Identify, Profile, and Radio-quantitate the Metabolites of [^{14}C]-Nefopam in Male and Female Rats after a Single Oral Administration

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INTRODUCTION

Nefopam hydrochloride is a dual reuptake inhibitor of norepinephrine and serotonin with potential uses in psychiatry and analgesia. Nefopam is extensively metabolized but the complete metabolic profile is unknown. At least one of the three known metabolites of nefopam has been reported to be pharmacologically active. This study characterized the pharmacokinetics, distribution, metabolism, and excretion properties of nefopam in rats following a single oral administration to male and female Wistar Han (WH) and Long-Evans (pigmented) rats. We report here the metabolite identification and profiling in plasma and excreta of WH rats.

MATERIALS AND METHODS

Animal dosing and sample collection: Each rat received a single 20 mg/kg oral dose of [^{14}C]-nefopam (200 µCi/kg, 10 mL/kg) as a solution in sterile water. Urine (at 0-8 h, 8-24 h, and every 24-h interval subsequently) and feces (at 24-h interval) samples were collected up to 168 h post-dose from intact rats (n = 3/sex) while serial plasma samples were collected up to 24 h post-dose from a separate group of jugular vein-cannulated rats (n= 6/sex).

Sample pooling for metabolite profiling and radio-quantitation: Plasma samples were pooled by equal volume at each time point and between 0-8 h across animals of the same gender. Urine and fecal samples were pooled by fixed percentage of weight/volume at each time interval and between the selected time intervals across all animals of the same gender. The pooled samples for metabolite profiling represented greater than 90% of the radioactivity contained in the specified matrix sample

Analytical Sample Preparation: Pooled plasma and fecal homogenates were extracted with acetonitrile/methanol and the supernatants were concentrated under a stream of nitrogen. The residues were reconstituted in water/acetonitrile and the mixtures were centrifuged prior to analysis. The pooled urine samples were centrifuged before analysis.

Metabolite identification and radio-quantitation: The HPLC-MS/RFD system consisted of a HTC PAL autosampler (CTC Switzerland), a Surveyor HPLC pump (Thermal Scientific, MA), an LTQ mass spectrometer (Thermal Scientific, MA) and a β-RAM Model 3 (LabLogic System, FL) radio flow-through detector (RFD). The HPLC eluent was split between the RFD and mass spectrometer with a ratio of 4 to 1. An ARC stop flow analyzer (AIM Research, DE) was also used to measure the low level radioactivity samples. High resolution mass spectra were obtained using an LTQ Orbitrap mass spectrometer (Thermal Scientific, MA).

RESULTS

Following oral administration of [^{14}C]-nefopam to rats, the compound was well absorbed with greater than 97% of the administered dose recovered within 168 h for both genders. Among the radioactivity recovered in the excreta, there was 57.6% in urine and 39.6% in feces for the males and 80.3% in urine and 19.3% in feces for the females.

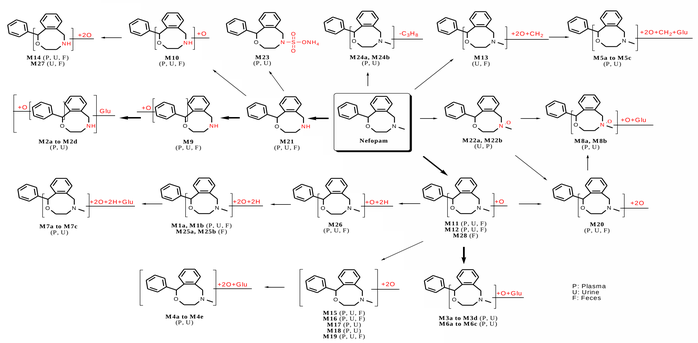
The radioactivity recoveries for extraction and reconstitution process for plasma and fecal homogenates and for urine centrifugation process are listed in Table 1.

Table 1. Average Radioactivity Recovery for Sample Preparation

Radioactivity recovery	Plasma	Feces	Urine
Extraction (%)	96.1 ± 2.9	93.1 ± 0.6	NA
Reconstitution (%)	98.4 ± 2.4	96.7 ± 0.7	NA
Overall (%)	94.6 ± 1.5	90.0 ± 0.8	NA
Centrifugation (%)	NA	NA	100.2

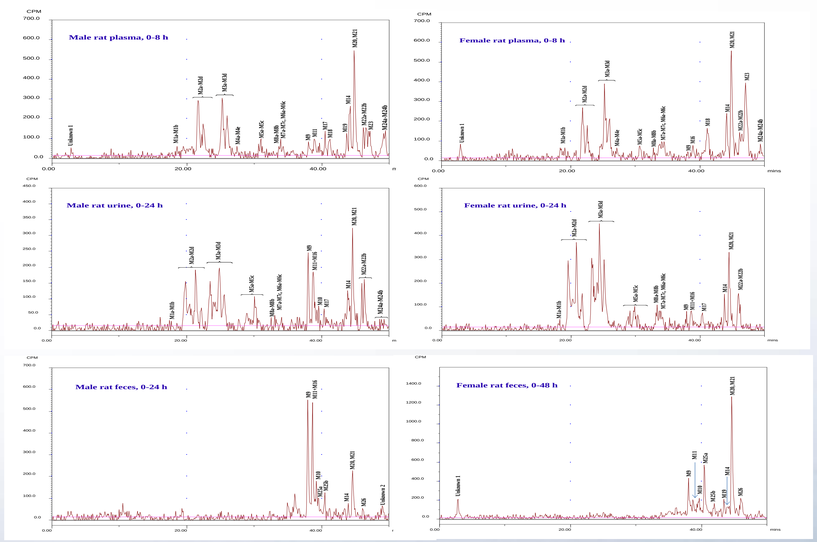
This study found that nefopam is biotransformed in the rat by pathways including *N*-demethylation followed by hydroxylation, sulfation, and glucuronidation of the corresponding hydroxylated products resulting in the formation of M21, M9, M10, M14, M27, M23, and M2a to M2d; hydroxylation of nefopam followed by glucuronidation to yield M11, M12, M15, M16, M17, M18, M19, M20, M25, M26, M28, M1a to M1b, M3a to M3d, M6a to M6c, M7a to M7c, and M4a to M4e; *N*-oxidation followed by hydroxylation and glucuronidation to form M22a to M22b, M20, and M8a to M8b; hydoxylation at the methyl group followed by methylation and glucuronidation generated M13 and M5a to M5c (Figure 1). In addition, an oxidation at the benzoxazocine moiety followed by a loss of C3H8 moiety from nefopam led to the formation of M24a to M24b.

Figure 1. Proposed Structures of Metabolites and Biotransformation Pathways of Nefopam



Metabolite profiles of nefopam in plasma, urine, and feces between male and female rats were qualitatively similar in the corresponding matrices (Figure 2). Nefopam was present at minor or trace levels in plasma and excreta of male and female rats. The major circulating metabolites in male rats were M3a to M3d, M2a to M2d, M20, M24a to M24b, M14, while in female rats the major metabolites were M3a to M3d, M2a to M2d, M23, and M20 (co-elute with M21) (Figures 2). The major urinary metabolites were M3a to M3d, M2a to M2d, and M20 for both genders (Figures 2). The major fecal metabolites included M11 and M9 in male rats, but only M20 in female rats.

Figure 2. Metabolite Profiles of Nefopam in Rat Plasma, Urine, and Feces



The mean combined AUCs of nefopam, M2a to M2d, M3a to M3d, hydroxylated nefopam (M20), *N*-desmethyl nefopam (M21), and nefopam *N*-oxides (M22a and M22b) accounted for approximately 60% of the total radiocarbon in circulation and urine (Table 2).

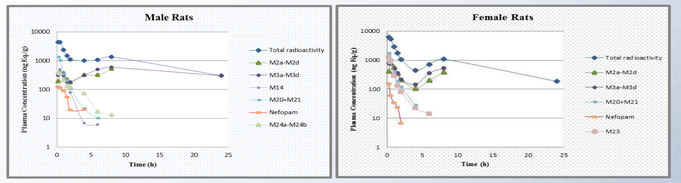
Table 2. Radio-quantitation of [^{14}C]-Nefopam and Its Metabolites in Rats

Metabolite	[M+H]$^+$ (m/z)	Male rats			Female rats		
		Plasma (% Total AUC$_{0\text{-}8h}$)	Urine (% Dose)	Feces (% Dose)	Plasma (% Total AUC$_{0\text{-}8h}$)	Urine (% Dose)	Feces (% Dose)
Unknown 1		1.40	ND	BQL	1.20	ND	0.41
M1a and M1b	288	3.70	0.28	BQL	3.20	0.61	0.17
M2a to M2d	432	**21.60**	**13.74**	ND	**19.90**	**23.39**	ND
M3a to M3d	446	**25.10**	**17.81**	ND	**25.60**	**30.65**	ND
M4a to M4e	462	1.50	1.33	ND	0.40	1.65	ND
M5a to M5c	476	3.80	4.29	ND	2.90	4.24	ND
M6a to M6c, M7a to M7c, M8a to M8c	446, 464, 462	1.80	0.93	ND	**8.20**	2.81	ND
M27	272	ND	BQL	0.28	ND	BQL	BQL
M15	286	BQL	BQL	1.70	BQL	BQL	0.18
M9	256	1.50	2.02	**13.03**	0.30	0.91	1.75
M11 and M16	270, 286	1.50	2.52	**13.33**	1.30	0.91	0.96
M13	300	1.30	BQL	1.72	0.40	0.08	0.18
M10	256	BQL	0.70	1.28	BQL	0.2	1.19
M17	286	2.20	1.04	ND	2.00	1.19	ND
M12	270	0.40	BQL	1.06	BQL	0.18	3.33
M25	288	ND	ND	BQL	ND	ND	0.26
M18	286	1.30	0.19	ND	3.10	0.3	ND
M28	270	ND	ND	0.30	ND	ND	0.72
M19	286	1.00	0.92	BQL	0.50	0.41	0.67
M14	272	5.00	1.98	0.32	3.80	2.2	1.12
M20 and M21	286 and 240	**10.00**	**5.70**	4.76	**9.90**	**5.84**	**6.27**
Nefopam	254	1.60	0.04	BQL	0.80	0.35	0.22
M22a to M22b	270	4.20	3.84	ND	3.70	4.03	ND
M23	337	3.4	BQL	ND	**9.50**	BQL	ND
M26	272	BQL	BQL	BQL	BQL	BQL	1.24
Unknown 2	254	ND	ND	0.60	ND	ND	0.17
M24a to M24b	210	**6.4**	0.18	ND	0.90	0.29	ND
Cumulative results		100	57.61	39.58	100	80.31	19.33

BQL: Below radio quantitation limit but could be detected by LC-MS/MS method. ND: not detected by MS spectrometry method. Values > 5% are shown in bold.

The overall concentration-time profiles of M2a to M2d and M3a to M3d in plasma mirrored that of total radioactivity (Figure 3). The plasma concentrations of nefopam, hydroxylated nefopam (M20) and *N*-desmethyl nefopam (M21) declined faster than total radioactivity. The apparent longer half-life of total radioactivity concentration in plasma may reflect the presence of one or more metabolites with a longer half-life than that of nefopam, M21, or M20. Indeed, the plasma kinetics of the major metabolites M2a to M2d and M3a to M3d were complex and characterized by concentrations approaching that of total radioactivity 6-8 h after dosing.

Figure 3. Plasma Radioactivity Concentration *Versus* Time Profiles



Previously, it was thought that *N*-desmethyl nefopam (M21) and nefopam *N*-oxides (M22a and M22b) were the major metabolites of nefopam. Our study revealed much higher abundance of hitherto unknown metabolites in plasma and urine relative to nefopam and its known metabolites. It would be of interest to characterize the biological activity of these newly identified metabolites in the brain.

CONCLUSIONS

This study demonstrates that the oral dose of nefopam to rats is well absorbed, rapidly and extensively metabolized to numerous metabolites, and excreted more in urine than in feces with some gender differences. Differences were noted in the relative abundance of the individual metabolites between genders. However, the overall metabolism of nefopam in rats is qualitatively similar for both genders.

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