

Presumed Phase I Metabolites of N-Propionitrile Chlorphine

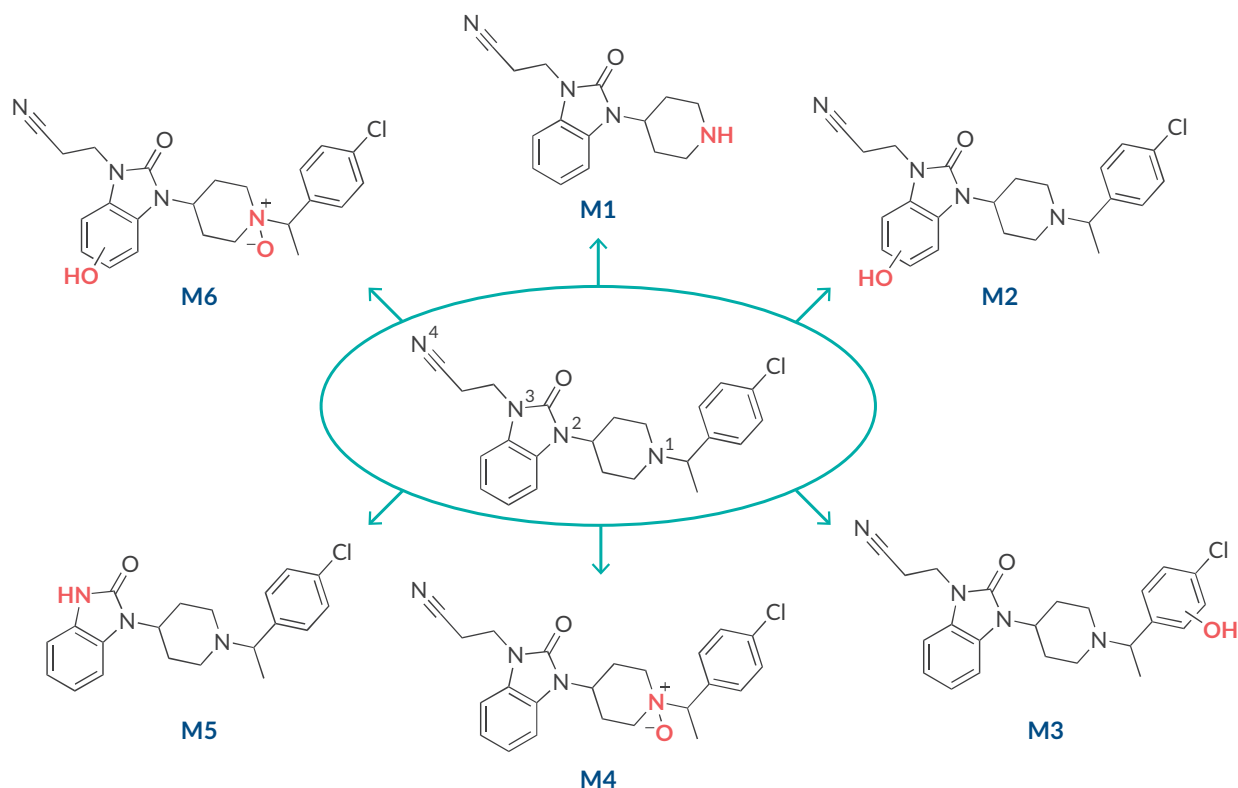


Figure 1. Presumed phase I metabolites of N-propionitrile chlorphine (formed by HLMs), detected using LC-MS/MS. Red text indicates the positions at which metabolic changes occur. Nitrogens are labeled N1-N4 for ease of reference throughout the text.

N-Propionitrile Chlorphine Metabolite Formation & Detection

The parent compound N-propionitrile chlorphine was incubated with HLMs to form phase I metabolites. The structures of the tentatively identified metabolites, as well as the parent compound, can be seen in **Figure 1**. The incubation mixtures were quenched with acetonitrile, centrifuged, and injected into an Ultra Performance Liquid Chromatography (UPLC) system coupled to an Orbitrap mass spectrometer. Potential metabolites were analyzed using high mass resolution positive-ion MS and data-dependent MS/MS. The accurate m/z values, the retention times, and the MS/MS fragmentation data were all used to identify the presumed metabolites formed during HLM incubation. The total-ion chromatogram (TIC) (**Figure 2**) shows three main peaks at retention times 2.77, 3.57, and 3.72 min. However, the latter large peak, dominated by the unreacted parent material, masks the signals of several potential metabolites, which can be revealed by the extracted ion chromatograms (EICs). A total of six potential metabolites were observed – their EICs, including the EIC for the parent compound, are shown in **Figure 3**. The mass spectra associated with the EIC signals display fragmentation similar to the fragmentation of brophrine, reported by Grafinger *et al.*³ The complete list of all m/z values that were extracted are summarized in **Table 1**. Structural information supporting the tentative identification of these metabolites was provided by their MS/MS spectra, as discussed below for each one. None of these signals were observed in blank incubations without the parent compound. For comparison, the MS/MS spectrum of the parent N-propionitrile chlorphine is shown in **Figure 4**.

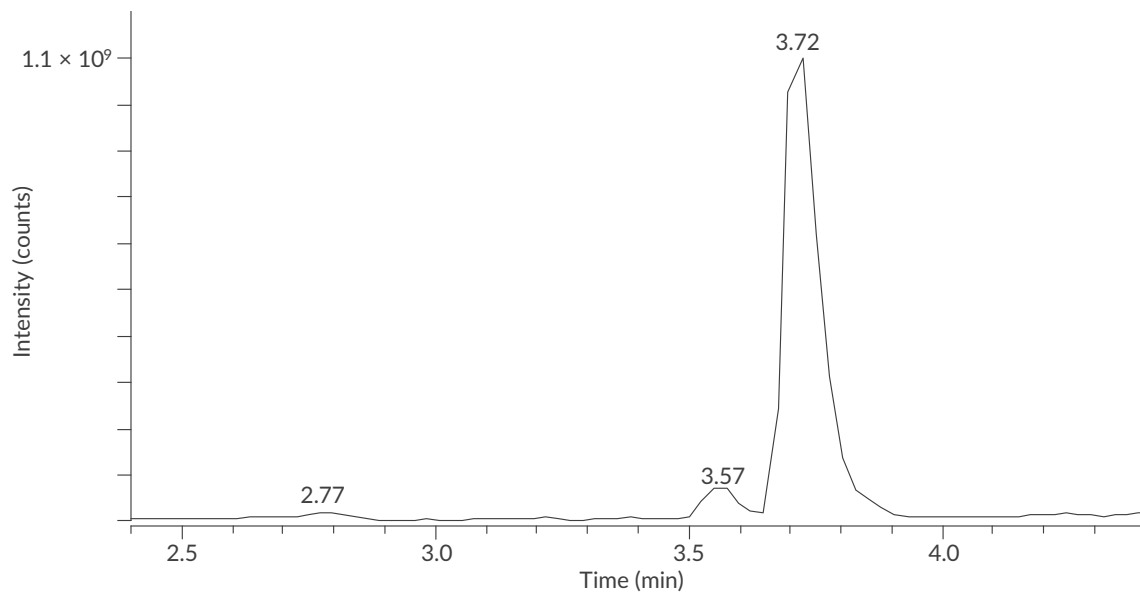


Figure 2. TIC of the products of N-propionitrile chlorphine formed upon HLM incubation.

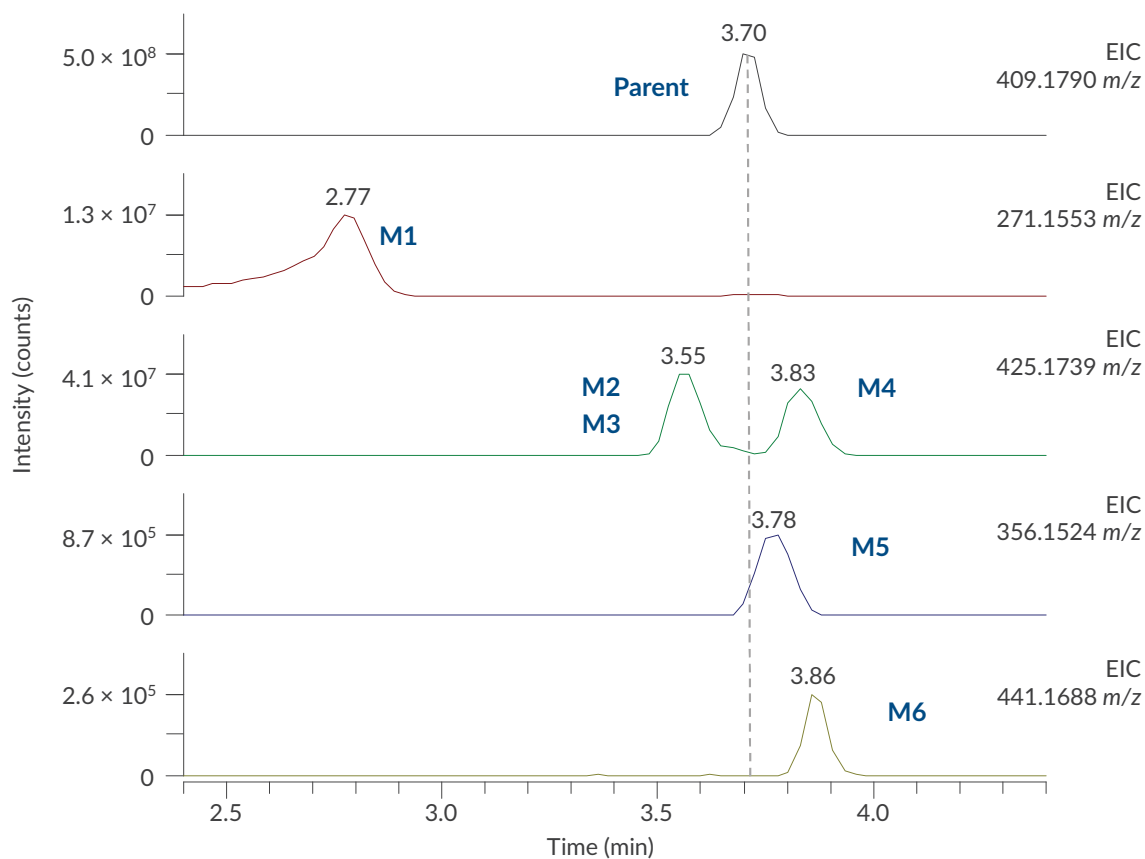
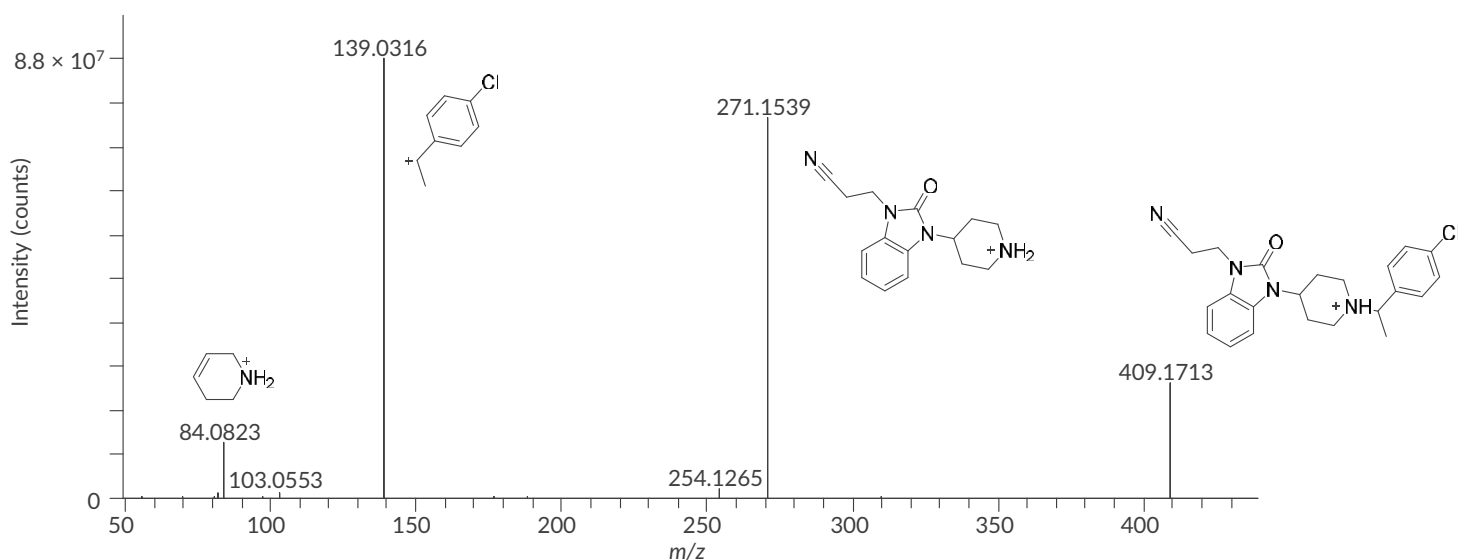


Figure 3. EICs of the potential metabolites of N-propionitrile chlorphine, post-incubation with HLMs. Dashed vertical line has origin at middle point of parent EIC signal.

Table 1. *m/z* values extracted to investigate the presence of potential metabolites of N-propionitrile chlorphine.

Transformation	<i>m/z</i> of [M+H] ⁺	Identification
None	409.1790	Parent
N-Dealkylation from piperidine nitrogen, N1	271.1553	M1
Hydroxylation of benzimidazolone ring	425.1739	M2
Hydroxylation of chlorobenzyl ring	425.1739	M3
N-Oxidation of piperidine nitrogen, N1	425.1739	M4
N-Dealkylation at N3	356.1524	M5 , chlorphine
N-Oxidation of N1 and hydroxylation of benzimidazolone ring	441.1688	M6
Hydroxylation and O-methylation	439.1895	Not found
N-Dealkylation and hydroxylation	287.1503	Not found
N-Dealkylation and two hydroxylations	303.1452	Not found
Nitrile oxidation	442.1892	Not found
Two hydroxylations and O-methylation	455.1844	Not found

**Figure 4.** High-resolution MS/MS spectrum of N-propionitrile chlorphine (*m/z* 409), with proposed fragment structures.

Metabolite M1

The first eluting metabolite at 2.77 min, **M1**, yielded the MS/MS spectrum shown in **Figure 5**. The intact ion peak at *m/z* 271 is consistent with N-dealkylation at the piperidine nitrogen, N1. Although this matches one of the major fragments of the parent compound (**Figure 4**), the much earlier retention time makes it clear that this is not the result of in-source fragmentation; the absence of a peak of *m/z* 409 further supports this. The fragment at *m/z* 84 likely corresponds to the piperidine group; this fragment is also observed in the parent compound spectrum and in the spectrum for bromphine.³

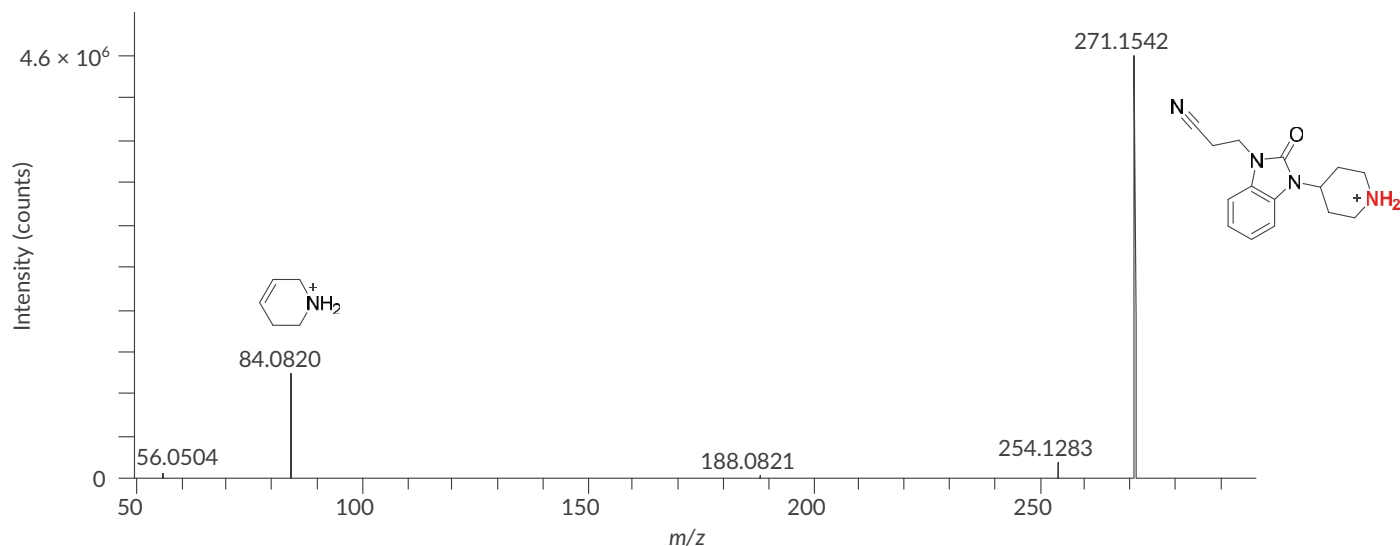


Figure 5. High-resolution MS/MS spectrum of the N-dealkylated metabolite **M1** (m/z 271).

Metabolites M2, M3, & M4

When extracting the chromatogram for m/z 425 (which may indicate the addition of an oxygen functionality such as a hydroxyl group or N-oxide), we detected two peaks eluting at 3.55 min and 3.83 min. The MS/MS spectrum associated with the peak eluting at 3.55 min (**Figure 6**) shows a fragment of m/z 287, which likely indicates hydroxylation on the benzimidazolone ring. This result is similar to what has been reported regarding the metabolism of bromphine.³ The fragment at m/z 139, likely indicative of the cleaved chlorobenzyl group with no attached hydroxyl group, corroborates this. Additionally, this spectrum contains a prominent fragment at m/z 155 which would suggest the addition of a hydroxyl group on the chlorobenzyl group. Thus, we propose that this chromatographic peak contains two co-eluting monohydroxylated metabolites, one containing a hydroxyl group on the benzimidazolone ring (**M2**) and one containing a hydroxyl group on the chlorobenzyl ring (**M3**). The MS/MS spectra do not allow one to determine the actual positions of the hydroxyls on the ring, and this uncertainty is reflected on the displayed structures.

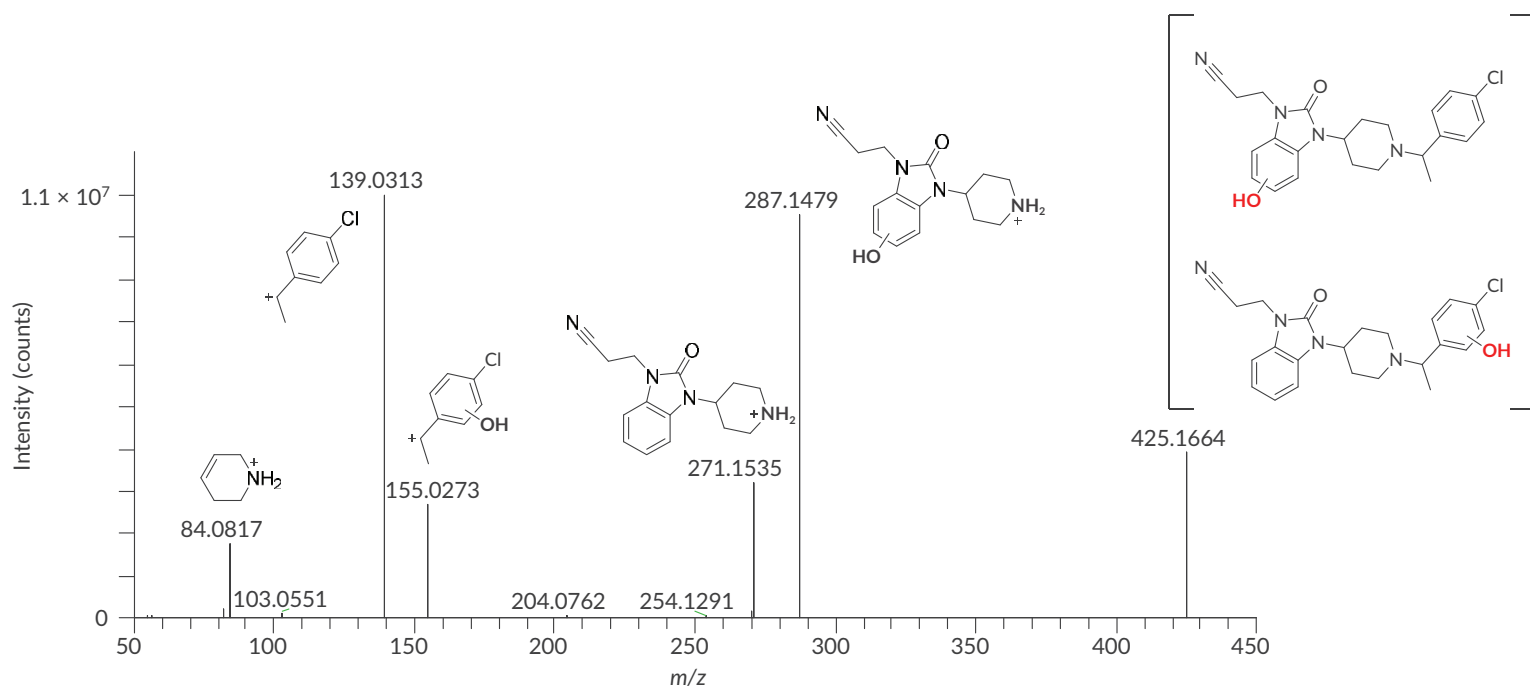


Figure 6. High-resolution MS/MS spectrum of the two proposed hydroxylated metabolites **M2** and **M3** (m/z 425).

The MS/MS spectrum (**Figure 7**) of the metabolite eluting at 3.83 min (**M4**) appears to indicate N-oxide formation. This is suggested by the fragment ions at m/z 82 (loss of H_2O from the piperidine ring nitrogen, N1) and m/z 287 (dealkylation with retained N-hydroxyl at N1). The retention time is also slightly later than that of the parent compound, which would be expected. These observations are consistent with the previously reported bromphine metabolite LC-MS analysis.³

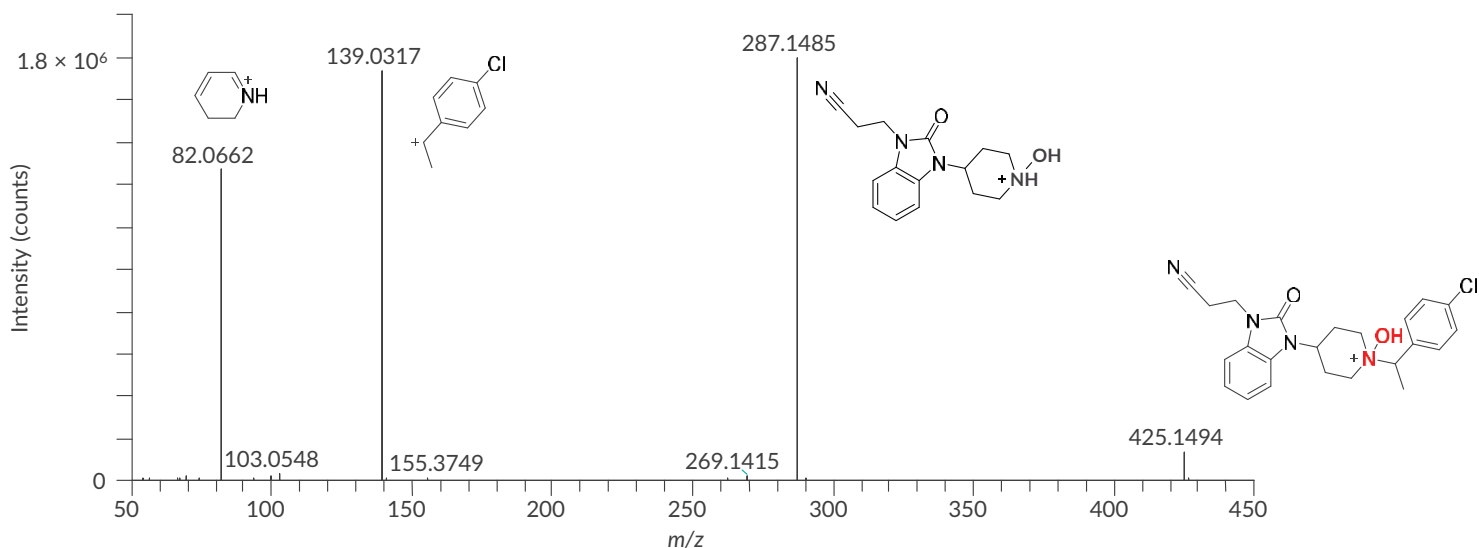


Figure 7. High-resolution MS/MS spectrum of N-oxide metabolite **M4** (m/z 425).

Metabolite M5

The MS/MS spectrum of metabolite **M5**, displayed in **Figure 8**, matches the spectrum of chlorphine, a known synthetic opioid for which a reference standard is available. The m/z of 356 is consistent with dealkylation of the propionitrile moiety at the benzimidazolone nitrogen, N3. Similar to the parent compound, the fragments at m/z 139 and m/z 218 are likely the result of dealkylation at N1, and the piperidine fragment of m/z 84 further supports the proposed structure.

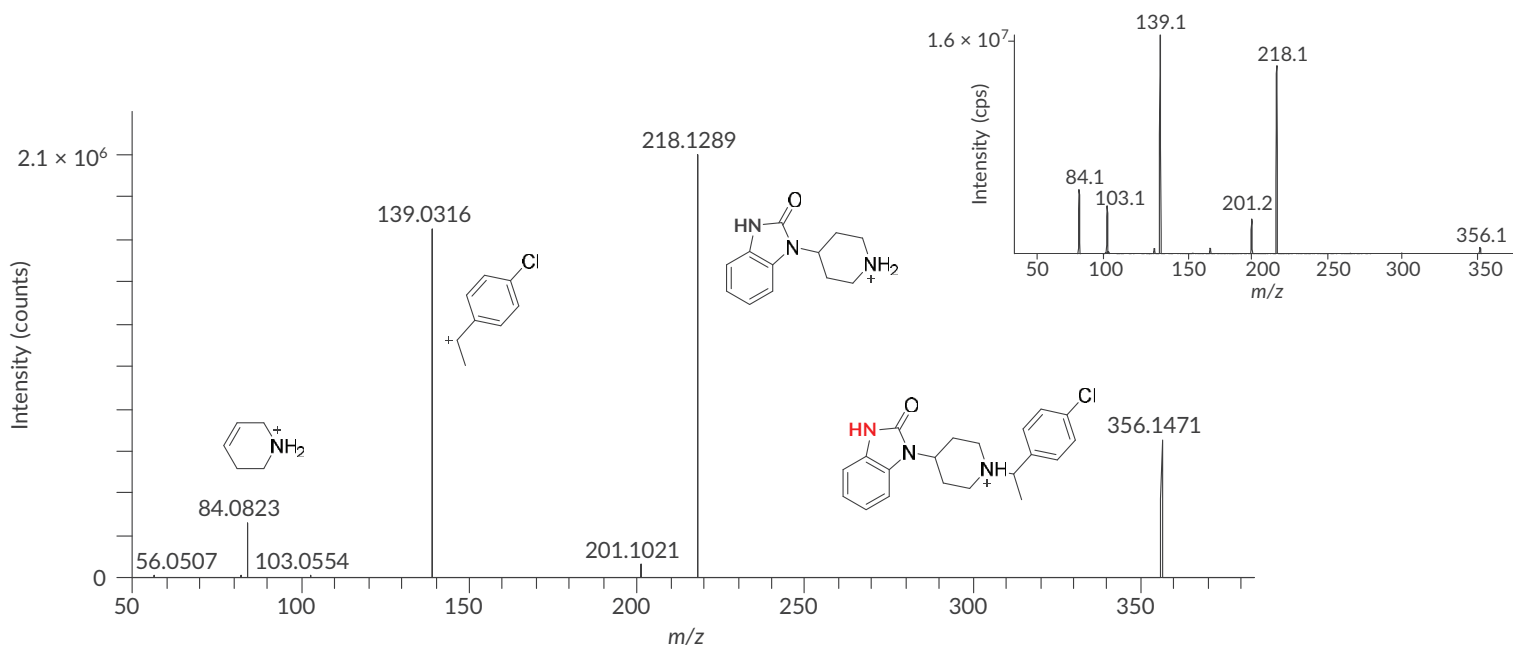


Figure 8. High-resolution MS/MS spectrum of metabolite **M5**, with the proposed fragment structures. The upper right inset shows the low-resolution MS/MS spectrum of an authentic chlorphine standard for comparison.

Metabolite M6

The MS/MS spectrum of metabolite **M6**, at m/z 441, is displayed in **Figure 9**. As shown above for **M4**, the ion at m/z 82 suggests the presence of an N-oxide group at N1, while the pair of fragments at m/z 303 and m/z 285 are consistent with the presence of a hydroxyl group on the benzimidazolone ring.

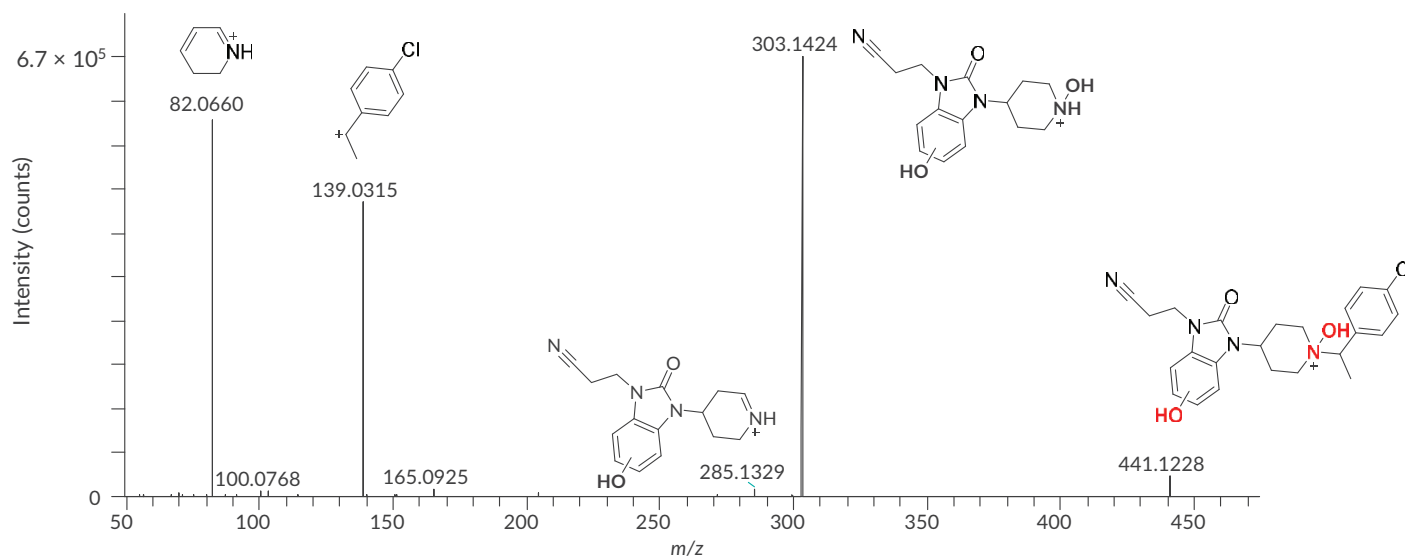


Figure 9. High-resolution MS/MS spectrum of metabolite **M6** (m/z 441), with proposed fragment structures.

Conclusion

Incubation of N-propionitrile chlorphine with human liver microsomes resulted in the formation of six potential phase I metabolites. One of the metabolites, **M5**, is the known opioid chlorphine. For the other metabolites described here, unequivocal structural confirmation will only be possible when authentic standards become available. However, these results may provide aid to forensic toxicologists regarding the interpretation of data resulting from suspected orphine exposure.

Methodology

Following reported experimental procedure, 79.8 μ L of phosphate-buffered saline (pH 7.4), 4.3 μ L superoxide dismutase (200 units/mL in buffer), and 1 μ L drug substrate solution (1 mg/mL in methanol) were added to 1.5 mL Eppendorf tubes containing 5 μ L of HLMs (20 mg/mL protein, previously aliquoted and stored at -80°C).³ The mixtures were vortexed and pre-incubated for 5 min at 37°C . Then, 10 μ L NADPH regeneration system (1.5 mL diluted with 3.5 mL deionized water) was added to achieve the following reaction concentrations: 1 mM NADP, 5 mM glucose-6-phosphate, and 1 unit/mL glucose-6-phosphate dehydrogenase. All samples were vortexed again prior to incubation at 37°C for 60 min. Reaction samples were quenched with 100 μ L ice-cold acetonitrile, then 10 μ L of 10 M ammonium formate solution was added. Samples were vortexed again and then centrifuged at 13,200 rpm for 15 min (room temperature). The organic phases (60 μ L) were removed and transferred to HPLC vials for analysis by LC-MS. These experiments were performed in triplicate.

A blank sample was prepared which contained HLMs, superoxide dismutase, buffer, and NADPH regeneration system without drug substrate. Negative control samples were prepared containing the drug substrate in buffer with superoxide dismutase and NADPH regeneration system. These samples were pre-incubated and incubated alongside the reaction samples and treated with ice-cold acetonitrile and 10 M ammonium formate as described above.

Materials & Instrumentation

Table 2. Reference standards used in this study (Cayman Chemical).

Item No.	Product Name
40326	N-Propionitrile Chlorphine (hydrochloride)
36301	Chlorphine

Table 3. Materials used for *in vitro* metabolism experiments.

Material	Supplier
Pooled Human Liver Microsomes	XenoTech
NADPH Regeneration System	BioIVT
Superoxide Dismutase (bovine erythrocytes)	Sigma Aldrich
PBS	Cayman Chemical

Table 4. Instrumentation used in this study.

Instrument	Supplier
Dionex™ UltiMate™ 3000 UPLC System	Thermo Fisher Scientific
Q Exactive™ Plus Orbitrap MS System	Thermo Fisher Scientific
Acquity BEH C18 50 mm x 2.1 mm x 1.7 μm	Waters
Xcalibur™ Software	Thermo Fisher Scientific

References

1. <https://www.cfsre.org/nps-discovery/monographs/n-propionitrile-chlorphine>
2. Janssen, P.A., Derivatives of benzimidazoliny l piperidine. *Janssen Pharmaceutica N.V.*, **3,318,900** (1967).
3. Grafinger, K.E., Wilde, M., Otte, L., *et al.* Pharmacological and metabolic characterization of the novel synthetic opioid brorphine and its detection in routine casework. *Forensic Sci. Int.*, **327**, 110989, (2021).