Monograph: Issue 5

Cayman Novel Psychoactive Substances Metabolism Monograph

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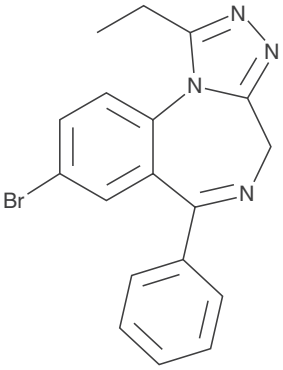
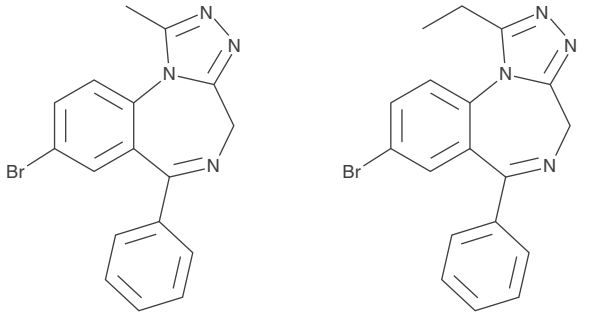
Ethylbromazolam

Item No. 42723 | Novel Designer Benzodiazepine



Ethylbromazolam is a novel psychoactive substance (NPS) and designer benzodiazepine that has been detected in toxicological samples beginning in the summer of 2025 (Center for Forensic Science Research & Education).[1] It is structurally similar to bromazolam, a benzodiazepine first patented in 1976 as a candidate therapeutic that was never approved for medical use.[2] Investigation of the *in vitro* metabolism of bromazolam with pooled human liver microsomes (pHLMs) has been conducted by research groups such as Tang et al.[3] and Wagmann et al.[4]. The goal of this monograph is to identify potential major phase I metabolites of ethylbromazolam using pHLMs. To the best of our knowledge, this is the first report of the *in vitro* metabolism of ethylbromazolam.

The Tang and Wagmann teams each reported the detection of multiple metabolites of bromazolam including hydroxylation at the α-position and the 4-position, among others. As can be seen in **Figure 1**, the chemical structure of ethylbromazolam differs from bromazolam only in the chain length of the alkyl group attached to the triazolo ring system (bromazolam contains a methyl group, while ethylbromazolam contains an ethyl group). It was thus hypothesized that the metabolism of ethylbromazolam would follow similar pathways and yield metabolites similar to those reported for bromazolam. Hydroxylation at the β-position of ethylbromazolam was a possibility we considered, along with other presumptive metabolites such as those resulting from dihydroxylation, dealkylation, or reductive deamination. The structures of these are represented in **Figure 2**.



Bromazolam **Ethylbromazolam**

Figure 1. Chemical structures of bromazolam and ethylbromazolam.

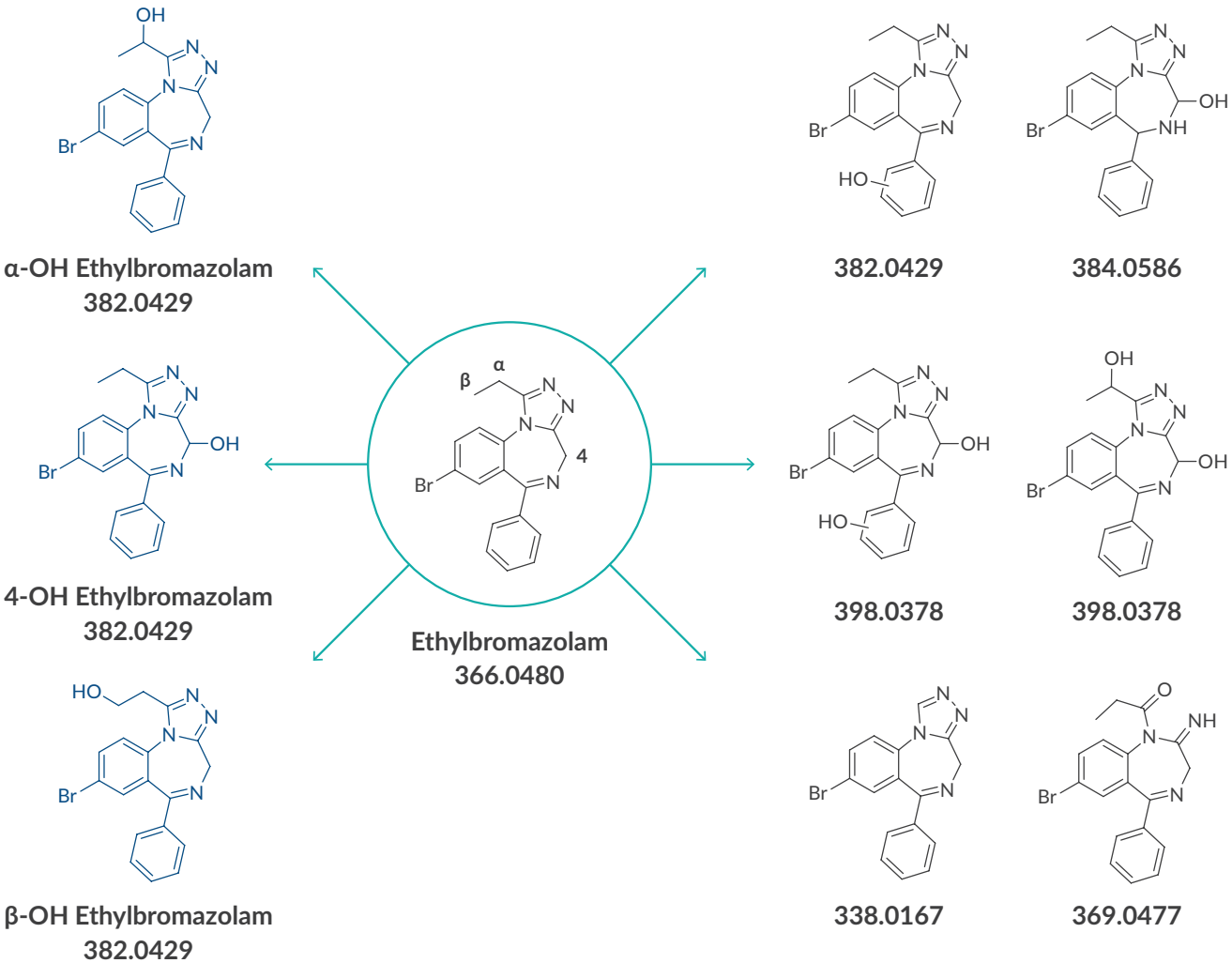


Figure 2. Ethylbromazolam (center) and presumptive metabolites (anticipated major metabolites on the left) along with their corresponding neutral masses.

Results

We used authentic standards of ethylbromazolam and three probable metabolites to determine their chromatography and mass spectrometry behavior. As shown in **Figure 3**, each analyte exhibited unique MS/MS fragments. The α-OH and 4-OH ethylbromazolam isomers were not resolved chromatographically.

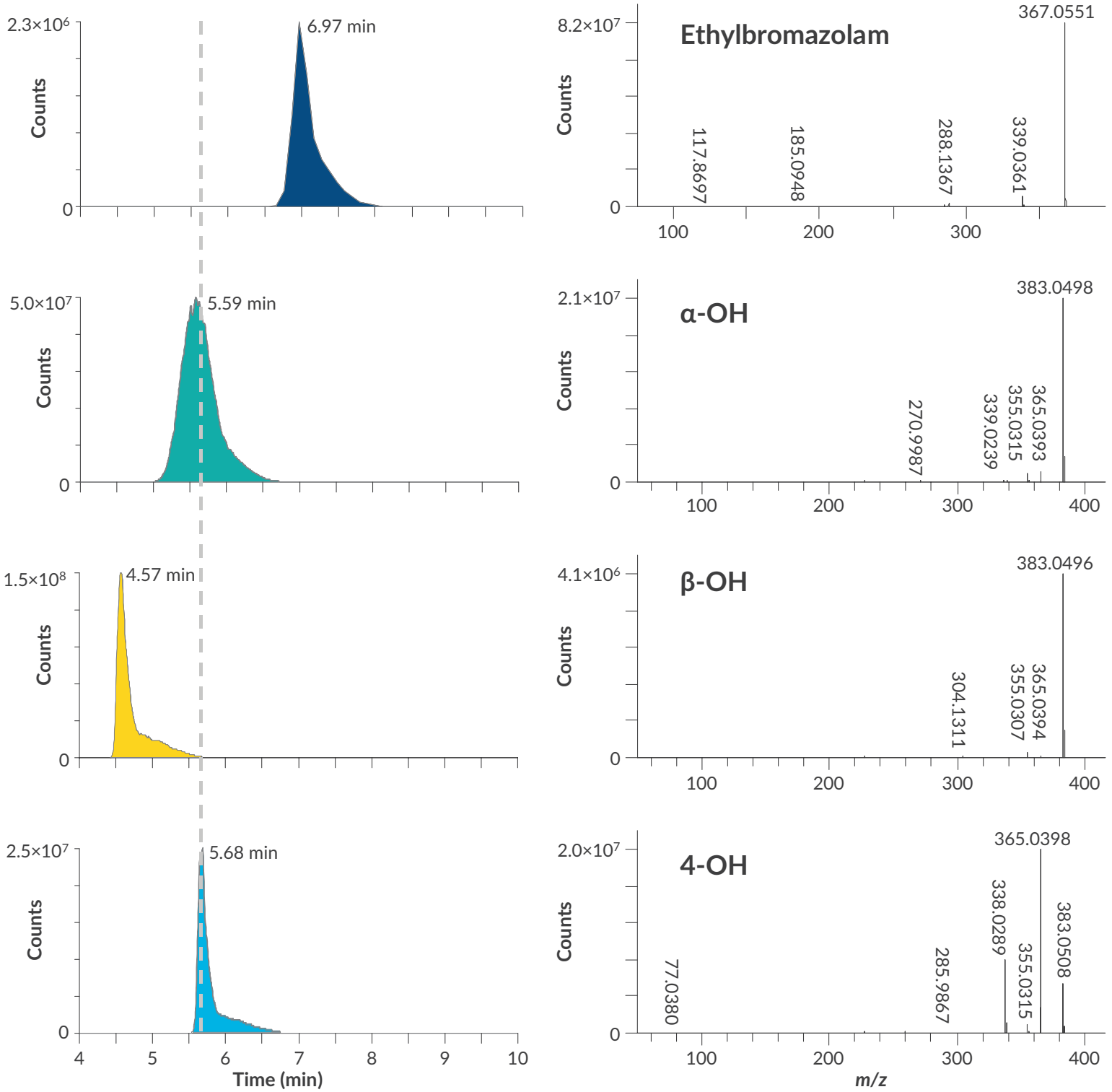


Figure 3. Chromatograms (left panels) and tandem mass spectra (right panels) corresponding to the $[M+H]^+$ ions of reference standards of ethylbromazolam and three hydroxy ethylbromazolam isomers.

Next, selected ion monitoring (SIM) was used to investigate the presence of the analytes shown in **Figure 2** after incubation of ethylbromazolam with pHLMs. As shown in **Figure 4**, only *m/z* signals corresponding to the parent ethylbromazolam and potential hydroxylated metabolites were detected.

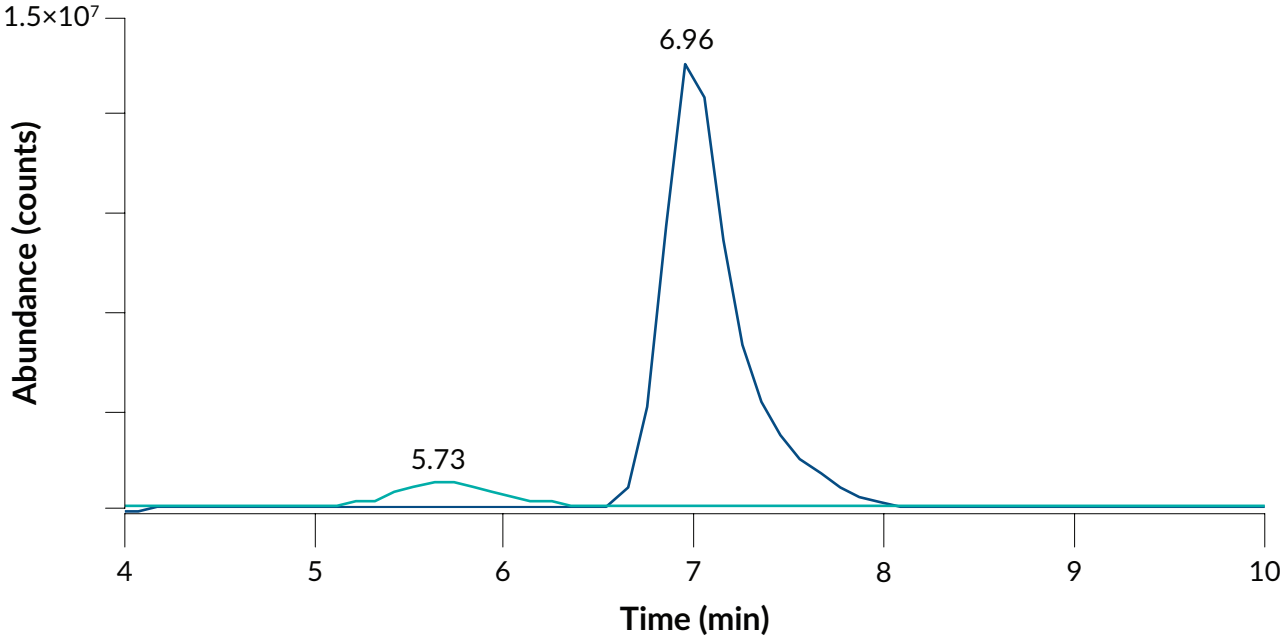


Figure 4. LC-MS chromatogram showing the $[M+H]^+$ ion signals from ethylbromazolam (blue) and hydroxylated ethylbromazolam (teal), resulting from incubation with pHLMs.

Unreacted ethylbromazolam eluted at 6.96 min, whereas a signal with an *m/z* value 16 Da higher, consistent with the addition of one hydroxyl group, was observed as a broad peak between 5.3 min and 6.6 min. To try to further identify this signal, we performed MS/MS across this chromatographic peak, as shown in **Figure 5**.

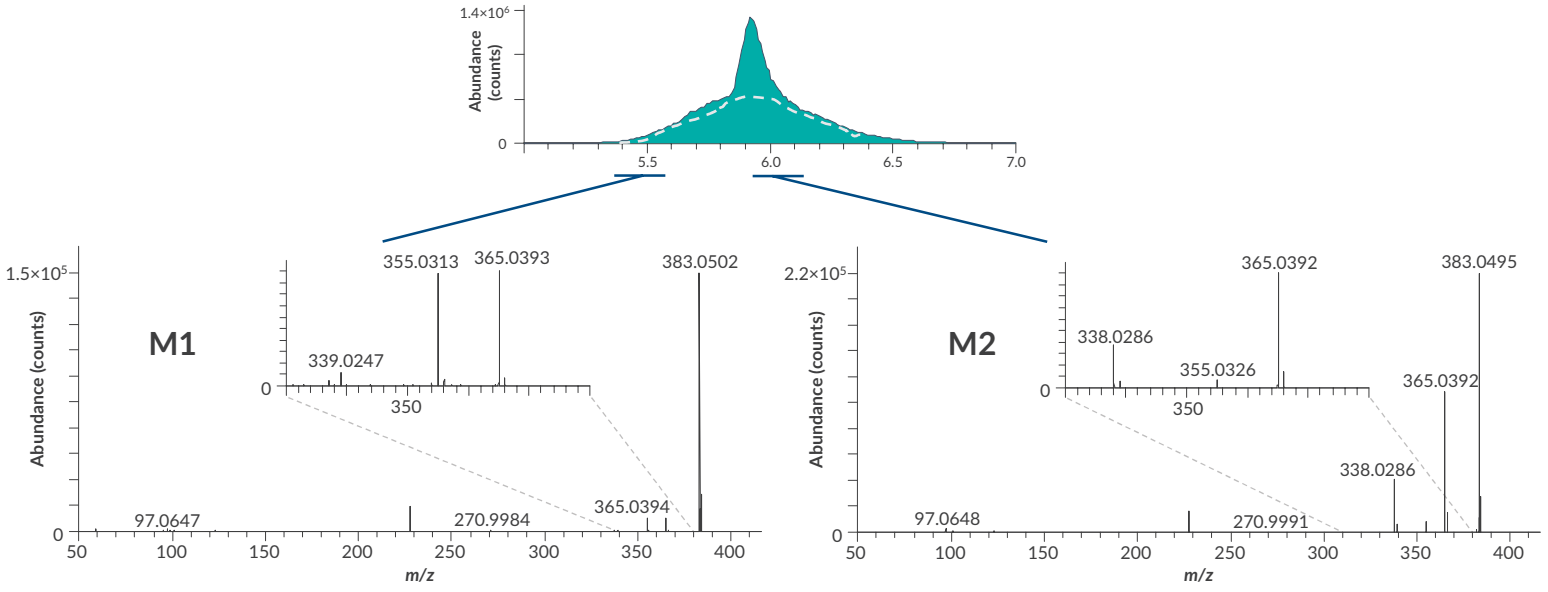


Figure 5. Chromatogram (top) and MS/MS spectra (bottom) corresponding to the signal at *m/z* 383.05 from the *in vitro* metabolism experiment. The white dashed outline in the chromatogram indicates the presence of two coeluting peaks of different shapes.

Analysis of the LC-MS/MS data indicated that the experimental conditions employed produced two confirmed metabolites: α-OH ethylbromazolam (**M1**) and 4-OH ethylbromazolam (**M2**). These metabolites coeluted, exhibiting a profile consistent with two overlapping peaks of differing shapes, similar to the corresponding standards that were prepared synthetically, as shown in **Figure 3**. The MS/MS spectra contain fragment ion *m/z* values particular to each metabolite (355 for α-OH and 338 for 4-OH), confirming the presence of two distinct materials. The structures of these are consistent with those reported by the Tang and Wagmann groups for bromazolam. There is no signal at the retention time of the β-OH standard. Other monohydroxylated species, such as those where a hydroxyl group is attached to the phenyl ring, may also reside under this signal. However, because reference standards are not currently available, their presence cannot be conclusively confirmed or ruled out. None of the other putative metabolites, such as dihydroxylation, were observed. These results are summarized in **Table 1**.

Table 1. Summary of the results of this study regarding the presumptive metabolites of ethylbromazolam that were investigated.

Transformation	*m/z* of $[M+H]^+$	*m/z* of Fragment Ions	Identification
None	367.0553	339.0361, 288.1367	Parent
α-Hydroxylation	383.0502	365.0393, 355.0315, 339.0239	**M1**
4-Hydroxylation	383.0502	365.0398, 338.0289, 355.0315	**M2**
β-Hydroxylation	383.0502	355.0307, 365.0394, 304.1311	Not found
Phenyl-Hydroxylation	383.0502		Uncertain
Dealkylation	339.0240		Not found
Dihydroxylation	399.0451		Not found
Imine Reduction	385.0659		Not found
Oxidative Deamination	370.0550		Not found

Conclusion

Incubation of ethylbromazolam in the presence of pHLMs resulted in the formation of two primary phase I metabolites: α-OH ethylbromazolam and 4-OH ethylbromazolam. These findings were confirmed by the direct comparison of chromatographic retention times and tandem mass spectral data to those of synthesized authentic reference standards. The precursor and fragment ion *m/z* signals may be useful as biomarkers when assessing the presence of ethylbromazolam.

Methodology

To a 1.5 mL Eppendorf tube containing 10 µL pHLMs (20 mg/mL protein) was added 176 µL PBS. Next, 2 µL of ethylbromazolam solution (1 mg/mL in methanol) was added, followed by 12 µL of NADPH regeneration system to reach a final volume of 200 µL. NADPH system parts A and B were used in a 5:1 volume ratio (10 µL A:2 µL B). After incubation for 1 h at 37°C, samples were quenched with 200 µL ice cold acetonitrile and then centrifuged at 13,000 rpm for 5 min. The supernatant was transferred to another tube, dried down under nitrogen at 40°C, and then taken up in mobile phase for analysis by LC-MS/MS.

Materials & Instrumentation

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

Item No.	Product Name
42723	Ethylbromazolam
45236	4-hydroxy (4-OH) Ethylbromazolam
45237	α-hydroxy (α-OH) Ethylbromazolam
45238	β-hydroxy (β-OH) Ethylbromazolam

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

Instrument	Supplier
Pooled Human Liver Microsomes	Xenotech
NADPH Regeneration System	BioIVT
Phosphate Buffered Saline (PBS)	VWR

Table 4. Instrumentation used in this study.

Instrument	Supplier
Vanquish™ UHPLC System	Thermo Fisher Scientific
Orbitrap Exploris™ 240 MS System	Thermo Fisher Scientific
Kinetex C18, 1.7 µm, 100 mm x 2.1 mm	Phenomenex
Xcalibur™ Software	Thermo Fisher Scientific

References

1. CFSRE NPS Discovery – New Drug Monograph. Ethylbromazolam. https://www.cfsre.org/nps-discovery/monographs/ethylbromazolam
2. Critical Review Report: Bromazolam. Expert Committee on Drug Dependence, 45th Meeting, Geneva, 10-14 October 2022. https://cdn.who.int/media/docs/default-source/controlled-substances/45th-ecdd/bromazolam_draft.pdf?sfvrsn=f1bc761e_1
3. Tang Y., Zhao, J., Zhang, M., *et al.*, Characterization of *in vivo* and *in vitro* metabolites of the benzodiazepine drug bromazolam. *Chinese J. Applied Chem.* **41(12)**, 1770-1779 (2024). https://doi.org/10.19894/j.issn.1000-0518.240179
4. Wagmann, L., Manier, S.K., Felske, C., *et al.*, Flubromazolam-derived designer benzodiazepines: Toxicokinetics and analytical toxicology of clobromazolam and bromazolam. *J. Anal. Toxicol.* **45(9)**, 1014-1027 (2021). https://doi.org/10.1093/jat/bkaa161

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