Understanding the Metabolism of Ethylbromazolam Using Human Liver Microsomes

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KEY FINDING

***In vitro* metabolism of Ethylbromazolam coupled with comparison to authentic reference standards resulted in the identification of hydroxylated metabolites.**

INTRODUCTION

Ethylbromazolam is a novel psychoactive substance (NPS) and designer benzodiazepine that has been detected in toxicological samples beginning in the summer of 2025.[1] It is structurally similar to bromazolam, a benzodiazepine first patented in 1976 as a candidate medication that was never approved for use.[2] While *in vivo* investigation into the metabolic products of bromazolam with pooled human liver microsomes (HLMs) has been studied, to the best of our knowledge, this is the first report of the *in vitro* metabolism of ethylbromazolam.

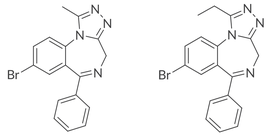


FIGURE 1 – Chemical structures of Bromazolam and Ethylbromazolam.

POTENTIAL PHASE I METABOLITES

Based on the published bromazolam metabolism work of Tang *et al.* and Wagmann *et al.*, we anticipated that hydroxylations at the α-position and the 4-position were likely metabolic outcomes.[3,4] Given the introduction of the ethyl moiety on the triazolo ring of ethylbromazolam (as compared to a methyl group in bromazolam), we postulated that hydroxylation at the β-position could possibly occur as well. Note that all three of these possibilities have the same neutral mass (382.0429).

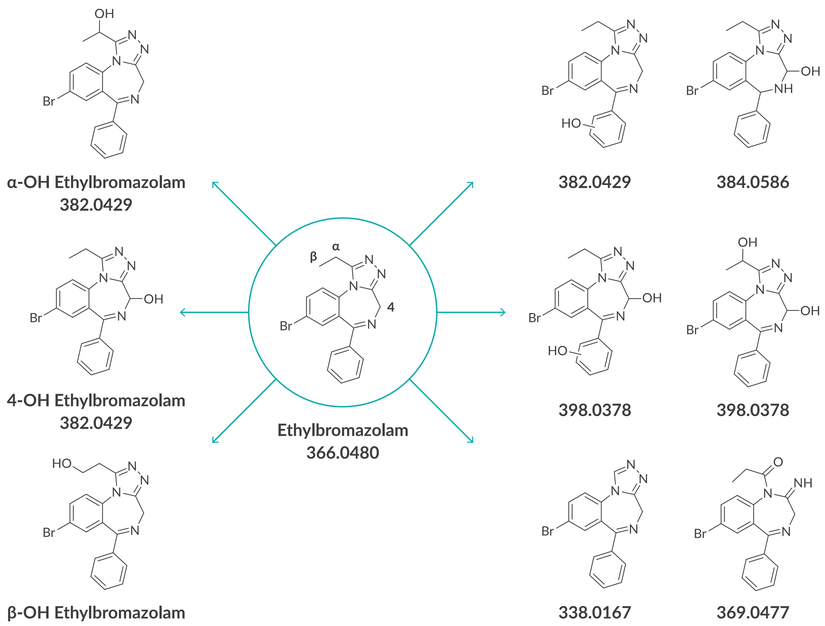


FIGURE 2 – Ethylbromazolam (center) and presumptive metabolites (anticipated major metabolites on the left) along with their corresponding neutral masses.

RESULTS & DISCUSSION

Ethylbromazolam was incubated with pooled HLMs. Metabolites were analyzed using UPLC coupled to the electrospray ionization (ESI) source of an Orbitrap Exploris mass spectrometer scanning in selected ion monitoring (SIM) mode with MS2 for targeted analysis of specific *m/z* of anticipated metabolites. The potential accurate *m/z* values of interest and the corresponding MS/MS spectra were extracted based on anticipated methods of metabolism for other related benzodiazepines.

TABLE 1 – Summary of results.

Transformation	*m/z* of $[M+H]^+$	*m/z* of Fragments	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

Upon analysis of the LC-MS/MS data, the experimental conditions we employed resulted in intact parent ethylbromazolam along with 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 that of the corresponding standards that were prepared synthetically. 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. Other mono-hydroxylated species, such as those where a hydroxyl group is attached to the phenyl ring, may also reside under this signal. But as such standards are not currently available, their presence cannot be conclusively confirmed or discarded. There is no signal at the retention time of the β-OH reference standard. None of the other putative metabolites, such as dihydroxylation, were observed.

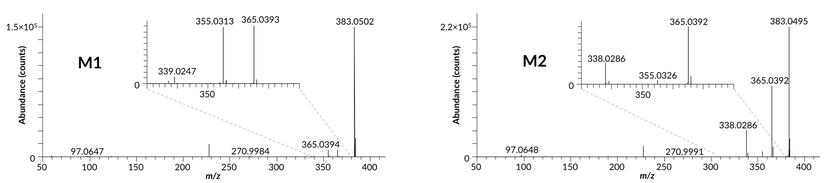
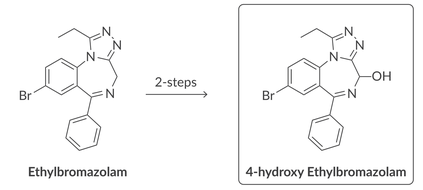


FIGURE 3 – MS/MS spectra of M1 and M2.

Learn more about this study in our NPS Metabolism Monograph

SYNTHESIS OF REFERENCE STANDARDS

Authentic reference standards of presumptive metabolites α-OH ethylbromazolam, 4-OH ethylbromazolam, and β-OH ethylbromazolam were synthesized and used to support our HLM findings. We were able to make 4-OH ethylbromazolam in two steps from our existing ethylbromazolam reference standard while both α-OH ethylbromazolam and β-OH ethylbromazolam were each made in two steps from our existing desalkylgidazepam standard. Note that ethylbromazolam and desalkylgidazepam are individually made after several synthetic steps from commercially available precursor chemicals.



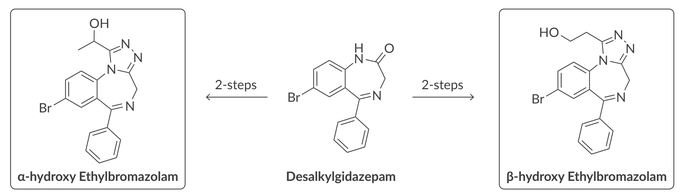


FIGURE 4 – Generic scheme showing the synthesis of metabolite reference standards.

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

CONCLUSIONS

- Ethylbromazolam metabolizes like bromazolam, with mono-hydroxylation occurring at the α-position or the 4-position on the benzodiazepine scaffold yielding isobaric structures sharing a common *m/z* of 383.05.
- Unique fragment ions help differentiate α-OH ethylbromazolam (*m/z* = 355.0307) and 4-hydroxy ethylbromazolam (*m/z* = 338.0289).

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.
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).
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).