Pharmacokinetics, distribution, metabolism, and excretion of the dual reuptake inhibitor \[^{14}\text{C}\]-nefopam in rats

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Abstract

1. This study examined the pharmacokinetics, distribution, metabolism, and excretion of \[^{14}\text{C}\] nefopam in rats after a single oral administration. Blood, plasma, and excreta were analyzed for total radioactivity, nefopam, and metabolites. Metabolites were profiled and identified. Radioactivity distribution was determined by quantitative whole-body autoradiography.

2. The pharmacokinetic profiles of total radioactivity and nefopam were similar in male and female rats. Radioactivity partitioned approximately equally between plasma and red blood cells. A majority of the radioactivity was excreted in urine within 24 hours and mass balance was achieved within 7 days.

3. Intact nefopam was a minor component in plasma and excreta. Numerous metabolites were identified in plasma and urine generated by multiple pathways including: hydroxylation/oxidation metabolites (M11, M22a and M22b, M16, M20), some of which were further glucuronidated (M6a to M6c, M7a to M7c, M8a and M8b, M3a to M3d); N-demethylation of nefopam to metabolite M21, which additionally undergoes single or multiple hydroxylations or sulfation (M9, M14, M23), with some of the hydroxylated metabolites further glucuronidated (M2a to M2d).

4. Total radioactivity rapidly distributed with highest concentrations found in the urinary bladder, stomach, liver, kidney medulla, small intestine, uveal tract, and kidney cortex without significant accumulation or persistence. Radioactivity reversibly associated with melanin-containing tissues.

Keywords

Disposition, mass balance, metabolite identification, metabolite profiling, Nefopam, radio-labeled

Introduction

Nefopam [(±)-3,4,5,6-tetrahydro-5-methyl-1-phenyl-1H-2,5-benzoxazocine hydrochloride] (Figure 1) is a widely used potent, centrally acting, non-opioid analgesic with potential safety advantages over NSAIDs and opiates. Antinociception mediated by nefopam is likely due to potent inhibition of the synaptosomal reuptake of norepinephrine and serotonin (\(K_i < 50\) nM), and dopamine to lesser degree, resulting in augmentation of the descending pain inhibitory pathway (Hunskaar et al., 1987; Gregori-Puigjané et al., 2012). The involvement of glutamatergic pathways via inhibition of voltage-sensitive sodium and calcium channels has been reported (Verleye et al., 2004; Novelli et al., 2005). With the exception of 5-HT\(_{2C}\) (\(K_i = 56\) nM), nefopam has either weak or no direct affinity for opioidergic, serotoninergic and adrenergic receptors (Girard et al., 2006; Gray et al., 1999; Gregori-Puigjané et al., 2012; Mather et al., 2000). As an inhibitor of serotonin and norepinephrine reuptake (SNRI), nefopam may have therapeutic utility in multiple indications.

SNRIs are used in the therapy of disorders such as major depression, anxiety, and chronic pain, including fibromyalgia and neuropathic pain. The advantages and limitations of current SNRIs have been reviewed (Dell’Osso et al., 2010; Lee & Chen, 2010; Taylor et al., 2013; Thase, 2008). Nefopam inhibits proliferation in cultures of human fibroblasts from aggressive fibromatosis tumors and from hypertrophic cutaneous wounds likely by reducing supra-physiological levels of β-catenin (Poon et al., 2012). Nefopam suppressed the neoplastic phenotype in murine aggressive fibromatosis tumors (Poon et al., 2012).

Early studies in rat, rabbit and human liver S9 preparations suggested that N-desmethyl nefopam was a potential metabolite of nefopam (Bolt et al., 1974). Nefopam was reported to form an intermediate complex with cytochrome P450 (CYP450) in phenobarbital-induced rat liver microsomes in vitro, but not in vivo (Leurs et al., 1987, 1989). Repeated administration of nefopam to rats does not change the glutathione status in liver cytosol or alter CYP450 activities (Leurs et al., 1989). The metabolism of nefopam is not induced and toxicokinetics remained unchanged in a 4-week repeated-dose study of nefopam up to 100 mg/kg in the rat (unpublished results, Impax).
In humans, 87% of a single intravenous (IV) dose of radio-labeled nefopam is excreted in urine within 5 days and 8% in feces (Heel et al., 1980). Less than 5% of a single IV or oral dose is detected as unchanged parent in urine (Chawla et al., 2003). The oral bioavailability of nefopam in humans is low (Aymard et al., 2003). Following an oral dose of nefopam, peak plasma concentrations (C_{max}) are attained 1–2 h after dosing and eliminated with an apparent terminal half-life (t_{1/2}) of 3–8 h. Seven putative metabolites have been reported in humans three of which were identified as N-desmethyl nefopam, nefopam-N-oxide, and its N-glucuronide; most were excreted in urine (Heel et al., 1980). However, the full extent of its biotransformation along with the identity and exposures of metabolites is unknown. The pharmacokinetics, mass balance, tissue distribution, excretion, and in vivo metabolic fate of nefopam in any nonclinical species have not been reported.

The pharmacological activities of N-desmethyl nefopam, nefopam N-oxide, or other metabolites have not been fully characterized. Route-dependent differential effects such as analgesia, sedation and drowsiness after administration of equal IV and oral doses of nefopam in humans imply that the metabolites are biologically active (Aymard et al., 2003; Chawla et al., 2003). The in vitro pharmacological profiles of N-desmethyl nefopam and the diastereomers of nefopam N-oxide [(1R,5R)/(1S,5S), 1R,5S]/(1S,5R)] mirror that of nefopam (unpublished results, Impax). However, studies on the analgesic activities of N-desmethyl nefopam and nefopam N-oxide in rodents have yielded conflicting results (Heel et al., 1980; Kirchherr & Christ, 1984). It is therefore of interest to identify all nefopam-derived metabolites and estimate their systemic exposures in the rat as a species used in pharmacology and safety-toxicology assessments.

The objectives of this study were to (1) characterize the mass balance, total radioactivity, and nefopam in plasma, urine, and feces of male and female rats following a single oral dose of 20 mg/kg [14C]-nefopam administered as the hydrochloride (HCl) salt, (2) identify the elimination routes for nefopam, its metabolites, and total radioactivity, (3) profile and identify the metabolites of nefopam in plasma and excreta, and (4) determine the tissue distribution of nefopam-related material in the pigmented and non-pigmented rat. Gender-related differences in the disposition and metabolism of nefopam were also examined because preliminary studies indicated that female rats had higher exposures and greater sensitivity to the effects of nefopam.

**Materials and methods**

**Chemicals and reference compounds**

GMP-grade nefopam HCl was provided by Impax Laboratories (Hayward, CA). [14C]-Nefopam HCl (Figure 1A) was synthesized by Ricerca Biosciences LLC (Cleveland, OH). The test drug was prepared as a dry blend of both radio-labeled and unlabeled nefopam HCl. The specific activity of the final blend was 58 mCi/mmol with a purity of >99% determined by high-performance liquid chromatography (HPLC) and radio flow-through detection (RFD). On the day of dosing, blended [14C]-nefopam HCl was dissolved in sterile water at a final concentration of 2 mg/mL for dosing by oral gavage. Prior to dosing, the concentration, homogeneity, and radio-purity of the dosing solution was confirmed to be >97% pure with a specific activity of 10.21 μCi/mg of [14C]-nefopam. Reference standard of nefopam was procured from TLC Pharmachem Inc. (Ontario, Canada). Formic acid (FA) and ammonium formate were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Acetonitrile (B & J Brand, carbonyl free, for applications sensitive to trace aldehyde and ketone), water (B & J Brand, for GC, HPLC and spectrophotometry), and methanol (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). All other materials were of HPLC or analytical grade.

**Mass balance and quantitative Whole-body autoradiography (QWBA) study design**

The in-life study design, purpose, dosing and sample collection schedules are summarized in Tables 1 and 2. All animals in Groups 1 and 5 were fitted with an indwelling jugular vein cannula (JVC) to facilitate serial blood sampling. Animals were acclimated in individual suspended stainless steel wire mesh cages for at least 2 days prior to dosing. Food (Certified Rodent Diet #5002, PMI Nutrition International, Brentwood, MO) and water was provided ad libitum, except when fasted overnight before dosing and up to 4 hours after dosing. Animals in Groups 1 and 2 were housed in metabolism cages,
and animals in Groups 3, 4 and 5 were housed in plastic shoebox cages after dosing and throughout the study.

**Blood and plasma sample collection (groups 1, 3, 4 and 5)**

Serial blood samples were collected from the JVC of Group 1 and 5 rats into tubes with K₂EDTA as an anticoagulant. Non-terminal sample volumes were 0.50 mL. Blood was replaced by transfusing 0.40 mL of blank donor rat blood via the JVC after each non-terminal sample collection prior to the 24 h post-dose collection. The blood sampling schedules are shown in Table 2. Aliquots (0.05 mL) of each blood sample were saved for analysis of total radioactivity, and plasma was separated from the remaining blood within 30 min of collection. Plasma was collected for liquid scintillation counting (LSC) analysis and stored at \(-20^\circ C\).

**Excreta sample collection**

Urine samples were collected over dry ice from each animal in Group 2. Each sample was weighed and frozen at \(-70^\circ C\) until analysis. Feces specimens were collected over dry ice and the total weight of each sample was documented, and samples were stored at approximately \(-70^\circ C\) until analysis. Cage residue specimens were collected. Cages were rinsed following daily post-dose excreta collection beginning at 24 h post-dose, total weight of each rinsate and wash specimen recorded, and stored at \(-20^\circ C\). Carcasses of animals in Group 2 were weighed and stored at \(-20^\circ C\) but were not analyzed because >90% of the administered radioactivity was recovered in excreta.

**QWBA sample collection**

One male WH rat (Group 3) each was euthanized immediately after blood collection at 0.5, 4, 24, and 168 h post-dose. One male LE rat (Group 4) each was euthanized immediately after blood collection at 0.5, 1, 4, 8, 24, 72 and 168 h post-dose. One female LE rat (Group 4) each was euthanized immediately after blood collection at 1, 8, 24, 72, and 168 h post-dose. Each rat was deeply anesthetized by isoflurane inhalation, a blood sample was collected by cardiocentesis (approximately 5 mL) in K₂EDTA tubes, and each was euthanized and frozen in a hexane dry ice bath. Aliquots of blood (0.05 g) were stored at 4°C and analyzed for total radioactivity. Residual blood samples were maintained at 4°C and centrifuged to obtain plasma within 30 min of collection. Aliquots (100 μL) of plasma were analyzed for total radioactivity using LSC and residual samples saved at \(-70^\circ C\) for metabolite analysis.

**Analysis of total radioactivity in blood, plasma, urine, and feces**

Triplicate weighed aliquots (approximately 0.100 g) of blood samples were combusted in a Packard Sample Oxidizer (Model 307, Tri-Carb Oxidizer, PerkinElmer, Waltham, MA),
followed by LSC analysis. Feces were homogenized and analyzed for total radioactivity. Weighed feces samples from each rat were homogenized in approximately three volumes of water, total weight of each homogenate determined, and triplicate weighed aliquots (~0.5 g) were combusted in a Packard Sample Oxidizer, followed by LSC analysis. Preweighed portions of each feces homogenate were placed into a Combust-o-Cone® that contained a Combust-o-Pad® (PerkinElmer, Waltham, MA) and each was burned completely in the sample oxidizer. Samples weighing less than 0.5 g were oxidized entirely without homogenization. Residual feces homogenates were maintained at ~70°C after analysis for metabolite analysis. The 14CO2 liberated by combustion was trapped in a solution of Carbo-Sorb® and Permafluor® (PerkinElmer, Waltham, MA) and the radioactivity was determined by LSC. Blank cones that contained a known amount of radioactivity (0.100 mL Spec-Chec® [PerkinElmer, Waltham, MA]) were oxidized and compared to non-combusted standards to determine recovery. Blank cones (without added radioactivity) were burned to evaluate the radioactivity carryover between samples. Plasma, urine, cage rinse, cage wash, and cage wipe extract samples were analyzed by LSC in Ultima Gold scintillation cocktail (10 mL, PerkinElmer, Waltham, MA). Radioactivity (counts per minute) in each sample was converted to disintegrations per minute (DPM) by means of an external standardization and a quench curve. All samples were counted for at least 5 minutes or at least 100 000 counts. Recovery of radioactivity in each sample was expressed as a percentage of the total radioactivity dose. For data values below the limit of quantification (BQL), a value of zero was assigned for calculations of means. Any sample that was less than two times the background DPM was assigned a value of zero.

**QWBA**

One male and one female pigmented rat were euthanized at various times as listed in Table 2 by deep anesthesia via isoflurane inhalation. Each rat was immediately prepared for QWBA by immersion into a freezing chamber containing dry ice and hexanes for at least 15 min. The carcass was embedded, cut into a number of sagittal whole-body sections (40 µm thick, using a Leica CM3600 CryoMacrotome, Nussloch, Germany). Whole-body sections from each rat were mounted on cardboard along with [14C]-blood calibration standards and exposed to phosphor imaging plates (Fuji Biomedical Inc., Stamford, CT) for 4 days at room temperature (RT). Digital phosphor images were acquired using a validated Typhoon 9410 image acquisition system (GE Molecular Dynamics, Sunnyvale, CA) and tissue radioactivity in the whole-body auto-radiograms were quantified by densitometry using an MCID image analysis software (v. 7.0, MCID Image Analysis Software Solutions for Life Sciences, Cambridge, UK). Radioactivity concentrations were expressed as µg equivalents of [14C]-nefopam per gram sample (µg equiv/g). A single lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were based on the low (0.00033720 µCi/g) and high (5.38346310 µCi/g) standards used for the calibration curves, which were each divided by the specific activity of the radioactivity in the formulation used on this study. The LLOQ and ULOQ were determined to be 0.033 and 527.026 µg equiv/g of tissue, respectively.

**Sample collection, preparation, extraction and analysis of metabolites in biological matrices**

Samples collected for metabolite identification, profiling, and radio quantitation

Samples from two groups of WH rats were used for metabolite identification, profiling and radio quantitation: one group of intact rats (n = 3 per gender) and one group of JVC rats (n = 6 per gender). Blood, urine and feces were collected from the groups as shown in Table 2. Blood was centrifuged for 15 min at 4°C, and the resulting plasma was transferred to a new tube. All samples were stored at ~70°C until analysis.

**Biological sample pool and metabolite extraction**

For metabolite profiling, urine samples between 0 and 24 h post-dose for intact rats across all animals of the same gender were pooled proportional to the volumes of urine collected at each time point. The pooled urine accounted for greater than 90% of the excreted radioactivity. For radio quantitation of metabolites, individual samples of urine from the same time interval were pooled across animals of the same gender subgroup. The pooled urine samples were centrifuged (3000 rpm, 10 min), and the supernatants were directly injected onto HPLC column for analysis.

The feces homogenates between 0–24 h post-dose for male intact rats and between 0 to 48 h post-dose for female intact rats were pooled separately, in proportion to the weight of feces homogenates at each time point. The pooled feces homogenates accounted for greater than 90% of the excreted radioactivity. For radio quantitation of metabolites, individual samples of feces homogenates from the same time interval were pooled across animals of the same gender. Aliquots of the pooled feces homogenates (~5 g) were suspended in 15 mL of acetonitrile/methanol (4:1, v/v), vortexed (5 min), sonicated (5 min), and centrifuged at 3000 rpm for 10 min. The pellet was re-suspended in 15 mL water/acetonitrile (4:1, v/v) by vortexing followed by sonication. The process was repeated once. The supernatants from three extractions were combined and evaporated to dryness in a TurboVap LV evaporator at 22°C under a nitrogen stream. The residues were reconstituted in 0.50 mL of water/acetonitrile (4:1, v/v) and were centrifuged. Aliquots of the supernatants were injected onto the LC-MS/RFD system for analysis.

For metabolite profiling, plasma samples from individual time points between 0 to 8 h post-dose were pooled by equal volume across all animals of the same gender. For radio quantitation of metabolites, equal volume of plasma from each of the same time point was pooled across animals of the same gender. Aliquots of the pooled plasma were extracted by three volumes of acetonitrile/methanol (2:1, v/v). The mixture was then centrifuged and the supernatant was transferred to a clean tube. The extraction was repeated two more times. The supernatants from three extractions were combined and
evaporated in a TurboVap LV evaporator at RT under a nitrogen stream. The residues were reconstituted in water/acetonitrile (9:1, v/v) and centrifuged at 3000 rpm for 10 min. Aliquots of the supernatants were injected onto the LC-MS/RFD system for analysis.

**HPLC-MS/RFD system**

The HPLC-MS/RFD system for metabolite profiling and identification consisted of a HTC PAL autosampler (CTC, Switzerland), a Surveyor HPLC pump (Thermo Fisher Scientific, Waltham, MA), an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) and a β-RAM Model 3 (LabLogic, FL) radio flow-through detector (RFD).

Chromatography was performed on a Waters XSELEC HSS T3 C18 column (150 × 4.6 mm) with a mobile phase of water containing a mixture of 10 mM ammonium formate and 0.1% FA (solvent A), and 10% water in acetonitrile containing 10 mM ammonium formate and 0.1% FA (solvent B). The mobile phase was initially composed of solvent A/solvent B (95:5) and held for 5 min, then followed by a linear solvent gradient, running from 5% B to 10% B in 23 min, 10% B to 40% B in 18 min and 40% B to 100% B over the next 2 min, held at 100% B for 6 min, and returned to 5% B over 1 min. The column was allowed to equilibrate at 5% B for 8 min before the next injection. The flow rate was 0.8 mL/min and the separation was achieved at ambient temperature. The effluent from the HPLC column was split, and ~160 μL/min was introduced to mass spectrometer electrospray ion (ESI) source. The remaining effluent was directed to the flow cell of β-RAM RFD.

Quantitation of [14C]-nefopam and its metabolites was carried out by measuring radioactivity in the individual HPLC-separated peaks using β-RAM. The β-RAM was operated in the homogeneous liquid scintillation counting mode with addition of 2 mL/min Ultima-FloTM-M scintillation cocktail. When the radioactivity level in a sample was low, an accurate radioisotope counting system (AIM Research, Hockessin, DE) was used to control externally the HPLC effluent and the scintillation cocktail that directed into the flow cell of β-RAM RFD. Based on the integration of peaks detected on the LC-Radio-chromatograms and the total radioactivity concentration or dose recovered in the corresponding sample, the concentrations of [14C]-nefopam and its metabolites in urine and feces samples were calculated and converted to percent of dose administered to animals. Data measured in plasma samples were reported as ng nefopam equivalent/mL or as μM for metabolites.

Mass spectrometry analyses were conducted using a standard ESI source operating in positive-ionization mode. Source operating conditions were 4.5 kV ion spray voltage, 300°C heated capillary temperature, 20 V capillary voltage, and sheath and auxiliary gas flow at 50 and 20 arbitrary units of the manufacturer, respectively. For LTQ Orbitrap, the full-scan and MS/MS product ion scan mass spectra were acquired at a resolving power of 15 000 (at m/z 400), and the data were centered. The product ion scan activation parameters used an isolation width of 2 Da, normalized collision energy of 35%, and an activation time of 30 ms. The predicted chemical formula and calculated accurate mass were obtained from ChemDraw Std 8.0 (CambridgeSoft Corporation, Cambridge, MA) on the basis of the proposed fragmentation pathways and the putative fragment structures. The data were filtered in Qual Browser (Thermo Fisher Scientific, Waltham, MA) based on accurate mass thresholds. In addition, the Qual Browser chemical formula calculator was used to provide chemical formula and experimental error values for molecular ions and product ions of nefopam and its metabolites. Fragmentations were proposed on the basis of plausible protonation sites, subsequent isomerization, and even electron species, as well as bond saturation. Comparison between the parent and metabolite product ion spectra further aided in the identification of metabolite structures and modification site(s) in the parent molecule.

**Metabolite identification**

Metabolites were identified by LC-MS/Radiochromatogram. First, a product ion mass spectrum of authentic nefopam reference standard was acquired on an LTQ Orbitrap mass spectrometer. Then their major fragmentation patterns were proposed and the elemental compositions of the corresponding fragment ions were confirmed by accurate mass spectra. Second, the retention times of the metabolites observed on LC-Radio-chromatograms were compared to the corresponding retention times on LC-MS chromatogram operating in a full scan positive ionization mode and the molecular ions of the metabolites were determined. Product ion mass spectra were then acquired for the molecular ions of the potential metabolites. Accurate mass spectra were also acquired on a LTQ Orbitrap high-resolution mass spectrometer to confirm the chemical formulas of the proposed molecular ions and their product ions. The plausible fragmentation pathways and the putative metabolite structures were proposed.

**Pharmacokinetic analysis**

Plasma pharmacokinetic (PK) parameters were determined for individual animals in Group 3 using nominal sampling times by standard non-compartmental analysis (NCA) using WinNonlin (v5.2; Pharsight Corporation, Mountain View, CA). Apparent elimination rate constant (k el) was estimated by linear regression of the log-linear plasma concentration-time curve utilizing a minimum of three data points (excluding C max). Apparent terminal half-life (t 1/2) was calculated as 0.693/k el. Area under the concentration time curve (AUC) extrapolated from time t to infinity (AUC 0–∞) was estimated as AUC 0–t + C last/k el, where C last is the last measurable concentration in plasma. Pharmacokinetic parameters were estimated based on the actual dose of nefopam administered to each animal. Samples with radioactivity levels BQL were considered zero for calculation of mean and SD. Tissue concentration data (μg equiv/g) in pigmented rats were used to calculate individual tissue PK parameters using NCA, which included: C max, time of maximal concentration (T max), t 1/2, AUC all [μg equiv.h/g], and AUC 0–∞ [μg equiv.h/g]. Concentrations that were BQL were treated as zero.
Results

Mass balance and excretion

Over 98% of the administered radioactive dose was recovered in excreta within 7 days in both male and female rats. The major route of excretion after oral administration of [14C]-nefopam in male and female rats was in the urine, which accounted for an average of 57.6 and 80.3% of the administered dose, respectively. The time course of excretion of total radioactivity in male and female rats is shown in Figure 2. Most of the radioactivity was recovered in the urine of male and female rats within 24 h of dosing indicating rapid elimination. An average of 39.6 and 19.3% of the administered dose was recovered in feces of male and female rats, respectively. The extent of radioactivity excreted in urine suggests that nefopam is well absorbed.

Pharmacokinetics of total radioactivity in whole blood and plasma

The pharmacokinetic profiles of concentrations of total radioactivity in blood and plasma of male and female WH rats (Group 1) are shown in Figure 3. The time course of unchanged [14C]-nefopam and selected major metabolites in pooled plasma samples WH rats (Group 5) are shown in Supplementary Figure 1.

The $C_{\text{max}}$ of total radioactivity in plasma was noted within 30 min of dosing and was higher in female rats $(6.49 \pm 0.33 \mu g \text{ equiv/mL; mean } \pm \text{ SD})$ than in males $(4.07 \pm 0.65 \mu g \text{ equiv/mL; mean } \pm \text{ SD})$. The $AUC_{\text{last}}$ (mean $\pm$ SD) of total radioactivity in plasma was comparable in males $(20.44 \pm 3.77 \mu g \text{ equiv h/mL})$ and females $(17.53 \pm 1.61 \mu g \text{ equiv h/mL})$. As in plasma, mean $C_{\text{max}}$ of total radioactivity in blood occurred within 30 min was higher in females $(5.32 \pm 0.39 \mu g$
equiv/mL) than in males (3.70 ± 0.70 µg equiv/mL). The AUC_last (mean ± SD) of total radioactivity in blood were similar for male (15.19 ± 2.43 µg equiv h/mL) and female (14.09 ± 0.97 µg equiv h/mL) WH rats. The apparent terminal t1/2 of total radioactivity in plasma and blood could not be estimated reliably within the sampling period of 24 h. The blood to plasma ratio (mean ± SD) was 0.823 ± 0.091 and 0.832 ± 0.051 in male and female rats, respectively, and remained uniform within the sampling period.

Unchanged nefopam was present in plasma at very low levels relative to total drug-derived radioactivity, reached peak concentrations at approximately 15 min post-dose, and tended to decrease exponentially. Plasma concentrations of the major metabolites M2a to M2d and M3a to M3d showed a more complex profile and were sustained through 24 h (Supplementary Figure 1).

Metabolite profiles

Plasma

Plasma samples were pooled by equal volume between 0–8 h across animals of the same gender. The pooled samples represented average 98.3 and 99.0% of the total radioactivity contained in the plasma of male and female rats, respectively. The HPLC radio-chromatograms for plasma pooled from 0–8 h samples in male and female rats are shown in Figure 4. There were no qualitative differences in the circulating metabolite profiles between male and female rats. The relative percentage of metabolites in relation to the total circulating radioactivity in pooled plasma from 0 to 8 h (% AUC_{0–8h} of the plasma total radioactivity) is listed in Table 3. Multiple radioactive peaks were observed in rat plasma. Parent [^{14}C]nefopam was a very-minor radioactive component in the plasma of both male and female rats, accounting for 1.6 and 0.8%, respectively, of the total radioactivity AUC_{0–8h}. Several minor and trace metabolites were also identified in plasma. In plasma of male rats, the major radioactive peaks M3a to M3d, M2a to M2d, M20 (co-eluted with M21), M24a to M24b, and M14 accounted for 25.1, 21.6, 10.0, 6.4, and 5.0%, respectively, of the total radioactivity AUC_{0–8h}. As in the males, several minor and trace metabolites were also identified in plasma.

Urine

Urine samples were pooled between 0 to 24 h post-dose and across all animals of the gender, which represented average 90.7 and 92.8% of the total radioactivity contained in the urine of male and female rats, respectively. The HPLC radio-chromatograms for 0–24 h urinary metabolites of [^{14}C]nefopam in male and female rats are shown in Supplementary Figure 2. There were no qualitative differences in the urinary metabolite profiles between male and female rats. The mean percentages of urinary metabolites in relation to the administered dose excreted from the urine 168 h post dose of male and female rats are presented in Table 3. Multiple radioactive peaks were observed in rat urine. Parent [^{14}C]nefopam accounted for less than 1% of the administered dose in male and female rats. In the urine of male rats, major radioactive peaks M3a to M3d, M2a to M2d, M23, M6a to M8b, and M20 (co-eluted with M21) accounted for 25.6, 19.9, 9.9, 9.5, and 8.2%, respectively, of total radioactivity AUC_{0–8h}. As in males, several minor and trace metabolites were identified in urine.

Feces

Fecal homogenate samples from male rats at 0–24 h and 0–48 h samples from female rats were pooled separately for metabolite profiling, which covered average 90.3 and 94.9%, respectively, of the total radioactivity contained in feces of male and female rats. The HPLC radio-chromatograms for fecal metabolites of [^{14}C]nefopam in male (0–24 h) and female (0–48 h) rats are shown in Supplementary Figure 3. There were no qualitative differences in the fecal metabolite profiles between male and female rats. The mean percentages...
of fecal metabolites in relation to the administered dose excreted from the feces 168 h post dose of male and female rats are presented in Table 3. Intact [14C]nefopam accounted for less than 1% of the administered dose for female rats and was not quantifiable for male rats. In the feces of male rats, major radioactive peaks M11 and M9 accounted for 13.3 and 13.0%, respectively, of the administered dose. The major radioactive peak in feces of female rats was M20 and accounted for 6.3% of the administered dose. As in plasma and urine, several minor or trace metabolites were detected in feces from both male and female rats.

Mass spectra fragmentation of nefopam

Nefopam had a retention time of ~45.0 min on the LC-Radio-chromatogram. The collision-induced dissociation (CID) mass spectrum of the protonated molecular ion of nefopam at m/z 254 resulted in product ions at m/z 181, 179 and 166 as shown in Figure 5. The major product ion at m/z 181 resulted from a loss of C₃H₇NO moiety from the benzoxazocine ring. The ion at m/z 179 resulted from a loss of two hydrogen atoms from the ion of m/z 181. A loss of a CH₃ moiety from the ion of m/z 181 led to the formation of a radical cation of m/z 166. Accurate mass measurement of these ions confirmed their chemical formulas. The proposed fragmentation ions were applied toward the structural elucidation of the metabolites of nefopam.

Metabolite identification

The structures of the metabolites were characterized with respect to mass, chemical formula and fragmentation pattern by liquid chromatography tandem mass spectrometry (LC-MS/MS), and accurate mass measurement techniques. The assignments and proposed structures of metabolites that constituted greater than 8% of the total radioactivity in plasma or excreta along with metabolites M14, M22a and M22b are shown in Table 4.

Metabolites M2a to M2d

Metabolites M2a, M2b, M2c and M2d were detected in rat urine and plasma and eluted around 19.0 ~22.3 min. They all
had molecular ions at \( m/z \) 432 (Figure 6). The extracted-ion chromatogram (XIC) of the molecular ions at \( m/z \) 432 showed four peaks. Since the mass spectra of all the four metabolites were the same, so only those of the M2a are shown in Figure 6. Accurate mass measurement of this ion provided a chemical formula of \( \text{C}_{23}\text{H}_{38}\text{NO}_{8}^+ \), with mass errors from \(-1.5 \) to \(-2.1 \) ppm, suggesting an addition of \( \text{C}_5\text{H}_6\text{O}_7 \) moiety to the parent molecule (Table 4). The product ion spectra of M2a indicated a major ion at \( m/z \) 256, which was formed through a loss of a glucuronic acid moiety. Accurate mass

| Metabolite | \([\text{M}+\text{H}]^+ (m/z)\) | Plasma (0–168 h) | Urine (0–168 h) | Feces (0–168 h) | Plasma (0–168 h) | Urine (0–168 h) | Feces (0–168 h) |
|-----------|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Nefopam   | 254             | 1.6            | 0.04           | BQL            | 0.8            | 0.35           | 0.22           |
| M1a and M1b | 288         | 3.7            | 0.28           | BQL            | 3.2            | 0.61           | 0.17           |
| M2a to M2d | 432           | 21.6           | 13.74          | ND             | 19.9           | 23.39          | ND             |
| M3a to M3d | 446           | 25.1           | 17.81          | ND             | 25.6           | 30.65          | ND             |
| M4a to M4e | 462           | 1.5            | 1.33           | ND             | 0.4            | 1.65           | ND             |
| M5a to M5c | 476           | 3.8            | 4.29           | ND             | 2.9            | 4.24           | ND             |
| M6a to M6c, M7a to M7c, 446, 464, 462 | 1.8 | 0.93 | ND | 8.2 | 2.81 | ND |
| M9        | 256             | 1.5            | 2.02           | 13.03          | 0.3            | 0.91           | 1.75           |
| M10       | 256             | BQL            | 0.7            | 1.28           | BQL            | 0.2            | 1.19           |
| M11 and M16 | 270, 286      | 1.5            | 2.52           | 13.33          | 1.3            | 0.91           | 0.96           |
| M12       | 270             | 0.4            | BQL            | 1.06           | BQL            | 0.18           | 3.33           |
| M13       | 300             | 1.3            | BQL            | 1.72           | 0.4            | 0.08           | 0.18           |
| M14       | 272             | 5              | 1.98           | 0.32           | 3.8            | 2.2            | 1.12           |
| M15       | 286             | BQL            | BQL            | 1.7            | BQL            | BQL            | 0.18           |
| M17       | 286             | 2.2            | 1.04           | ND             | 2              | 1.19           | ND             |
| M18       | 286             | 1.3            | 0.19           | ND             | 3.1            | 0.3            | ND             |
| M19       | 286             | 1              | 0.92           | BQL            | 0.5            | 0.41           | 0.67           |
| M20 and M21 | 286 and 240 | 10 | 5.7 | 4.76 | 9.9 | 5.84 | 6.27 |
| M22a to M22b | 270      | 4.2            | 3.84           | ND             | 3.7            | 4.03           | ND             |
| M23       | 337             | 3.4            | BQL            | ND             | 9.5            | BQL            | ND             |
| M24a to M24b | 210      | 6.4            | 0.18           | ND             | 0.9            | 0.29           | ND             |
| M25       | 288             | ND             | ND             | BQL            | ND             | ND             | 0.26           |
| M26       | 272             | BQL            | BQL            | BQL            | BQL            | BQL            | 1.24           |
| M27       | 272             | ND             | BQL            | 0.28           | ND             | BQL            | BQL            |
| M28       | 270             | ND             | ND             | 0.3            | ND             | ND             | 0.72           |
| Unknown 1 | 1                | 1.4            | ND             | BQL            | 1.2            | ND             | 0.41           |
| Unknown 2 | ND               | ND             | 0.6            | ND             | ND             | ND             | 0.17           |
| 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 LC-MS/MS method.

Table 3. Mass balance of \([^{14}\text{C}]\)-nefopam and its metabolites in rats following a single 20 mg/kg oral dose.

Figure 5. CID product ion mass spectrum of nefopam at \( m/z \) 254 and the proposed fragmentation pathways.
Table 4. LC retention times (Rt), chemical formulae, accurate masses, and postulated structures of nefopam metabolites identified in rats.

| Compound | R<sub>t</sub>* (min) | Chemical formula | Measured mass (Da) | Calculated mass (Da) | Mass error (ppm) | Major fragment ions | Structure and fragmentation |
|----------|----------------------|------------------|-------------------|---------------------|------------------|---------------------|---------------------------|
| Nefopam  | 45.0                 | \([C_{17}H_{20}NO]^+\) | 254.1534          | 254.1539            | -2.2             | 181, 179, 166       | ![Structure and fragmentation of Nefopam] |
| M2a to M2d | 19.0–22.3             | \([C_{22}H_{26}NO_8]^+\) | 432.1644 ~ 432.1646 | 432.1653            | -1.5 ~ 2.1       | 256, 197, 195, 179 | ![Structure and fragmentation of M2a to M2d] |
| M3a to M3d | 22.8–25.8             | \([C_{23}H_{28}NO_8]^+\) | 446.1802          | 446.1809            | -1.6 ~ -1.8      | 270, 197, 195, 179 | ![Structure and fragmentation of M3a to M3d] |
| M6a to M6c | 33.4–34.9             | \([C_{23}H_{30}NO_9]^+\) | 464.1905          | 464.1909            | 1.0              | 270, 197, 195, 167 | ![Structure and fragmentation of M6a to M6c] |
| M7a to M7c | 33.2–34.6             | \([C_{23}H_{30}NO_9]^+\) | 464.1910 ~ 464.1909 | 464.1915            | -1.0 ~ -1.5      | 402, 226, 197, 195 | ![Structure and fragmentation of M7a to M7c] |
| M8a and M8b | 32.5–33.8             | \([C_{23}H_{28}NO_9]^+\) | 462.1752          | 462.1759            | -1.4             | 286, 176, 225, 211, 195, 193 | ![Structure and fragmentation of M8a and M8b] |
| M9       | 37.9                 | \([C_{16}H_{18}NO_2]^+\) | 256.1329          | 256.1332            | -1.3             | 197, 195, 179, 162 | ![Structure and fragmentation of M9] |

(continued)
| Compound | $R_t$ (min) | Chemical formula | Measured mass (Da) | Calculated mass (Da) | Mass error (ppm) | Major fragment ions | Structure and fragmentation |
|----------|-------------|------------------|--------------------|---------------------|-----------------|---------------------|--------------------------|
| M11      | 38.6        | [C$_{11}$H$_{20}$NO$_2$]$^+$ | 270.1486           | 270.1489            | −0.9            | 197, 195, 179      | ![Diagram for M11]       |
| M16      | 38.7        | [C$_{17}$H$_{20}$NO$_3$]$^+$ | 286.1435           | 286.1438            | −1.0            | 176                | ![Diagram for M16]       |
| M14      | 43.8        | [C$_{16}$H$_{18}$NO$_3$]$^+$ | 272.1280           | 272.1281            | −0.6            | 196                | ![Diagram for M14]       |
| M20      | 44.5        | [C$_{17}$H$_{20}$NO$_3$]$^+$ | 286.1433           | 286.1438            | −1.5            | 210                | ![Diagram for M20]       |
| M21      | 44.6        | [C$_{15}$H$_{18}$NO]$^+$    | 240.1380           | 240.1383            | −1.1            | 222, 181, 179      | ![Diagram for M21]       |
| M23      | 47.1        | [C$_{16}$H$_{21}$N$_2$O$_4$S]$^+$ | 337.1215           | 337.1216            | −0.3            | 320, 240, 181, 179 | ![Diagram for M23]       |
| M22a and M22b | 45.8, 46.2 | [C$_{17}$H$_{20}$NO$_2$]$^+$ | 270.1487           | 270.1489            | −0.4            | 252, 197, 195, 181, 179, 176, 169 | ![Diagram for M22a and M22b] |

*LC retention times.
measurement of this product ion confirmed its chemical formula. Further, fragmentation of the ion at m/z 256 yielded ions at m/z 197, 195, 179 and 162. The product ions of m/z 197 resulted from a loss of C₂H₅NO moiety from the ion of m/z 256. The ions at m/z 195 and 179 resulted from a loss of two hydrogen atoms and a H₂O moiety, respectively, from the ion of m/z 197. The ion at m/z 162 was formed through a loss of the phenyl ring from the ion of m/z 256. Mass spectra suggested that the ion at m/z 256 was a hydroxylated nefopam ion. Therefore, M2a was likely a glucuronide of both hydroxylation and demethylation product of nefopam. M2a to M2d were likely diastereomers and/or regioisomers due to the facts that nefopam is a racemic mixture, hydroxylation could be at multiple positions, and there exists the possibility of O- or N-glucuronidation.

Metabolites M3a to M3d

Metabolites M3a, M3b, M3c and M3d were detected in rat urine and plasma and eluted around 22.8 – 25.8 min. The XIC of these metabolites showed four peaks at m/z 446 (Figure 7). Since the mass spectra of all the four metabolites were the same, only those of M3a are shown in Figure 7. Accurate mass measurement of these ions provided a chemical formula of C₂₃H₂₈NO₈⁺, with mass errors from −1.6 to −1.8 ppm, suggesting an addition of C₆H₄O₇ moiety to the parent
molecule (Table 4). CID of the molecular ion of M3a resulted in a major fragment ion at m/z 270, which formed through loss of a glucuronic acid residue from the molecular ion. Accurate mass measurement of these product ions confirmed their chemical formulas. MS³ spectra showed major ions at m/z 197 and 179. The ion at m/z 197 resulted from a loss of C₃H₇NO moiety from the benzoxazocine ring. The ion at 179 resulted from a loss of two hydrogen atoms from the ion of m/z 197. Mass spectra suggested that the ion at m/z 270 was a hydroxylated-nefopam ion. Therefore, M3a was likely a glucuronide of the hydroxylated nefopam. M3a to M3d were likely diastereomers and/or regioisomers.

Metabolite M21

Metabolite M21 was detected in rat plasma, urine, and feces and eluted at 44.6 min. The XIC of this metabolite showed a molecular ion at m/z 240 (Figure 8). Accurate mass measurement of this ion provided a chemical formula of C₁₆H₁₈N₂O₇, with a mass error of −1.1 ppm, suggesting a loss of CH₂ moiety from the parent molecule (Table 4). CID of the molecular ion resulted in major ions at m/z 181 and m/z 179. The ion at m/z 181 resulted from a loss of C₃H₇NO moiety from the benzoxazocine ring. The ion at m/z 179 was formed through a loss of two hydrogen atoms from the ion of m/z 181.
The mass spectra of M21 suggested that it was N-desmethyl nefopam.

Metabolites M22a and M22b
Metabolites M22a and M22b were identified in rat urine and plasma and eluted at 45.8 and 46.2 min, respectively. The XIC of these metabolites showed two peaks at m/z 270 (Supplementary Figure 4). Accurate mass measurement of these ions provided a chemical formula of C_{17}H_{20}NO_{2}^{+}, with mass error of -0.4 ppm, suggesting an addition of O moiety to the parent molecule (Table 4). CID of these molecular ions resulted in major ions at m/z 195, 181, 179 and m/z 176. The ions at m/z 195 and 181 were formed through a loss of C_{3}H_{9}NO and C_{3}H_{7}NO_{2} moiety, respectively, from the molecular ion. The ion at m/z 176 was formed through the loss of the phenyl group and a H_{2}O moiety from the molecular ion. The mass spectra of M22a and M22b suggested they were N-oxide diastereomers of nefopam.

Metabolites M6a to M6c
Metabolites M6a to M6c were identified in rat plasma and urine and eluted around 33.5 ~ 35.0 min. The XIC of these metabolites showed three peaks at m/z 446 (Supplementary Figure 5). Since the mass spectra of all the three metabolites were the same only those of M6b are shown in Supplementary Figure 5. Accurate mass measurement of these ions provided a chemical formula of C_{23}H_{28}NO_{8}^{+}, with mass error from -1.0 to -1.5 ppm, suggesting an addition of C_{6}H_{10}O_{8} moiety to the parent molecule (Table 3). CID of these molecular ions resulted in ions at m/z 402, suggesting a loss of a C_{2}H_{6}O_{2} moiety. CID of the ion at m/z 402 resulted in product ion at m/z 226, corresponding to a loss of a glucuronic acid residue from the ion of m/z 402. The mass spectra of M6a to M6c were similar and suggested they were the glucuronides of hydroxylated nefopam. These metabolites were likely diastereomers and/or regioisomers.

Metabolites M7a to M7c
Metabolites M7a, M7b, and M7c were identified in rat plasma and urine and eluted around 33.0 ~ 34.6 min. The XIC of these ions showed three peaks ions at m/z 464 (Supplementary Figure 6). Since the mass spectra of all the three metabolites were the same, only those of M7b are shown in Supplementary Figure 6. Accurate mass measurement of these ions provided a chemical formula of C_{23}H_{30}NO_{9}^{+}, with mass errors from -1.0 to -1.5 ppm, suggesting an addition of C_{6}H_{10}O_{8} moiety to the parent molecule (Table 3). CID of these molecular ions resulted in ions at m/z 402, suggesting a loss of a C_{2}H_{6}O_{2} moiety. CID of the ion at m/z 402 resulted in product ion at m/z 226, corresponding to a loss of a glucuronic acid residue from the ion of m/z 402. The mass spectra of M7a to M7c were similar and suggested they were the glucuronides of the hydroxylated nefopam with benzoxazocine ring opened. They were likely diastereomers and/or regioisomers.

Metabolites M8a and M8b
Metabolites M8a and M8b were identified in rat plasma and urine, and eluted around 32.0 ~ 33.6 min. The XIC of these ions showed two peaks at m/z 462 (Supplementary Figure 7). Since the mass spectra of both metabolites were the same, only those of M8a are shown in Supplementary Figure 7. Accurate mass measurement of these ions provided a
chemical formula of $\text{C}_{23}\text{H}_{28}\text{NO}_9^+$, with mass errors between $-1.4$ to $-1.5$ ppm, suggesting an addition of $\text{C}_6\text{H}_8\text{O}_8$ moiety to the parent molecule (Table 4). CID of these molecular ions resulted in major ions at $m/z$ 286 and 176, corresponding to a loss of a glucuronic acid residue and a $\text{C}_{12}\text{H}_{14}\text{O}_8$ moiety, respectively, from the molecular ion. Accurate mass measurement of these product ions confirmed their chemical formulas. Mass spectra of M8a and M8b were similar and suggested they were the glucuronides of the di-oxidation product of nefopam. Their regiochemistry is unknown.

Metabolite M9

Metabolite M9 was identified in rat plasma, urine, and feces and eluted at 37.8 min. The XIC of this ion showed one peak at $m/z$ 256 (Supplementary Figure 8). Accurate mass
measurement of these ions provided a chemical formula of C_{16}H_{18}NO_2^+, with a mass error of -1.3 ppm, suggesting an addition of O and a loss of CH_2 moiety from the parent molecule (Table 4). CID of the molecular ion of M9 resulted in major ions at m/z 197, 195, 179 and 162. The ions at m/z 197 and 162 were resulted from a loss of C_3H_7NO moiety and a loss of C_6H_6O moiety, respectively, from the molecular ion. The ion at m/z 195 was formed through a loss of two hydrogen atoms from the ion of m/z 197. The mass spectra of M9 suggested it was the product of hydroxylation as well as demethylation of nefopam.

**Metabolite M11**

Metabolite M11 was identified in rat plasma, urine, and feces and eluted at 38.5 min. The XIC of this ion showed one peak at m/z 270 (Supplementary Figure 9). Accurate mass measurement of this ion provided a chemical formula of C_{17}H_{20}NO_3^+, with a mass error of -0.3 ppm, suggesting an addition of O atom to the parent molecule (Table 4). CID of the molecular ion resulted in major ions at m/z 197 and m/z 179, corresponding to a loss of C_3H_7N and C_3H_9NO_2 moiety, respectively, from the molecular ion. The mass spectra of M11 suggested it was a hydroxylated nefopam.

**Metabolite M14**

Metabolite M14 was identified in rat plasma, urine, and feces and eluted at 43.8 min. The XIC of this ion showed one peak at m/z 272 (Supplementary Figure 10). Accurate mass measurement of this ion provided a chemical formula of C_{16}H_{18}NO_3^+, with a mass error of -0.6 ppm, suggesting an addition of O atom from the parent molecule (Table 4). CID of the molecular ion resulted in major ions at m/z 197 and m/z 179, corresponding to a loss of C_3H_7N and C_6H_6O moiety, respectively, from the molecular ion. The mass spectra of M14 suggested it was a product of both demethylation and hydroxylation of nefopam.

**Metabolites M16 and M20**

Metabolites M16 and M20 were identified in rat plasma, urine, and feces and were eluted at 38.7 and 44.5 min, respectively. The XIC of these metabolites showed molecular ions at m/z 286 (Figure 9 and Supplementary Figure 11). Accurate mass measurement of these ions provided a chemical formula of C_{17}H_{20}NO_3^+, with mass errors of -1.0 and -1.5 ppm, respectively, for M16 and M20, suggesting an addition of two O atoms to the parent molecule (Table 4). CID of the molecular ion of M16 resulted in a major ion at m/z 176, corresponding to a loss of C_6H_6O_2 moiety from the molecular ion. CID of the molecular ion of M20 resulted in a major ion at m/z 210, corresponding to a loss of C_2H_4O_3 moiety from the molecular ion. Accurate mass measurement of these product ions confirmed their chemical formula. The mass spectra of M16 and M20 suggested both of them were hydroxylation products of nefopam.

**Metabolite M23**

Metabolite M23 was identified in rat plasma and eluted at 45.9 min. The XIC of this metabolite showed one peak at m/z 337 (Figure 10). Accurate mass measurement of this ion provided a chemical formula of C_{16}H_{21}N_2O_4S^+, with a mass error of -0.3 ppm, suggesting a loss of C atom from, and an addition of NHSO_3 moiety to the parent molecule (Table 4). CID of this ion resulted in major ions at m/z 320 and m/z 240, corresponding to a loss of NH_3 moiety and loss of both NH_3 and SO_3 moieties, respectively, from the molecular ion. The mass spectra of M23 suggested it was a sulfate of desmethyl nefopam. It likely formed an ammonium adduct in the ion source of the mass spectrometer.

**Estimation of the AUC_0–8h of M20 and M21**

LC-Radio-chromatogram analysis of plasma samples indicated that M20 and M21 co-eluted. A method was developed
to approximately estimate their relative contributions to the total radioactivity AUC. Since the skeletal structure of N-desmethyl nefopam (M21) is the same as that of nefopam, assuming their ionization energy are similar, the ratio of radio peak area to the mass response of nefopam in plasma was employed as a correction factor to back calculate the concentration of M21 based on its mass response. Subsequently, the concentration of M20 was estimated by subtracting the concentration of M21 from the combined concentration of the two metabolites measured from radio peak integration. The results revealed that the AUC_{0-8h} of M20 and M21 was 1089 and 67 ng equiv/h/mL, respectively.
for male rats and 845 and 206 ng equiv-h/mL, respectively, for female rats.

The proposed structures of the metabolites and biotransformation pathways of nefopam are depicted in Figure 11.

*Tissue distribution*

The tissue distribution of nefopam-derived radioactivity was investigated using QWBA in male albino rats as well as male

Figure 13. Representative whole-body autoradiogram of radioactivity distribution in a male pigmented LE rat at 8 h following a single oral dose of 20 mg/kg [14C]nefopam.

*Note: Three sagittal sections obtained at 3 different levels are shown*
and female pigmented rats. Representative whole-body autoradiograms showing radioactivity distribution in tissues at 0.5 h and 8 h in two separate male LE rats are illustrated in Figures 12 and 13. The key pharmacokinetic parameters of total radioactivity in various tissues of male and female LE rats are summarized in Table 5.

### Male pigmented rats

$C_{\text{max}}$ of total radioactivity in tissues were noted at 0.5–1.0 h post-dose. Peak tissue concentrations >15.0 μg equiv/g were noted in the urinary bladder (320.827 μg equiv/g), liver (45.086 μg equiv/g), kidney medulla (28.634 μg equiv/g), small intestine (27.007 μg equiv/g), eye uveal tract (19.840 μg equiv/g), and kidney cortex (17.471 μg equiv/g). The $C_{\text{max}}$ of total radioactivity in regions of the brain ranged from 1.8 to 2.1 μg equiv/g. The highest overall concentrations were observed in alimentary canal ($C_{\text{max}}$ ranged from 8.479 μg equiv/g in stomach at 0.5 h to 1943.303 μg equiv/g in contents of the small intestine at 1 h), urinary bladder contents (167.473 μg equiv/g at 1 h), and in bile (760.054 μg equiv/g at 0.5 h), which reflected the major route of elimination for the drug-derived radioactivity after an oral dose. Radioactivity concentrations decreased steadily thereafter but was not eliminated from all tissues by 168 h. Low amounts of drug-derived radioactivity was present in approximately 72% of the tissues at 168 h. The tissues with the highest terminal concentrations (>0.15 μg equiv/g) at 168 h were eye uveal tract (1.135 μg equiv/g), thyroid (0.749 μg equiv/g), liver (0.712 μg equiv/g), kidney medulla (0.309 μg equiv/g).

#### Table 5. Pharmacokinetics of total radioactivity in individual tissues of male and female Long-Evans (pigmented) rats after a single oral dose of 20 mg/kg [14C]-nefopam.

| Organ/Tissue (with notes) | $C_{\text{max}}$ (μg equiv/g) | AUC$_{0-\infty}$ (μg equiv⋅h/g) | $t_{1/2}$ (h) |
|---------------------------|-------------------------------|---------------------------------|--------------|
|                           | M | F | M | F | M | F | M | F |
| Adipose (brown)           | 3.06 | 3.69 | 23.70* | 53.50* | 77.8* | 214.8* |
| Adipose (white)           | 1.05 | 1.72 | 6.60* | 9.48* | 10.4* | 8.3* |
| Adrenal gland             | 6.69 | 7.20 | 46.87* | 83.83 | 61.8* | 108.3 |
| Blood (cardiac)           | 3.34 | 3.19 | 31.49 | 35.00* | 18.1 | 30.8* |
| Bone                      | 0.28 | 0.18 | 1.38 | 6.16* | 3.1 | 24.0* |
| Bone marrow               | 3.21 | 6.44 | 20.96 | 41.59 | 25.0 | 33.0 |
| Brain (cerebellum)        | 1.77 | 4.19 | 7.53* | 19.67* | 16.1* | 9.0* |
| Brain (cerebrum)          | 2.08 | 5.56 | 7.12* | 23.04* | 9.1* | 1.3* |
| Brain (medulla)           | 1.90 | 4.41 | 7.44* | 19.70* | 14.1* | 9.9* |
| Cecum                     | 8.33 | 3.81 | 256.89 | 70.16 | 19.4 | 14.2 |
| Epididymis                | 2.29 | NA | 14.48 | NA | 6.9 | NA |
| Esophagus                 | 7.45 | 25.28 | 33.16 | 161.91 | 18.4 | 4.9 |
| Eye (lens)                | 0.27 | 0.36 | 8.23 | 3.33* | 50.4 | 18.0* |
| Eye (uveal tract)         | 19.84 | 67.54 | 723.83 | 1395.44 | 72.7 | 104.5 |
| Harderian gland           | 6.33 | 20.85 | 41.93* | 117.40* | 48.5* | 145.9 |
| Heart                     | 2.61 | 3.79 | 22.79 | 48.64* | 23.6 | 146.0 |
| Kidney cortex             | 17.47 | 19.17 | 198.85 | 256.90 | 59.0 | 86.2 |
| Kidney medulla            | 28.63 | 35.61 | 413.17 | 686.95 | 51.7 | 35.0 |
| Large intestine           | 2.77 | 7.10 | 24.28 | 60.56 | 9.3 | 15.9 |
| Liver                     | 45.09 | 22.26 | 527.95 | 395.49 | 67.5 | 67.5 |
| Lung                      | 4.80 | 9.88 | 48.03* | 76.69 | 66.9* | 71.1 |
| Lymph node                | 3.91 | 6.34 | 27.89 | 45.71 | 20.1 | 150.4 |
| Mammary gland region      | 2.12 | 1.46 | 7.66 | 32.05* | 7.8 | 333.6 |
| Oral mucosa               | 3.37 | 3.36 | 23.52 | 42.89* | 19.4 | 23.2 |
| Ovary                     | NA | 6.26 | NA | 52.96* | NA | 72.7* |
| Pancreas                  | 4.39 | 5.99 | 48.93* | 50.58* | 238.6* | 151.4* |
| Pituitary gland           | 6.11 | 10.76 | 38.40 | 98.37* | 65.4 | 165.4 |
| Prostate gland            | 3.27 | NA | 17.06 | NA | 19.6 | NA |
| Salivary gland            | 7.70 | 9.67 | 23.79 | 165.29* | 7.8 | 167.3 |
| Seminal vesicles          | 2.22 | NA | 12.50* | NA | 15.1* | NA |
| Skeletal muscle           | 1.63 | 4.23 | 12.87 | 55.68* | 25.8 | 304.2 |
| Skin (non-pigmented)      | 3.06 | 3.95 | 15.15 | 38.49* | 5.8 | 140.2 |
| Skin (pigmented)          | 3.11 | 3.95 | 75.99 | 231.76* | 87.5 | 126.9 |
| Small intestine           | 27.01 | 77.21 | 134.78* | 725.42* | 10.3* | 27.8 |
| Spinal cord               | 1.87 | 3.85 | 7.09* | 16.27* | 12.0* | 1.5* |
| Spleen                    | 4.70 | 8.28 | 50.82 | 162.76* | 104.0 | 303.1 |
| Stomach                   | 8.48 | 113.33 | 50.48* | 477.93* | 14.8* | 58.8 |
| Testis                    | 1.65 | NA | 11.19 | NA | 7.8 | NA |
| Thymus                    | 3.05 | 4.73 | 11.55 | 31.54* | 9.1 | 57.2 |
| Thyroid                   | 6.58 | 8.50 | 389.27 | NA | 167.1 | ND |
| Urinary bladder           | 320.83 | 21.83 | 1661.95* | 154.93* | 4.5 | 54.3 |
| Uterus                    | NA | 3.78 | NA | 42.54* | NA | 46.5 |
| Vagina                    | NA | 4.25 | NA | 38.01* | NA | 67.8 |

NA, not applicable;  
*Value is an estimate due to insufficient data points (<3 used for $t_{1/2}$ determination) and/or the $r^2$ value of the regression line used to determine $t_{1/2} \leq 0.85$.  

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equiv/g), kidney cortex (0.197 μg equiv/g). The remaining tissues had concentrations that were approaching the LLOQ suggesting that elimination was ongoing and near completion.

Female pigmented rats

Radioactivity reached peak levels in all tissues by 1 h post-dose. Peak concentrations of >15.0 μg equiv/g were found in the stomach (113.325 μg equiv/g), small intestine (77.205 μg equiv/g), eye uveal tract (67.541 μg equiv/g), kidney medulla (35.613 μg equiv/g), esophagus (25.277 μg equiv/g), liver (22.555 μg equiv/g), urinary bladder (21.829 μg equiv/g), Harderian gland (20.852 μg equiv/g), and kidney cortex (19.165 μg equiv/g). In female rats, Cmax of total radioactivity in regions of the brain were higher than in males and ranged from 4.2 to 5.6 μg.equiv/g. The highest overall concentrations were observed in the contents of the alimentary canal (Cmax ranged from 194.400 μg equiv/g in large intestine at 8 h to 1285.951 μg equiv/g in the small intestine at 1 h), urinary bladder contents (115.387 μg equiv/g at 1 h), and in bile (127.397 μg equiv/g at 1 h), which reflected the major route of elimination for the drug-derived radioactivity after an oral dose. As in male LE rats, tissue concentrations consistently declined over 7 days, but quantifiable amounts of radioactivity were evident in approximately 38% of the tissues at 168 h. The tissues with the highest terminal concentrations (>0.15 μg equiv/g) at 168 h were eye uveal tract (2.276 μg equiv/g), thyroid (1.411 μg equiv/g), liver (0.516 μg equiv/g), kidney medulla (0.373 μg equiv/g), pigmented skin (0.343 μg equiv/g), kidney cortex (0.322 μg equiv/g), and spleen (0.206 μg equiv/g).

Reliable tissue half-lives in male and female rats ranged from 3.1 h (in bone of males) to 167 h (in thyroid of males). Reliable t1/2 values were determined for 27 of 40 male tissues, and for 13 of 40 female tissues in accordance with acceptance criteria (i.e. at least 3 time points included in the determination of terminal t1/2 and the regression line must have an r² value ≥ 0.85), but t1/2 values were estimated for all tissues using the data available. Exposure (Cmax and AUC) in the urinary bladder of male rats was approximately 10-to 15-fold greater than in female rats. However, the uveal tract, gastric mucosa, and small intestine of female rats were exposed to higher radioactivity concentrations than in male rats. The association of [14C]-nefopam-derived radioactivity with the pigmented uveal tract was reversible with a t1/2 of 73 h and 104 h in pigmented male and females rats, respectively.

Tissue distribution in non-pigmented rats

The overall tissue distribution profile in the non-pigmented WH rats was qualitatively similar to that in pigmented rats. Concentrations in the uveal tract (Cmax = 19.840 μg equiv/g) of pigmented rats were substantially higher than that observed in the uveal tract of non-pigmented rats (Cmax = 1.606 μg...
balance following oral administration of $^{14}$C-nefopam was characterized in pigmented and non-pigmented rats. Mass metabolism, and excretion mass balance of nefopam was discussed and noted in pigmented and non-pigmented skin, but to a lesser extent.

**Discussion**

In this study, the pharmacokinetics, tissue distribution, metabolism, and excretion mass balance of nefopam was characterized in pigmented and non-pigmented rats. Mass balance following oral administration of $^{14}$C-nefopam was demonstrated. Nefopam-derived radioactivity was quickly excreted with approximately 90% of the administered dose recovered within 24 h in male and female rats. An additional 8–9% of the administered radioactivity was recovered between 2 and 7 days. The majority of the radioactivity was excreted in urine suggesting that urinary excretion was the primary route of elimination of $^{14}$C-nefopam-derived radioactivity in this species. The kinetic profile of total radioactivity in whole blood and plasma of male and female rats were generally similar but characterized by a higher C$_{\text{max}}$ in the latter. Total radioactivity concentrations in blood and plasma were comparable suggesting a lack of partitioning into cellular components of whole blood; no gender differences in plasma: blood partitioning were evident. Concentrations of nefopam in plasma reached a peak at the same time or earlier than total radioactivity.

Our findings in the rat are consistent with the mass balance and excretion pattern of oral $^{14}$C-nefopam in humans (to be published, Impax, Hayward, CA). For instance, over 90% of a single 75 mg oral dose of $^{14}$C-nefopam to male human volunteers was excreted within a week. The mean radioactivity recovered in urine and feces of the human subjects accounted for 73 and 27% of the administered dose, respectively, with a majority excreted within 24 h.

Radioactivity was well-distributed into tissues of pigmented male and female rats. Concentrations in most tissues were similar to blood at all time-points with the exception of excretory/metabolic tissues where radioactivity concentrations were generally higher than in blood. Tissue distribution of radioactivity into the urinary bladder and kidney medulla reflected the major route of excretion for nefopam-derived radioactivity after an oral dose. Drug-derived radioactivity reached a peak within 1 h of dosing and decreased steadily in all tissues but was not eliminated from all tissues by the end of study.

Overall, tissue distribution in male and female pigmented rats showed a similar profile with some differences in exposures to individual tissues. The distribution and tissue concentrations of radioactivity were very similar between non-pigmented and pigmented male rats except for radioactivity concentrations in the uveal tract. Nefopam-derived radioactivity reversibly associated with melanin-containing tissues/organs such as the uveal tract and pigmented skin.

Unchanged nefopam constituted a minor or trace fraction of the total radioactivity excreted suggesting that $^{14}$C-nefopam was extensively metabolized after absorption. The resulting metabolites were excreted primarily in urine. The presence of metabolites in feces suggests hepatobiliary excretion of drug-related radioactivity, non-biliary route(s) of excretion, or metabolism within the GI tract. The oral bioavailability of nefopam in rats is unknown; however, the relatively low amounts of intact nefopam in plasma and urine and extensive formation of metabolites are suggestive of it being low. A number of metabolites of nefopam were identified in the rat evidencing the extent of metabolism.

The oral bioavailability of nefopam in humans is poor (Aymard et al., 2003). Nefopam is relatively stable in human liver microsomes, human liver S9 fractions, and in human hepatocytes (unpublished data, Impax, Hayward, CA). Time- and cofactor-dependent formation of N-desmethyl nefopam and nefopam N-oxide, albeit at a low rate, is observed in microsomes and S9 preparations from the livers of rats, rabbits and humans (Bolt et al., 1974; Heel et al., 1980; unpublished results, Impax). In recombinant human CYP450 systems, nefopam is primarily metabolized by CYP2C19 and CYP2D6. Direct glucuronidation of nefopam or N-desmethyl nefopam by recombinant human uridine 5'-diphospho-glucuronosyltransferases was not observed (unpublished data, Impax, Hayward, CA).

Intact nefopam was present in plasma at concentrations that were much lower than total radioactivity indicating that metabolites accounted for a majority of the circulating radioactivity. The metabolic profiles of nefopam in plasma, urine, and feces between male and female rats were qualitatively similar in the respective matrices. Nefopam was extensively metabolized in rats to numerous metabolites. Most of them are formed through two metabolic pathways. Firstly, single or multiple oxidations of nefopam generate multiple hydroxylation/oxidation metabolites (M11, M22a and M22b, M16, and M20). Some of these metabolites are further metabolized through glucuronidation (M6a to M6c, M7a to M7c, M8a and M8b, and M3a to M3d). Secondly, N-demethylation of the nefopam results in metabolite M21, which further undergoes single or multiple hydroxylations or sulfation (M9, M14, and M23). Some of the hydroxylation metabolites are subsequently glucuronidated (M2a to M2d).

The major metabolites of nefopam in plasma and urine were glucuronides of mono-hydroxylated nefopam (M3a to M3d), glucuronides of mono-hydroxylation as well as N-demethylation metabolites of nefopam (M2a to M2d), and a dihydroxylation metabolite (M20). In addition, the combined exposure of three groups of glucuronides (M6a to M6c, M7a to M7c, and M8a to M8b) and the exposure of a sulfate of desmethyl nefopam (M23) were higher in female than in male rats. The unidentified peak (unknown 1) was less than 1.5% of the total circulating radioactivity AUC$_{0-\text{inf}}$.

The mean combined AUCs of nefopam, M2a to M2d, M3a to M3d, hydroxylated nefopam, N-desmethyl nefopam (M21), and nefopam N-oxides (M22a, M22b) accounted for approximately 60% of the total radiocarbon in circulation and urine. The relative abundance of previously unknown metabolites in plasma and in urine over nefopam, N-desmethyl nefopam, and nefopam N-oxides was unexpected. Until now, it was thought that N-desmethyl nefopam and nefopam N-oxide were the major metabolites of nefopam. A comparison of the metabolic profile of nefopam in the circulation and in excreta of humans (unpublished data, Impax, Hayward, CA) and rats did not reveal major human-specific metabolites. Therefore, these findings confirm that the rat is a suitable species to further characterize the safety and toxicology of nefopam and obviate.
the need to characterize the safety of one or more metabolites individually.

The overall concentration-time profiles of M2a to M2d and M3a to M3d in plasma mirrored that of total radioactivity (Supplementary Figures 1 and 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, N-desmethyl nefopam, or M20. Indeed, the plasma kinetics of the major metabolites M2a to M2d and M3a to M3d were complex with concentrations approaching that of total radioactivity 6–8 h after dosing. One may speculate that the late rise in plasma concentrations of M2a-M2d and M3a-M3d is due to entero-hepatic or other types of recycling from “peripheral” compartments, or biotransformation by enteric microflora followed by reabsorption.

Our findings suggest that nefopam is biotransformed in the rat by pathways including demethylation followed by hydroxylation, sulfation, and glucuronidation of the corresponding; hydroxylation of nefopam followed by glucuronidation; N-oxidation followed by hydroxylation and glucuronidation; and hydroxylation at methyl group followed by methylation and glucuronidation. By comparison, biotransformation of nefopam in humans occurs by hydrogenation coupled with glucuronidation; N-demethylation coupled with glucose conjugation; dehydration and glucuronidation; N-demethylation coupled with N-glucuronidation; and N-demethylation coupled with ring opening and carboxylic acid formation or di-oxidation (unpublished data, Impax, Hayward, CA).

Toxicokinetics of oral nefopam in preliminary safety and toxicology studies in rats had indicated that females had higher exposure to nefopam and displayed greater sensitivity to its biological effects. Significant gender differences in 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, N-desmethyl nefopam, or M20. Indeed, the plasma kinetics of the major metabolites M2a to M2d and M3a to M3d were complex with concentrations approaching that of total radioactivity 6–8 h after dosing. One may speculate that the late rise in plasma concentrations of M2a-M2d and M3a-M3d is due to entero-hepatic or other types of recycling from “peripheral” compartments, or biotransformation by enteric microflora followed by reabsorption.

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Toxicokinetics of oral nefopam in preliminary safety and toxicology studies in rats had indicated that females had higher exposure to nefopam and displayed greater sensitivity to its biological effects. Significant gender differences in the plasma PK profile of nefopam were not observed in this study (Supplementary Figures 1 and 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, N-desmethyl nefopam, or M20. Indeed, the plasma kinetics of the major metabolites M2a to M2d and M3a to M3d were complex with concentrations approaching that of total radioactivity 6–8 h after dosing. One may speculate that the late rise in plasma concentrations of M2a-M2d and M3a-M3d is due to entero-hepatic or other types of recycling from “peripheral” compartments, or biotransformation by enteric microflora followed by reabsorption.

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Toxicokinetics of oral nefopam in preliminary safety and toxicology studies in rats had indicated that females had higher exposure to nefopam and displayed greater sensitivity to its biological effects. Significant gender differences in the plasma PK profile of nefopam were not observed in this study. However, peak concentrations and AUC\textsubscript{0-8h} of the metabolites M6a to M8b, M23, and M3a to M3d were 3 to 14-fold higher in plasma of females than in males (Supplementary Figure 1 and Table 3). It is possible that these gender differences manifest only at higher toxicological doses or that one or more of the metabolites M6a to M8b, M23, and M3a to M3d may account for the increased sensitivity of female rats.

It is of interest to characterize the biological activity of the newly identified metabolites. Antinociception mediated by nefopam in rodent models of analgesia requires oral doses that are 10–20 fold higher than by intraperitoneal or IV routes (Girard et al., 2001; Mather et al., 2000). In contrast to the relatively-low potency of nefopam at voltage-sensitive Na and Ca channels (IC\textsubscript{50} > 5 \textmu M), parenteral nefopam has potent anticonvulsant effects in rodent models of seizure (Novelli et al., 2007; Verleye et al., 2004). These effects are observed at doses that are comparable to or lower than parenteral analgesic doses. One may hypothesize that the poor oral bioavailability of nefopam and extensive metabolism combined with potentially distinct pharmacological and pharmacokinetic properties of one or more of its major metabolites may account for the disparity (potency and activity) in its route-dependent effects in animal models.

In summary, this study demonstrated that oral [14C]-nefopam is well absorbed in the rat, widely distributed to tissues, and rapidly and extensively metabolized to numerous metabolites mainly via hydroxylation, glucuronidation, and N-oxidation. Nefopam-related radioactivity reversibly associated with melanin-containing tissues. A majority of the administered radioactivity in the rat was excreted within 24 h of dosing mainly via the urinary route. Intact nefopam was a minor component in the circulation. Urinary and fecal excretion of intact nefopam was negligible.

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Declaration of interest

A. Mittur and Nishit B. Modi are employees of Impax Laboratories, Inc. and hold Impax stock. The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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**Supplementary material available online**

Supplementary Figures S1–S11

Supplementary Tables