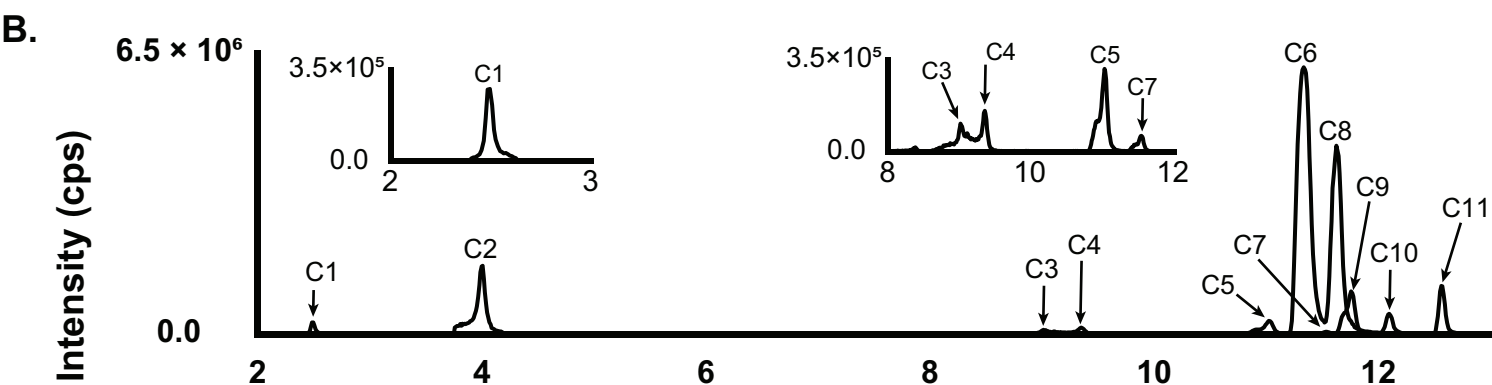
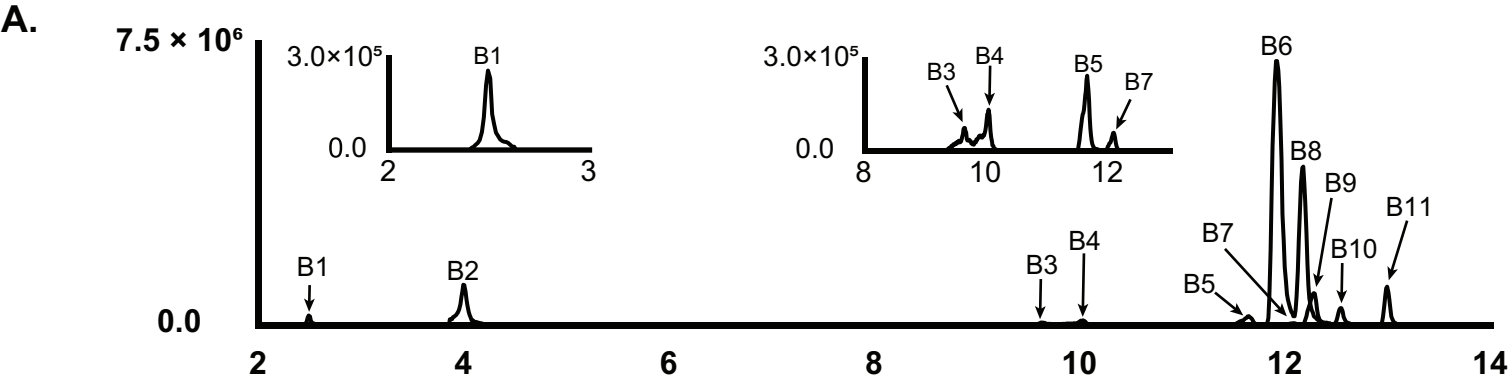


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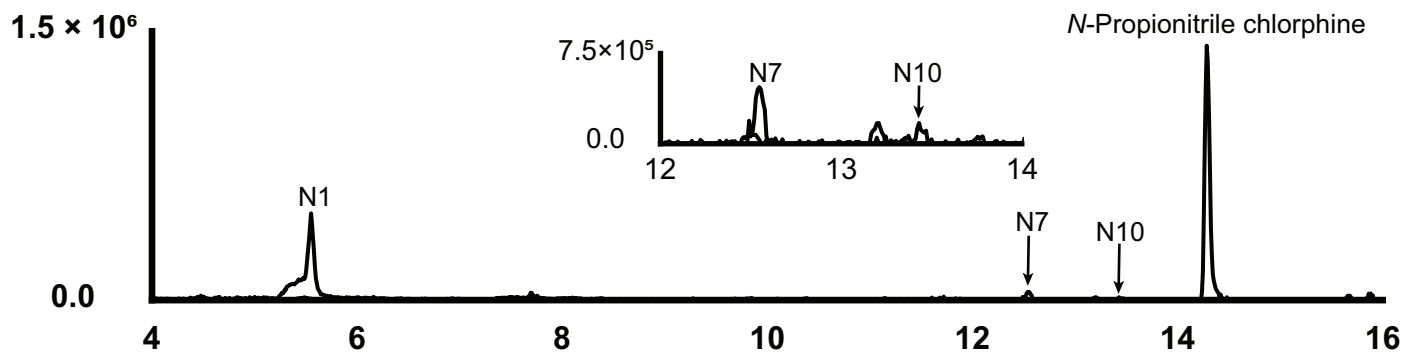


N-Propionitrile chlorphine

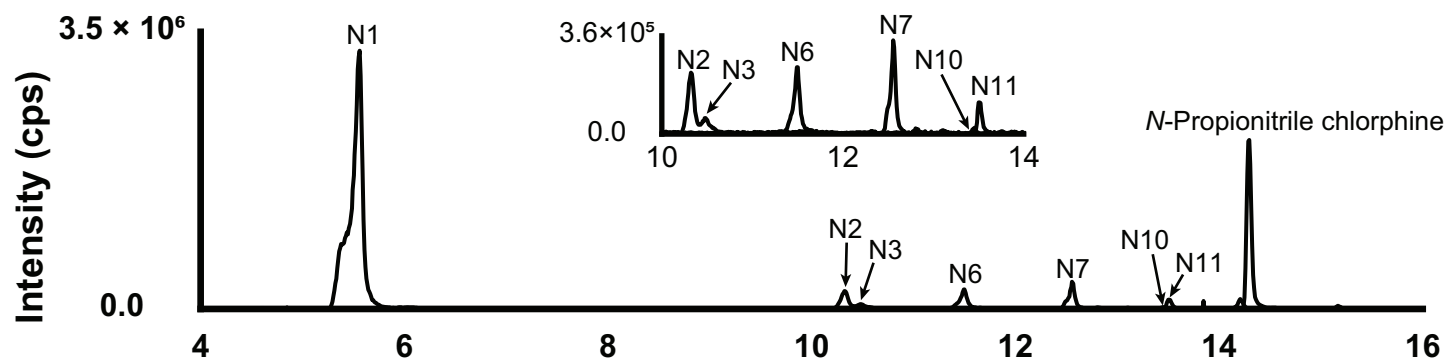




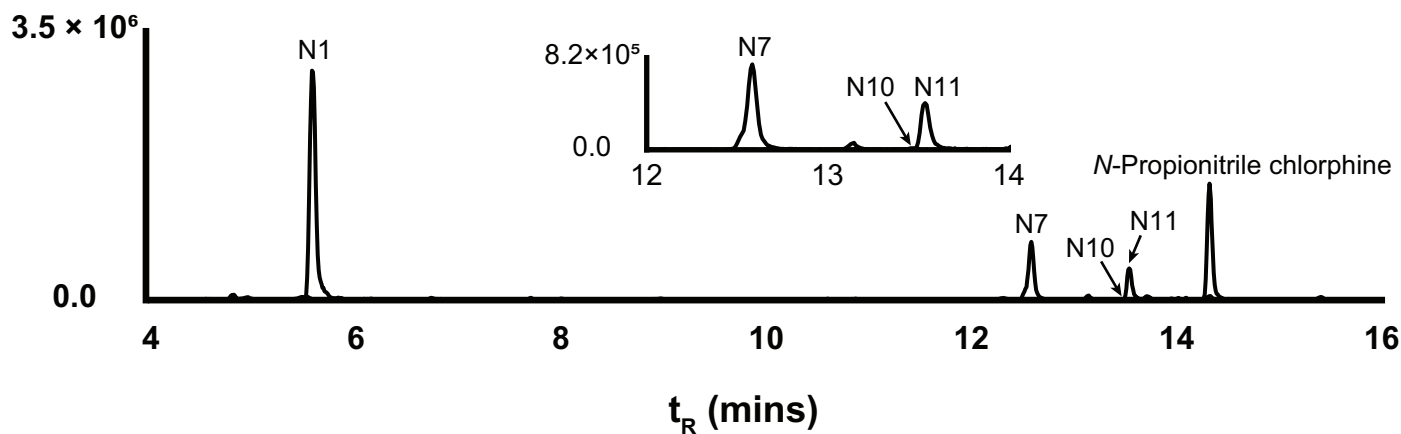
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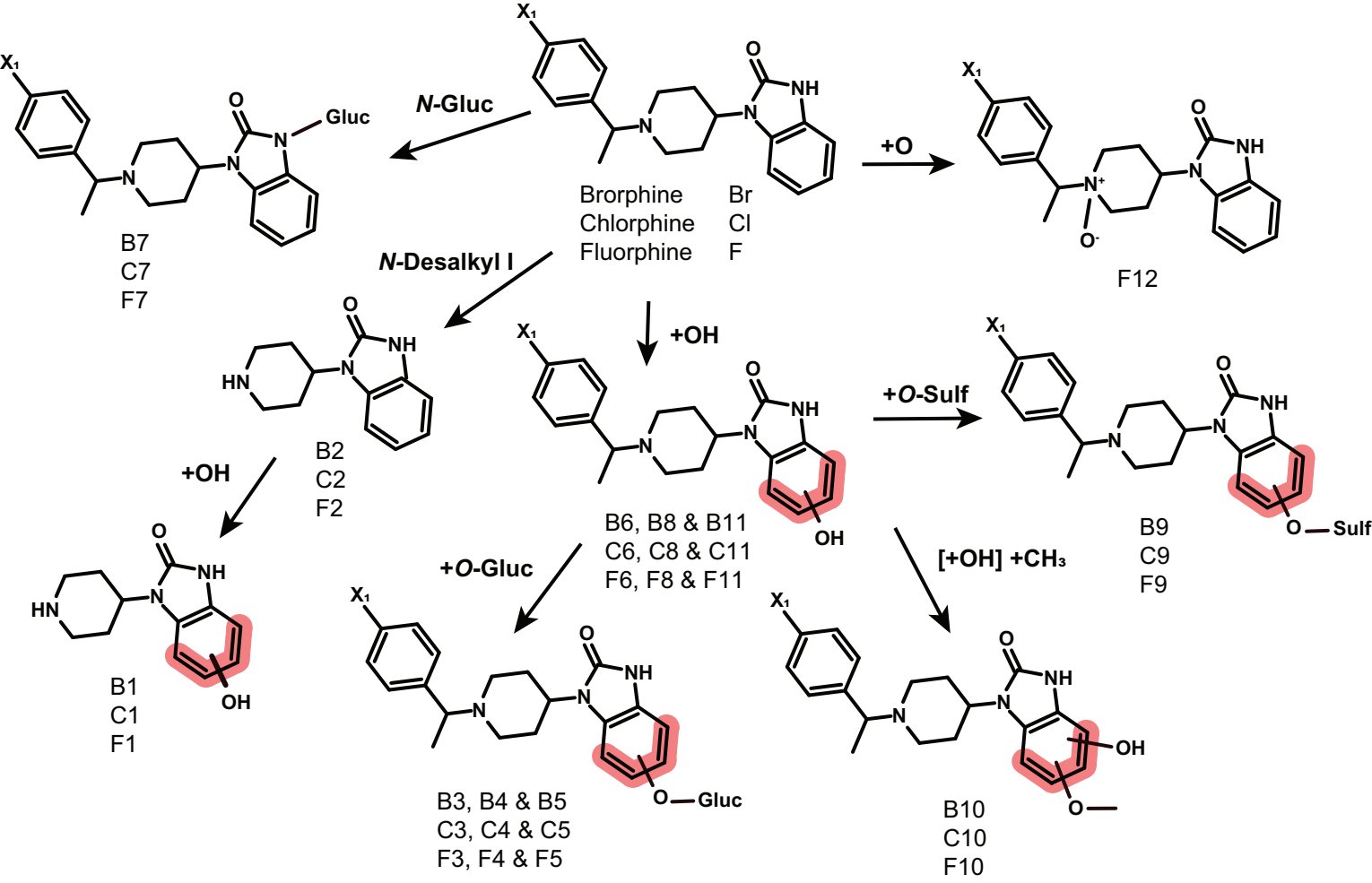


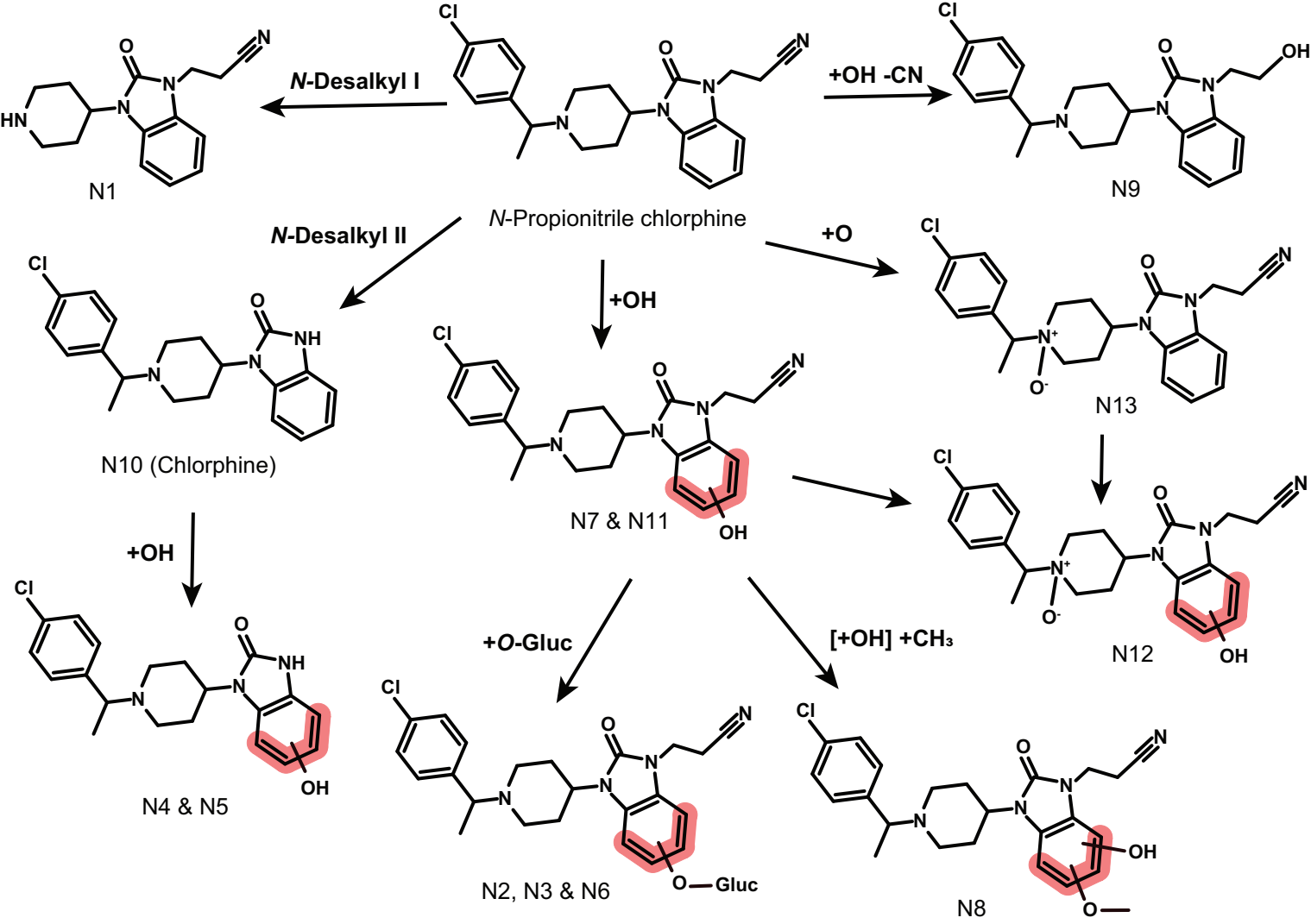
B.



C.







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