# Bioanalyses and Pharmacokinetics of Nafronyl in the Dog

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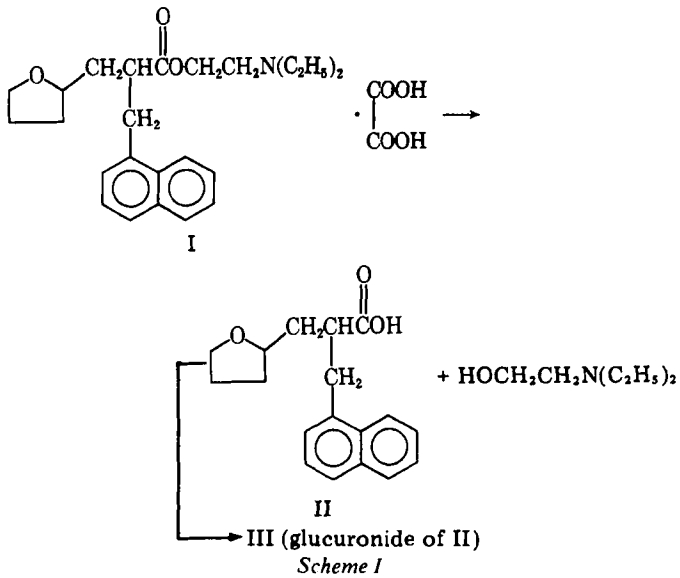
**Abstract** □ Improved specific and sensitive reverse-phase HPLC assays of nafronyl (I) and its acidic metabolite and hydrolysis product (II) in biological fluids were developed with sensitivities of 3–6 ng/mL using fluorometric detection with 225 nm excitation and 330 nm emission wavelengths. There were no significant differences in the stabilities and assays of I and II in plasma obtained using heparin, citrate phosphate dextrose solution, EDTA, citrate, or oxalate as anticoagulant. Inordinately high membrane binding did not permit the quantification of the high plasma protein binding of I by ultrafiltration; its instability precluded the use of equilibrium dialysis. Plasma protein binding of II by ultrafiltration was 76.4% and was not concentration dependent. The apparent red blood cell–plasma partition coefficients for I and II were 2.00 and 0.49, respectively, with almost all anticoagulants; the red blood cell–plasma water partition coefficient for II was 2.08 when corrected for plasma protein binding. Thus, both I and II had erythrocyte binding sites in addition to simple volume partitioning. Only heparin-treated blood gave anomalously low erythrocyte–plasma partition coefficients, indicating that heparin inhibited the partitioning of I and II into red blood cells from plasma water. The total body clearance of nafronyl (I) referenced to total plasma concentration [1295 ± 65(*SEM*) mL/min] was dose independent (35–70-mg range) and showed biphasic plasma half-lives (intravenous) of 12 and 100 min. Only 34% of the nafronyl appears as systemically circulating II in the plasma. Apparent volumes of distribution similarly referenced were 39.8 and 163 L for the central compartment and total body, respectively. Renal clearances referenced to total plasma concentration were 8.3 and 0.18 mL/min for I and II, respectively. The respective total urinary excretions of I, II, and the glucuronide of II (III) were 0.48, 0.021, and 0.32% of the administered intravenous doses. The respective total urinary excretions of I, II, and III for a bile-cannulated dog were 0.005, 0.16, and 0.40%. The total body clearance of intravenously administered II was 225 mL/min, with a renal clearance of 0.057 mL/min referenced to total plasma concentration. The respective total urinary excretions of II and III were 0.027 and 0.44% of the intravenous dose of II. Respective plasma half-lives of II (intravenous) were 2.5, 10.9, and 225 min. The apparent volume of distribution referenced to total plasma concentration was 2.2 L (9.1 L referenced to plasma water concentration). The apparent overall volume of distribution referenced to plasma concentration was 73 L. The bioavailability of nafronyl (I) was 0.3–2.7% of the oral 250-mg dose of I. The bioavailability of II was 10–17% of the oral 250-mg dose of I, indicating high presystemic and/or first-pass metabolism of I and II. Additional metabolites of nafronyl, probably the major ones, could be chromatographically identified and quantified. Their extraction and fluorescent properties indicated that they were hydroxylated naphthalenic compounds containing carboxylic acid groups and their glucuronides. Only one major unconjugated metabolite (IV) was found in significant quantities in plasma. In addition to IV, other organic extractable nonconjugated compounds (V, VI, and VII) appeared in urine and bile. Conjugates of VII were also present in urine and bile.

**Keyphrases** □ Nafronyl—pharmacokinetics in the dog, HPLC, red blood cell partitioning, plasma protein binding, effect of different anticoagulants, bioavailability □ Pharmacokinetics—nafronyl in the dog, HPLC, red blood cell partitioning, plasma protein binding, effect of different anticoagulants, bioavailability □ Bioavailability—nafronyl in the dog, HPLC, red blood cell partitioning, plasma protein binding, effect of different anticoagulants, pharmacokinetics

The vasodilator and metabolic activator nafronyl [2-diethylamino)ethyl tetrahydro-α-(1-naphthylmethyl)-2-furnpropionate (I) (1, 2) is prepared as the oxalate. Its smooth ıuscle relaxation properties, along with its ability to increase ɔripheral and cerebral blood flow, cerebral ATP concentraɔns, and glucose utilization may have value in the treatment senile brain disease (1–3). Tetrahydro-α-(1-naphthyl-ɔthyl)-2-furanpropionic acid (II), a hydrolysis product of ı (Scheme I), was observed by TLC in the plasma and urine of dogs, humans, and rats (4, 5) administered I, but was not




*Scheme I*

quantified. The glucuronide of II (III) was also observed in the bile and urine of dogs administered I intramuscularly (4).

Fontaine *et al.* (4) showed prolonged (at least through 5 h, but low, plasma levels of I (3.4–5.1 μg/mL of plasma) after administration of 40 mg/kg im of I to dogs. Detection of I in plasma was 80 ng/mL for three of six dogs at 24 h after intramuscular administration, with maxima of ~5 μg/mL at 1 h by the use of a nonspecific fluorometric assay (6). Minor amounts of the intramuscularly administered dose (0–0.4%) were excreted unchanged into dog urine, half within 6 h. As much as 20% of the dose was still present at the site of intramuscular injection at that time (4).

Bile collected over 12 h from bile-cannulated dogs contained 0.013% of the intramuscular dose of I and contained 0.42% of the injected dose as total I (free and liberated by β-glucuronidase), presumably fluorometrically analyzed; this agreed with the estimates of nafronyl equivalents obtained from measured spectrophotometric absorbance at 224 nm of acid extracts of ether-extracted alkalinized bile (4). The remaining aqueous phase after this extraction was acidified and extracted with ether. These ether extracts, back-extracted into 0.1 M NaOH and assayed spectrophotometrically at 224 nm, accounted for 33% of the dose in nafronyl absorbance equivalents, of which 22% was II, measured by quantifying TLC-separated amounts. After β-glucuronidase hydrolysis, the similarly treated bile accounted for 40% of the dose in nafronyl absorbance equivalents, of which 29% was II (free and liberated by β-glucuronidase). These ether extracts showed at least three identifiable TLC spots in addition to the one assignable to II. The IR chromatograms indicated the presence of both the naphthalenic and carboxyl groups in these metabolites, and two of the three contained phenolic hydroxyls. Since there was an increase in their presence after β-glucuronidase treatment, the glucuronide conjugates of these naphthols must also be metabolites of nafronyl (4).

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After oral administration of 150 mg of nafronyl to a human, plasma levels of I of 720 ng/mL at 1 h and 110 ng/mL at 6 h were claimed (4), and 0.6% of unchanged I was recovered in the urine within 2 d. After administration of 80 mg im of nafronyl to a human, plasma levels of I of 290 ng/mL at 1 h and 550 ng/mL at 3 h were noted, and 1.6% of unchanged I was recovered in the urine within 2 d (4). An apparent terminal plasma half-life of 90 min was observed for I when 100 mg of nafronyl was administered orally to two human subjects (7).

Since the above is the sum total of published studies on nafronyl pharmacokinetics, there is a need for systemically determined pharmacokinetics, disposition, and bioavailabilities. The purpose of this study was to determine the pharmacokinetic parameters of nafronyl after intravenous administration by quantitatively monitoring the time courses of both I and II and their possible conjugates in plasma, urine, and bile as a function of dose in the dog. Selected studies were conducted to estimate the oral bioavailability of nafronyl and to identify possible quantitative methods of monitoring the time courses of as yet unidentified metabolites.

The recent developments of HPLC methods for the bioanalyses of nafronyl (7) and nafronyl plus its derived metabolite, II (8), using UV detection at 224 nm after selective extractions from biological fluids, have provided specific and sensitive assays. These methods have been applied recently to the complete delineation of the solution stability of nafronyl as a function of pH and the optimization of analytical methods (8) in plasma. In this present report, further assay modifications and increased sensitivities with the use of fluorometric detection are given. Since questions had been raised as to the possible effects of different anticoagulants on the stability and assay of nafronyl in plasma, these factors were studied when various anticoagulants were used in blood procurement.

## EXPERIMENTAL

**Materials**—Acetic acid[1], sodium acetate[1], potassium and ammonium oxalate[1], dibasic and monobasic potassium phosphate[1], and volumetric concentrations of sodium hydroxide[2] and hydrochloric acid[2] were all analytical grade. Dichloromethane[3] and acetonitrile[3] were HPLC and UV grade. β-Glucuronidase, purified from *Escherichia coli*[4], (1,344,100 Fishman U/g, 1,816,490 U/g with $CHCl_3$) and beef liver[5] (50 Fishman U/g) were used as received. EDTA was a 2% solution of the disodium salt[6]. Citrate phosphate dextrose solution USP[6] contained 1.61 g of hydrous dextrose USP, 1.66 g of sodium citrate dihydrate USP, 0.19 g of anhydrous citric acid USP, and 0.14 g of sodium biphosphate USP dissolved to 63 mL with water for injection USP. The oxalate anticoagulant solution was 1.2 g of ammonium oxalate and 0.89 g of potassium oxalate in 100 mL of water for injection USP.

The pharmacokinetic studies used sodium chloride injection USP[6], sodium heparin injection USP[7], intravenous delivery system sets[6], evacuated blood collection tubes[8], disposable syringes[9], 5-French urethral catheters[9], three-way stopcocks[10], 16-gauge vein catheters, 14-gauge needles[11], and carbocaine hydrochloride[12]. Pure I and II were used as received[13].

Crystalline butacaine hemisulfate[4] was the internal standard for red blood cell partitioning, protein binding, plasma stability studies, the pharmacokinetic studies of bile-cannulated dog E, and most of the oral studies. Procaine hydrochloride[4] was the internal standard in all other pharmacokinetic studies.

**Apparatus**—The HPLC system[14] used for plasma stability, red blood cell partitioning, and protein binding studies contained a single pump, an alkyl phenyl column, and a manual injector. The variable-wavelength detector[15] was set at 225 nm, with a strip-chart recorder[16]. The system was modified for analyses in the intravenous pharmacokinetic studies to use a radial-compression module[17], an automatic injector[17], a fluorescence detector[18] with excitation at 225 nm and emission at 330 nm, and an integrator[19]. In the bile-cannulated dog E pharmacokinetic study, a different detector[20] and injector[20] were used.

**Liquid Chromatography**—*With Haloalkane Extraction (Method A)*—This method was used in the pharmacokinetic studies. Plasma (2 mL) was adjusted to pH 3.5 with 0.35 mL of 0.2 M HCl, vortexed, and extracted with 5.0 mL of dichloromethane with mild shaking. The samples were centrifuged at 3000 rpm for 10 min and 3 mL of the organic phase was evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 0.5 mL of acetonitrile–0.1 M acetate buffer pH 4.0 (80:20) containing 9.8 μg of procaine hydrochloride and 50 μL was injected into the HPLC (for method A1) or the residue was reconstituted with 0.5 mL of acetonitrile–0.1 M acetate buffer, pH 4.2, (10:90) containing 46 μg of butacaine hemisulfate, and 30 μL was injected into the HPLC (for method A2). Undiluted urine (1 mL) was adjusted to pH 3.5 with 0.175 mL of 0.2 M HCl, vortexed, extracted, evaporated, and reconstituted as above.

*Method A1*—This method was used for all intravenous pharmacokinetic studies except with the bile-cannulated dog study. Assay of I (retention time 5.4 min) used a mobile phase of acetonitrile–0.1 M acetate buffer pH 4.0 (80:20) through a radial cyano cartridge[21], with a flow rate of 3.0 mL/min. The assay of II (retention time 4.8 min) used a mobile phase of acetonitrile–acetate buffer pH 4.5 (50:50) through a radial $C_8$ cartridge[21], with a flow rate of 2.0 mL/min.

*Method A2*—In the oral and bile-cannulated dog studies only one system was used for the assay of I and II. A 5-μm $C_8$ column[22] (12 cm) maintained at 45°C was used, with a mobile phase of acetonitrile–0.05 M acetate buffer pH 4.0, (53:47) with a flow rate of 1.1 mL/min. A 25-μL aliquot of the 0.5-mL reconstituted residue was injected onto the HPLC. Peak area ratios to internal standard were used in all these pharmacokinetic studies for the assays.

*Acetonitrile Deproteinization (Method B)*—This method was used in the plasma stability, red blood cell partitioning, and protein binding studies. Acetonitrile (1.5 mL) containing 5.0 μg/mL of the internal standard butacaine sulfate was added to a 0.5-mL plasma sample, vortexed at high speed for 20 s, and centrifuged at 3000 rpm for 5 min; 50 μL of the supernatant was injected into the HPLC. The mobile phase of acetonitrile–0.1 M acetate buffer pH 4.5 (50:50) was passed through an alkyl phenyl column at a flow rate of 1.5 mL/min. Peak height ratios to internal standard were used in these *in vitro* studies.

*Glucuronide Assay in Urine (Method C)*—After extraction of the pH 3.5-adjusted urine with dichloromethane for assay of I and II as in method A1 and checking, in several instances, a second extraction to ascertain complete extraction, the urine samples were hydrolyzed with freshly prepared (100 Sigma or Fishman U/mL) β-glucuronidase (*E. coli*). Phosphate buffer at pH 7.0 (1 mL) and β-glucuronidase solution (0.2 mL) were added to 1 mL of the previously extracted urine. The mixture was incubated at 37.5°C for 30–40 min. An aliquot (1 mL) was adjusted to pH 2.5–3.0 with 0.5 mL of 0.2 M HCl, extracted with dichloromethane, and assayed for the aglycone using method A1. In one study, plasma was treated similarly to determine the presence of a generated aglycone, II. No significant II was found in plasma after β-glucuronidase treatment.

*Glucuronide Assay in Urine and Bile in Bile-Cannulated Dog E (Method D)*—Plasma and nonhydrolyzed urine and bile were extracted at pH 3.5, and the extracts were assayed by the single-injection HPLC method (method A2) in the bile-cannulated dog E study for the simultaneous determination I and II, using butacaine as the internal standard. The bile and urine were hydrolyzed by β-glucuronidase (*E. coli*) as in method C, except that the samples

[1] Mallinckrodt, Paris, Ky.
[2] Ricca Chemical Co., Arlington, Tex.
[3] Burdick and Jackson Laboratories, Muskegon, Mich.
[4] Sigma Chemical Co., St. Louis, Mo., β-glucuronidase (*E. coli*) was Type VII.
[5] Lot 042967; Aldrich Chemical Co., Milwaukee, Wis.
[6] McGaw Laboratories, Irvine, Calif.
[7] The Upjohn Co., Kalamazoo, Mich.
[8] Vacutainer blood collection tube with sodium heparin; Becton, Dickinson and Co., Rutherford, N.J.
[9] Monoject; Sherwood Medical, St. Louis, Mo.
[10] Pharmaseal, Inc., Toa Alta, P.R.
[11] Intracath; The Deseret Co., Sandy, Utah.
[12] Winthrop Laboratories, New York, N.Y.
[13] Nafronyl (I) LS 121, Lot 1956, IND 9571 and Lot 6260, analysis 23569; LS 74 (II) IND 9575, Lot 2357, Lipha Chemicals Inc., New York, N.Y.
[14] Model M 6000A solvent delivery system and Model U6k universal liquid chromatograph injector with a μBondapak column; Water Associates, Milford, Mass.
[15] LC 75 spectrophotometer detector for liquid chromatography; Perkin-Elmer Norwalk, Conn.
[16] Fisher recordall series 5000; Fisher Scientific Co., Pittsburgh, Pa.
[17] Wisp 710A automatic sampling device and RCM 100 module; Waters Associate Milford, Mass.
[18] Varian Fluorichrom fluorescence detector; Varian Instruments, Walnut Cree Calif.
[19] Model 3380A; Hewlett Packard, Avondale, Pa.
[20] PE models 420B and 630-10S; Perkin-Elmer, Norwalk, Conn.
[21] Waters Associates, Milford, Mass.
[22] Perkin Elmer HS 5-μm $C_8$ column; Perkin-Elmer, Norwalk, Conn.

were adjusted to pH 2.0 before extraction and the dried extracts were reconstituted in acetonitrile–0.1 M acetate buffer pH 4.2 (10:90) for injection because interfering impurities resulted from the plastic tips of the automatic injector[20] when the acetonitrile concentration was as high as that in the mobile phase (53% acetonitrile). The preextracted bile was also hydrolyzed with 0.2 M NaOH for 30 min at room temperature and adjusted to pH 2 with HCl before extraction and assay by method A2.

**Pharmacokinetic Studies in Dogs**—The dogs were healthy mongrels weighing 22–26 kg; dog E was female. All were negative for microfilaria. Initial hematocrits ranged between 0.43 and 0.38, with white blood counts of 11,500–15,000 (normal range).

The dogs were fasted for 24 h before each study, and a jugular vein catheter[12] was inserted *via* venipuncture using carbocaine as an anesthetic. The end of the catheter was fitted with the three-way stopcock[10]. The dead volume of the catheter was filled with 0.5 mL of heparinized sodium chloride solution (100 U/mL) to prevent clotting. The dog was placed in a clean metabolism cage overnight. On the day of the study the dog was placed in a dog sling[23] and an intravenous drip of 0.9% NaCl was started to keep the catheter open and to water-load the dog. A 5-French urethral catheter[9] was placed and the dog voided of urine prior to drug administration.

The drug was prepared by dissolving the appropriately weighed amount in 10 mL of isotonic saline. In the metabolite study, the appropriate amount of II was dissolved in 0.1 mL of propylene glycol before adding isotonic sodium bicarbonate to 10 mL. The percentage of propylene glycol was 0.67%.

Blood (60 mL) was drawn before drug injection for use in the plasma calibration curves. The drug solution (10 mL) was injected *via* the jugular catheter, and the stopcock and the catheter were flushed with 10 mL of isotonic saline; the time was measured from the end of the flush. For the oral studies, 250 mg of I was dissolved in 20 mL of deionized water. The solution was injected through a plastic feeding tube[9], which had been inserted orally to reach the stomach. It was followed by a 30-mL rinse of deionized water.

The normal time course for sampling in the intravenous pharmacokinetic studies was 2, 4, 6, 9, 12, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 110, 150, 180, 240, and 300 min. The catheter and stopcock were filled with 1.0 mL of fresh blood before sampling, which was then discarded. Blood (5 mL) was taken in a syringe and transferred to a heparinized tube. The sample was centrifuged at 3000 rpm for 5 min, and the plasma was extracted immediately for assay of I and II. The red blood cells were handled under sterile conditions, resuspended in isotonic saline, and infused back into the dog after the last urine sample was obtained. The dog was then treated with antibiotics to lower the risk of infection.

Urine samples were collected through the urethral catheter at the following times: 15, 30, 45, 60, 75, 90, 105, 120, 140, 160, 180, 210, 240, 300, and 360 min. The volume and pH were recorded, and the samples were placed in an ice bath. All samples were extracted after the last plasma sample had been obtained and were assayed for I, II, and glucuronide by the methods described previously.

The sampling times were modified for the oral studies. Blood samples were obtained at 10-min intervals up to 200 min and then at 30-min intervals up to 480 min. Urine was collected at 20-min intervals up to 240 min and thereafter at 30-min intervals up to 540 min. Bile was collected at 20-min intervals up to 220 min in the bile-cannulated dog study.

**Evaluation of β-Glucuronidase, Acid, and Alkaline Release of Conjugates of the Derived Acid**—Biological fluids to be assayed for conjugates of II (*i.e.*, III) were obtained from the nondosed animal and were used to establish calibration curves, with graded amounts of II added to dichloromethane-preextracted fluids prior to the solvolytic and analytical processes. A 1-mL aliquot of urine, plasma, or bile was extracted at pH 3.5 with 5 mL of dichloromethane for 10 min with shaking, the normal process in the assay of nonconjugated I or II. The aspirated aqueous phase was then hydrolyzed at 37.5°C with *E. coli* β-glucuronidase (40 Fishman U/mL) after adjustment to pH 7.0 with phosphate buffer or with beef liver β-glucuronidase (0.016 Fishman U/mL) after adjustment to pH 4.5 with acetate buffer. Finally, the solutions were adjusted to pH 3.5 and extracted with 10 mL of dichloromethane. The organic extract was evaporated under nitrogen and reconstituted in 500 μL of mobile phase, with 50 μL injected for assay by method A1.

Urine samples, taken at different times from dog A (dosed with 70 mg of *nafronyl*), were assayed several times to determine when complete release of the conjugates of II was obtained. Dichloromethane-preextracted urine (1.0 mL) from dog A was mixed 1:1 with 0.1 M NaOH, 0.2 M NaOH, 1 M HCl, and 6 M HCl. After 15 and 30 min at room temperature, the alkaline samples were adjusted to pH 2. The urine samples were extracted with 10 mL of dichloromethane and assayed by method A1 for II.

**Nafronyl Stability in Plasma Containing Various Anticoagulants**—The stability of nafronyl (I) was investigated in fresh dog plasma prepared with different anticoagulants. The amounts per milliliter of blood were: heparin, 0.5 U; citrate phosphate dextrose solution USP, 0.14 mL; 0.2% EDTA, 0.125 mL; 3.78% sodium citrate, 0.111 mL; and ammonium–potassium oxalate solution, 0.1 mL. Blood (50–80 mL) was withdrawn from the jugular vein using a 16-gauge needle and syringe containing the necessary amount of anticoagulant. The blood was centrifuged for 15 min at 3000 rpm; no hemolysis was observed. Clear plasma (5–17 mL) was transferred into a clean stoppered tube and equilibrated. Nafronyl (I) (0.075–0.27 mg) was added, and the mixture was vortexed 1 min to give final concentrations between 15 and 19 μg/mL. After the addition of plasma to I, a timer was started. Aliquots (0.5 mL) of the mixture were assayed with time by the acetonitrile deproteinization procedure (method B).

**Table I—Nafronyl Stabilities in Fresh Dog Plasma Prepared Using Various Anticoagulants** [a]

| Anticoagulant | $10^3 k$ [b], $min^{-1}$ | $t_{1/2}$, h | Amount Transformed at $t_\infty$, % |
|---|---|---|---|
| Heparin | 1.99 | 5.77 | 49.3 |
| Citrate phosphate dextrose solution | 4.40 | 2.63 | 40.7 |
| EDTA | 3.68 | 3.19 | 38.5 |
| Sodium citrate | 3.49 | 3.31 | 36.1 |
| Oxalate | 3.41 | 3.33 | 40.4 |

[a] All studies, except for heparin at 22°C, were performed at 37.5°C. The concentration of nafronyl was 16 μg/mL. [b] Obtained from the slopes of data plotted in accordance with Eq. 5.

Calibration curves were prepared (0.4–4 μg/mL of injected solution) from stock solutions of I, II, and butacaine dissolved in acetonitrile. In the EDTA, sodium citrate, and oxalate studies, the calibration curves were prepared from the same blank plasma that was used for the stability study, and the injected solutions had the same composition as those injections made in the studies. The plasma used for the calibration curves in the heparin study had been refrigerated overnight. The calibration curve for the citrate phosphate dextrose solution study was prepared from compounds in the mobile phase.

**Protein Binding**—The protein binding of I and II in the heparinized dog plasma was investigated using a cone[24] filtration technique (9). Fresh whole dog plasma was filtered to obtain the plasma water. Plasma water (1.5 mL) and plasma (1.5 mL) were spiked with 2–16-μg/mL concentrations of I and II. A 0.5-mL aliquot of each was assayed using acetonitrile deproteinization (method B) to determine the initial concentrations. The plasma water was then filtered at 3000 rpm for 20 min, and 0.5 mL of filtrate was assayed. The spiked plasma was then filtered through the same cone used to filter the plasma water, which contained substances at the same concentration.

**Red Blood Cell Partitioning**—Calibration curves for the assay of I, II, and mixtures of I and II were prepared at 0.2–16 μg/mL in whole blood and plasma obtained by centrifugation from the same blood. Calibration curves were established for mobile phases at 25% of these concentrations, since they were injected without the dilution by acetonitrile needed for deproteinization. The blood samples were obtained with different anticoagulants: heparin, oxalate, citrate phosphate dextrose solution, and 0.2% EDTA in concentrations given previously. The hematocrits ($H$) of the blood samples were measured and portions centrifuged to recover the plasma. The spiked blood samples were carefully mixed and centrifuged at 3000 rpm for 5 min, and the separated plasma samples were assayed by method B using acetonitrile denaturation. The final hematocrits of the spiked blood samples were corrected for the dilutions made by the addition of the compounds. The corrected hematocrits ($H'$) were 0.83 of those experimentally determined in the blood samples.

The spiked plasma and mobile phase were also assayed by this method. The concentrations per milliliter ($C_p$) in the separated plasma of spiked blood were ascertained from the calibration curve established for spiked plasma. The amount of drug in the plasma obtained from 1 mL of spiked blood is $A_{P-B} = (1 - H')C_p$. The amount of drug in the red blood cells obtained from 1 mL of spiked blood is $A_{RBC-B} = C_0 - A_{P-B}$, where $C_0$ is the concentration of spiked drug per milliliter of blood. The concentration of drug in the red blood cells of spiked blood is $C_{RBC} = A_{RBC-B}/H'$. Thus, the apparent red blood cell–plasma partition coefficient is:

$$D' = C_{RBC}/C_p = A_{RBC-B}/H'C_p = \frac{(C_0 - A_{P-B})/H'}{C_p} = \frac{C_0 - (1 - H')C_p}{H'C_p} = \frac{C_0}{H'C_p} - \frac{1 - H'}{H'} \quad \text{(Eq. 1)}$$

An alternate method to calculate the apparent red blood cell–plasma par-

[23] Dog Sling; Alice King Chatham, Medical Arts, Los Angeles, Calif.

[24] Centriflo Ultrafiltration Membrane Cones CF 50A; Amicon Corp., Lexington, Mass.

Table II—Parameters[a] for Calculation of the Apparent Red Blood Cell–Plasma Partition Coefficients, $D' = (1 - R + RH')/RH'$, of I and II

| Anti-coagulant | I | | | II | |
|---|---|---|---|---|---|
| | $H'$ | $R$ | $D'$ | $R$ | $D'$ |
| Citrate phosphate dextrose solution | 0.327[b] | 0.70 | 2.29 | 1.14 | 0.62 |
| | 0.275[c] | 0.83 | 1.75 | 1.13 | 0.58 |
| | 0.275[c] | 0.79 | 1.94 | 1.16 | 0.50 |
| | Mean ± *SEM* | | 2.00 ± 0.15 | | 0.57 ± 0.04 |
| Oxalate | 0.346[b] | 0.60 | 2.93 | 1.23 | 0.47 |
| EDTA | 0.300[c] | 0.89 | 1.41 | 1.19 | 0.46 |
| | 0.300[c] | 0.78 | 1.92 | 0.96 | 1.15 |
| | 0.330[c] | 0.65 | 2.67 | 1.30 | 0.30 |
| | Mean ± *SEM* | | 2.00 ± 0.37 | | 0.64 ± 0.26 |
| Heparin | 0.252[c] | 1.00 | 1.00 | 1.51 | −0.3 |
| | 0.252[b] | 1.00 | 1.00 | 1.56 | −0.1 |
| | 0.281[c] | 1.00 | 1.00 | 1.32 | 0.14 |
| | 0.281[b] | 0.81 | 1.85 | 1.28 | 0.23 |
| | Mean ± *SEM* | | 1.2 ± 0.2 | | 0.0 ± 0.1 |

[a] $D' = C_{RBC}/C_p = R = m/m'$, where $m$ is $\Delta PHR_p/\Delta C_p$ in the plasma concentration curve, $m' = \Delta PHR/\Delta C_0$ in the blood concentration curve, PHR is the peak height ratio of I and II, and $H'$ is the corrected hematocrit of the spiked blood samples. [b] Effected on mixtures of I and II, each 0.4–4.0 μg/mL of blood. [c] Separate studies for I and II.

tition coefficient, $D'$, is based on the HPLC calibration curves, established in spiked plasma or mobile phase, that pass through the origin and conform to:

$$PHR_p = mC_p \qquad \text{(Eq. 2)}$$

where $C_p$ is the spiked concentration in plasma or mobile phase that gives the peak height ratio, $PHR_p$. Calibration curves for drugs spiked in plasma and mobile phase were superimposable.

Apparent calibration curves were also prepared for the assay of drug in the plasma samples separated from spiked blood of different concentrations, $C_0$, obtained with different anticoagulants. This conformed to:

$$PHR_p = m'C_0 = m[(1 - H')C_p + H'C_{RBC}] = mC_p \qquad \text{(Eq. 3)}$$

Since $D' = C_{RBC}/C_p$ and $R = m'/m$, this equation can be rearranged to:

$$D' = \frac{1 - R + RH'}{RH'} \qquad \text{(Eq. 4)}$$

for the estimations of $D'$.

## RESULTS AND DISCUSSION

Unless specified all concentrations of I, II, and III are given as equivalent to nafronyl (as the oxalate).

**Improvements in Chromatographic Assays**—The HPLC assay methods developed herein substantially improved analytical sensitivity for nafronyl (I) and its derived acid metabolite (II) over those published previously (8) for a haloalkane extraction method using 224-nm spectrometric detection, where the standard errors of regression of concentration on peak area or peak height or their ratios to that of a butacaine internal standard were 100–200 ng/mL of plasma. The columns and conditions for these new studies with fluorometric detection (225-nm excitation, 335-nm emission) using method A1 and dichloromethane extraction gave calibration curves in the 0–300-ng/mL range, which indicated analytical sensitivities of 2–15 ng/mL in urine and plasma.

Some examples of linear regression equations with standard errors of the estimates for nafronyl (I) concentrations in plasma (ng/mL) as the oxalate in plasma are: $C \pm 4.0$ ng/mL = (522.6 ± 10.0)PHR − 11.9 ± 3.3, $r$ = 0.99960; $C \pm 6.2$ = (347.0 ± 2.5)PAR + 10.9 ± 3.1, $r$ = 0.99983; $C \pm 5.1$ = (10.27 ± 0.06) PH + 0.60 ± 2.60, $r$ = 0.9991. Some examples of linear regression equations with standard errors of the estimates of II concentrations in plasma (ng/mL) are: $C \pm 3.6$ = (8.56 ± 0.60)PH − 5.8 ± 5.2, $r$ = 0.9952; $C \pm 2.15$ = (4.744 ± 0.072)PA + 8.3 ± 1.6, $r$ = 0.99965. In urine for I: $C \pm 5.6$ = (3.78 ± 0.17)PH − 7.11 ± 5.10, $r$ = 0.9993; $C \pm 6.1$ = (894.0 ± 5.6) PHR − 6.4 ± 2.8, $r$ = 0.99986; $C \pm 7.1$ = (828.9 ± 20.6)PAR − 5.4 ± 9.8, $r$ = 0.9993. In urine for II: $C \pm 0.59$ = (2.363 ± 0.016)PH − 4.6 ± 0.6, $r$ = 0.9979; $C \pm 5.6$ = (3.78 ± 1.71)PH − 7.1 ± 5.1, $r$ = 0.996. Similar values were obtained for II in urine treated with β-glucuronidase for the assay of III.

The different mobile phases and columns selected for the respective assays of I and II in method A1 permitted the assay of I with a respectable retention time of 5.4 min. The faster moving II in this system eluted with large peaks for urine (0–2 min) and plasma components (0–1.5 min). The system for II increased its retention time to 4.74 min, well separated from interferences from plasma. It was difficult to observe any metabolite that had smaller retention times to I and II and would fall within the retention time intervals of interfering plasma and urine components.

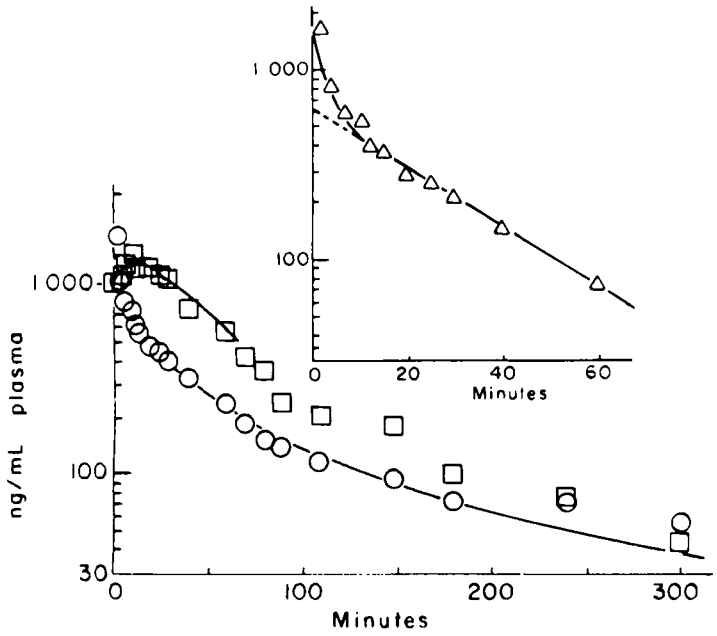


**Figure 1**—*Plasma concentrations of I (○) and II (□) in equivalents of nafronyl oxalate against time for dog A given a 70-mg iv bolus dose of I. The curve through I was calculated from the sum of exponentials (Eq. 10) using the parameters in Table III. The inset (△) is the feathered data for I with the curve calculated from the first two exponentials. The curve through II was calculated from Eq. 15 using the parameters in Table III.*

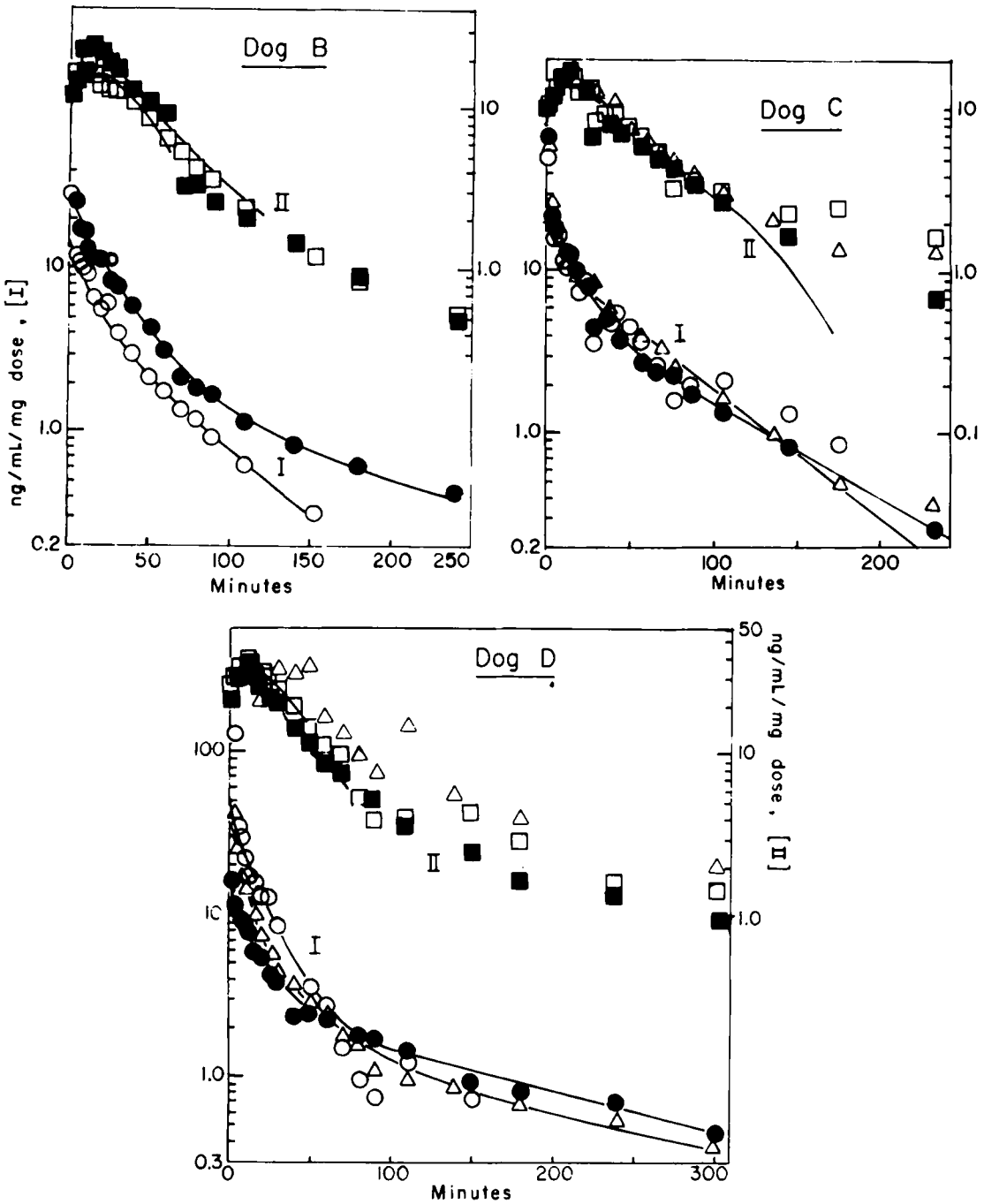


**Figure 2** *Plasma concentrations of I and II in equivalents of nafronyl oxalate per milligram of an intravenous dose of I against time for dogs B, C, and D. The curves through I were calculated from the sum of exponentials (Eq. 10) using the parameters in Table III. The curves through II were calculated from Eq. 15 using the parameters in Table III. Key: (○, □) 35 mg; (△) 53 mg; (●, ■) 70 mg.*

Examples of linear regression equations from HPLC method A2 for simultaneous assay of I (10.9 min) and II (3.9 min) with butacaine internal standard (6.1 min) (2.25-nm excitation, 330-nm emission) for plasma are $C_I \pm 21.4$ ng/mL = (51.8 ± 1.4)PA + 4.1 ± 14.7, $r = 0.9940$; $C_I \pm 21.8$ ng/mL = (537.4 ± 14.7)PAR + 4.7 ± 15, $r = 0.9992$; $C_{II} \pm 23$ = (37.6 ± 4.2)PA − 17.9 ± 24, $r = 0.9937$. For urine, they are: $C_{II} \pm 12.5$ ng/mL = (732 ± 40)PAR − 25 ± 10.6; $C_I \pm 8.4$ ng/mL = (70.9 ± 2.6)PA − 23 ± 7. For bile examples are: $C_{II} \pm 18$ ng/mL = (547 ± 44)PAR − 8.5 ± 14.4; $C_{II} \pm 29$ ng/mL = (58.4 ± 7.7)PA − 11.7 ± 23.5. The symbols PH and PA represent peak height and peak area, respectively, whereas PHR and PAR are the respective ratios to the internal standard.

**Release of Conjugates of Derived Acid from the Urine of a Nafronyl-Dosed Dog**—Preliminary studies of urine samples obtained from dog A treated with *E. coli* β-glucuronidase showed no significant change at 15, 30, and 60 min in the HPLC assay of generated II from dichloromethane-preextracted urine containing III. A similar study with β-glucuronidase (beef liver) showed no significant change in the HPLC assay of generated II at 3, 5.5, and 9 h.

Dichloromethane-preextracted urine (1.0 ml) from a nafronyl-dosed dog was hydrolyzed with 0.1 M NaOH, 0.2 M NaOH, 1 M HCl, and 6 M HCl. Significant peaks for II were observed from the alkaline hydrolyses, but none for the acid hydrolyses. There was a decided NaOH concentration effect. Peak heights of generated II were 19 and 11 mm after 15 and 30 min of hydrolysis, respectively, for 0.1 M NaOH and 40 and 49.5 mm after 15 and 30 min of hydrolysis, respectively, for 0.2 M NaOH. The latter peak height corresponds to 200 ng of II/mL of urine and compared well with the 251 and 192 ng/mL assayed after glucuronidase hydrolyses from bovine and *E. coli* (30 min at 37.5°C) sources, respectively.

**Effect of the Use of Different Anticoagulants on the Plasma Stability Studies of Nafronyl**—Previous studies of nafronyl stability at 1.25 μg/mL of fresh dog plasma at 24.5°C showed an apparent half-life of 3.2 h for the loss of 50% of nafronyl to II. The remaining 50% of nafronyl appeared to maintain its stability thereafter (see Figs. 10 and 11 in Ref. 8).

Table III—Pharmacokinetics of Intravenously Administered Nafronyl (I), Derived Acid (II), and Derived Acid Glucuronide (III)

| Parameter | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose ($D$)[a], mg | 70.0 | 70.2 | 35.07 | 71.52 | 53.6 | 35.4 | 70.0 | 53.0 | 35.6 | 70.46 | Mean ± *SEM* |
| Dog (Weight, kg) | A (22.7) | B (26.6) | B (27.0) | C (22.5) | C (23.0) | C (23.0) | D (25.0) | D (24.3) | D (23.0) | E (22.5)[p] | |
| Parameters[b] from plasma data | | | | | | | | | | | |
| $A$ ($+A'$)[c], ng/mL | 630 (+1000) | 1670 | 325 | 1460 | 1320 | 440 | 940 | 590 (+1200) | 1035 (+825) | 1920 | 1425 ± 168[r] |
| $B$ | 222 | 107 | 220 | 467 | 600 | 182 | 182 | 90 | 35[m] | 950 | 245 ± 78[r] |
| $10^2\,\alpha$, $min^{-1}$ ($t_{1/2}$, min) | 3.57 (19) | 4.06 (17) | 9.40 (7.4) | 7.41 (9.4) | 18.6 (3.7) | 4.13 (17) | 7.15 (9.7) | 3.87 (18) | 4.27 (16) | 29.0 (2.4) | 9.2 ± 2.6 (12 ± 2) |
| $10^3\,\beta$ | 5.77 (120) | 5.30 (131) | 21.3 (33) | 13.4 (52) | 17.6 (39) | 9.35 (74) | 5.79 (120) | 5.05 (137) | 5.1[m] | 26.0 (27) | 8.82 ± 2.2 (100 ± 18)[s] |
| Clearances, mL/min | | | | | | | | | | | |
| Total ($CL^{I}_{tot}$)[d] | 1190 | 1145 | 2546 | 1311 | 1302 | 1175 | 1570 | 1296 | 1031 | 1634 | 1295 ± 65[t] |
| Renal ($CL^{I}_{ren}$)[e] | 3.47 | 5.71 | 2.59 | 12.1 | 26.8 | 3.0 | 1.46 | 11.0 | —[n] | —[q] | 8.3 ± 3.0 |
| $10^{-4}\,AUC^{I}_{0}$[f] | 0 | −1.26 | 1.16 | 3.1 | −4.25 | 1.8 | 1.65 | −5.01 | —[n] | —[q] | |
| $CL^{II}_{ren}$[e] | 0.48 | 0.14 | 0.11 | 0.13 | 0.21 | 0.21 | 0.093 | 0.03 | 0.22 | —[q] | 0.18 ± 0.04 |
| $10^{-4}\,AUC^{II}_{0}$[f] | 5.8 | 3.4 | 2.1 | 0 | 2.8 | 2.1 | 3.0 | −3.0 | 2.7 | —[q] | |
| $CL^{I\to II}/V_{II}$[g] | 0.200 | 0.192 | 0.221 | 0.260 | 0.175 | 0.093 | 0.616 | 0.280 | 0.233 | 0.192 | 0.246 ± 0.044 |
| $CL^{II\to X}/V_{II}$ ($t_{1/2}$, min)[g] | 0.098 (7.1) | 0.125 (5.7) | 0.080 (8.7) | 0.131 (5.3) | 0.144 (6.1) | 0.071 (9.7) | 0.168 (4.1) | 0.116 (6.0) | 0.129 (5.7) | 0.135 (5.2) | 0.120 ± 0.009 (6.4 ± 0.5) |
| Recoveries, % | | | | | | | | | | | |
| $100\cdot\Sigma U^{I}_{330\,min}/D$ | 0.291 | 0.547 | 0.016 | 0.529 | 4.2 | 0.172 | 0.041 | 1.81 | —[n] | —[q] | 0.49 ± 0.24[u] |
| $100\cdot\Sigma U^{I}_{\infty}/D$[h] | 0.292 | 0.601 | 0.016 | 0.399[o] | 4.2 | 0.103[o] | 0.059 | 1.89 | —[n] | —[q] | 0.48 ± 0.25[u] |
| $100\cdot\Sigma U^{II}_{330\,min}/D$ | 0.0313 | 0.0126 | 0.0049 | 0.0146 | 0.0085 | 0.0134 | 0.0137 | 0.0056 | 0.0331 | —[q] | 0.015 ± 0.003 |
| $100\cdot\Sigma U^{II}_{\infty}/D$[h] | 0.0363 | 0.0128 | 0.0084 | 0.0162 | 0.0202 | 0.0234 | 0.0168 | 0.0131 | 0.0445 | —[q] | 0.021 ± 0.004 |
| $100\cdot\Sigma U^{III}_{330\,min}/D$ | 0.65 | 0.61 | —[n] | 0.025 | 0.039 | 0.025 | 0.090 | 0.151 | 0.441 | —[q] | 0.25 ± 0.09 |
| $100\cdot\Sigma U^{III}_{\infty}/D$[i] | 0.86 | 0.68 | —[n] | 0.024 | 0.050 | 0.037 | 0.114 | 0.189 | 0.562 | —[q] | 0.32 ± 0.12 |
| Other Parameters | | | | | | | | | | | |
| $10^3\,k^{i}_{III}$, $min^{-1}$ ($t_{1/2}$, min) | 4.52 (153) | 9.21 (75) | —[n] | 7.94 (87) | 5.16 (134) | 4.03 (172) | 2.91 (239) | 4.43 (156) | 4.73 (147) | —[q] | 5.37 ± 0.75 (145 ± 18) |
| $\Sigma U^{III}_{\infty}$, μg ($\Sigma U'_{\infty}{}^{III}$)[i] | 600 (497) | 480 (480) | —[n] | 17 (15) | 24 (28) | 13 (13) | 80 (55.5) | 600 (497) | 200 (200) | —[q] | |
| $10^{-4}\,AUC^{II}_{240\,min}$, ng·min/mL | 9.57 | 8.75 | 4.01 | 7.86 | 6.05 | 4.0 | 12.0 | 16.8 | 8.21 | 6.80 | 8.11 ± 0.33[v] |
| $10^{-4}\,AUC^{II}_{\infty}$[j] | 11.10 | 9.82 | 4.79 | 8.91 | 7.90 | 6.04 | 15.1 | 20.2 | 9.90 | 7.37 | 9.83 ± 0.57[v] |
| Apparent Volumes of Distribution | | | | | | | | | | | |
| $V^{I}_{c}$, L[k] | 37.8 | 39.5 | 64.4 | 37.1 | 27.9 | 56.9 | 62.4 | 28.2 | 18.8 | 24.6 | 39.8 ± 5.1 |
| $Vd^{I}$[l] | 206 | 216 | 119 | 98 | 74 | 126 | 271 | 256 | 202 | 63 | 163 ± 24 |

[a] As oxalate salt (I). [b] Estimated from best fit of experimental plasma levels, $[I] = A'e^{-\alpha' t} + Ae^{-\alpha t} + Be^{-\beta t}$ in ng/mL *versus* time $t$. [c] For most studies only the latter two exponentials were needed, *i.e.*, $A' = 0$. However a third exponential was needed in three cases; estimated values of $\alpha'$ in $min^{-1}$ ($t_{1/2}^{\alpha}$ in min) were: dog A 0.371 (1.87); dog D 0.153 (4.52); dog D 0.243 (2.85). [d] Ratio of dose to total area under plasma level of I–time curve, where the calculated $AUC_{\infty}^{I} = A'/\alpha' + A/\alpha + B/\beta$ and that calculated from the trapezoidal rule plus the quotient of the last observed plasma level, $p_n$, and $\beta$ were within 2% of each other in all cases. [e] Estimates from slopes of ng excreted renally/min against plasma level in ng/mL at the midtime of the interval of excretion agreed with estimates from the slope of cumulative amounts renally excreted at time $t$ plotted against the area under the plasma level–time curve ($AUC_t$) at that time in accordance with $\Sigma U = CL_{ren}^{I}$ ($AUC_t - AUC_0$) (see Figs. 3 and 4). [f] Value of AUC at $\Sigma U = 0$ from the best linear plot of prior data in Figs. 3 and 4. [g] Estimated from appropriate plots (Fig. 7) in accordance with $[II]/AUC_I = CL^{I \to II}/V_{II} - (CL^{II \to X}/V_{II})(AUC_{II}/AUC_I)$. [h] Calculated from $\Sigma U_{\infty} = CL_{ren}^{I}$ ($AUC_{\infty} - AUC_0$). [i] Determined by best fit of terminal urine data to $\Sigma U^{III} = \Sigma U_{\infty}^{III} - \Sigma U_{\infty} e^{-kIII t}$. [j] Estimated from the $AUC^{II}$ calculated from the trapezoidal rule plus the quotient of the terminal plasma level of II and the estimated terminal rate constant of $3.08 \times 10^{-3}$ $min^{-1}$. $kV_C^I = D/(A' + A + B)$. [k] $CL_{tot}^{II}/\beta$. [m] Data were widely scattered at the low level of assay detection; this value is only a crude estimate. [n] Due to equipment failure, urinary amounts could not be assayed. [o] Final urinary amounts excreted were higher than predicted from the best linear fit of $\Sigma U = CL_{ren}^{I}$ ($AUC - AUC_0$). [p] Bile-cannulated female dog. [q] Renal catheter improperly placed in female dog so that urine could not be obtained at timed intervals. [r] Average only for the 70-mg doses of all normal dogs; bile-cannulated dog E excluded. [s] Average excluded 35-mg doses and bile-cannulated dog E. [t] Average excluded outlier value for dog B at 35-mg dose. [u] Average excluded outlier dog C at 53.6-mg dose. [v] Average is $10^{-4}$ $AUC^{II}$ adjusted for 70-mg dose, *i.e.*, $10^{-4}$ AUC/70-mg dose; excludes the studies of dog D, which gave values significantly higher with average $10^{-4}$ AUC adjusted for 70-mg doses at 240 min and at infinity of 16.8 ± 3.0 and 20.4 ± 3.4, respectively. The overall averages, including dog D studies were 10.7 ± 1.5 and 13.0 ± 1.8 at 240 min and at infinity, respectively.

Plots in accordance with:

$$\ln (P_{\infty} - P) = -kt + \ln P_{\infty} \qquad \text{(Eq. 5)}$$

were linear for both I and II and of the same slope, where $P_{\infty}$ is the asymptotic peak height or area ratio of nafronyl or II to internal standard with time, $P$ is the peak height or area ratio of I or II at time $t$, and $t_{1/2} = 0.693/k$. The curves for nafronyl concentration *versus* time in fresh dog plasma were similar in the present studies when different anticoagulants were used. They decreased with decelerating rates to an asymptote, and changes in both I and II concentrations could be characterized by Eq. 5. The data are given in Table I. The use of different anticoagulants has no significant effect on the stability estimates of nafronyl in fresh dog plasma.

There were no interferences in the chromatograms of I and II by any of the anticoagulants used in these studies in the assay range of 0.4–4.0 μg/mL of injected solutions. Calibration curves constructed in plasma obtained from blood samples using the different anticoagulants did not show any significantly different regression statistics. A typical calibration curve by method B in the range of 0.4–4.0 μg/mL using a 50-μl sample of the supernatant from acetonitrile-denatured plasma showed a standard error of estimate for the assay from linear regression of ±43 and ±30 ng/mL for I and II, respectively. Correlation coefficients for six values were >0.9996.

There was no significant difference in the calibration curve statistics of I when materials were injected in the mobile phase or in the supernatant of acetonitrile-denatured plasma. However, II gave a 10% decrease in peak height ratio when injected in the supernatant of acetonitrile-denatured plasma than in mobile phase.

**Protein Binding**—Filtration (33%) of 2.0–16.0 μg/mL of I in spiked plasma water and plasma subsequently filtered through cones prefiltered with plasma containing the same concentration showed no detectable I in the respective filtrates. This inordinately high cone binding did not permit protein binding estimates of I by this method. Filtration (30%) of 2.0–16.0 μg/mL of II in plasma water gave the same concentrations of II in the filtrate as was in the plasma water, indicating insignificant cone binding of II. When the plasma containing II was filtered through the cone previously used to filter the plasma water, with the same concentration of the fraction bound, $f_b$, could be calculated from:

$$f_b = \frac{PHR_p - PHR_f}{PHR_p} \qquad \text{(Eq. 6)}$$

where $PHR_p$ and $PHR_f$ were the peak height ratios for the assay of the same volumes of each by method B in the plasma and filtrate, respectively. The respective plasma concentrations ($C_p$ in μg/mL) and fractions bound, $f_b$, were ($C_p$, $f_b$): 16.0, 0.762; 12.0, 0.777; 8.0, 0.751; 4.0, 0.767; and 2.0, and 0.815. If the last datum is excluded, since assay sensitivity was less at this lowest concentration, the average fraction of II bound to plasma protein is 0.764, and no concentration dependence is indicated.

**Red Blood Cell–Plasma Partition Coefficients**—The red blood cell-plasma partition coefficient $D'$ (Table II) of I was 2.00 when the anticoagulants citrate phosphate dextrose solution, oxalate, and EDTA were used. This indicated a specific binding to erythrocytes in addition to volume partitioning. When the blood samples were heparinized, there was a significant decrease in the partition coefficient to 1.2, indicating a possible competition between I and heparin for these erythrocyte binding sites.

The red blood cell–plasma partition coefficient of II (Table II) averaged 0.49 (excluding one outlier of 1.15) when the anticoagulants citrate phosphate dextrose solution, oxalate, and EDTA were used. If the fraction of II bound to protein, $f_b$, was taken as 0.764, the true partition coefficient, $D$, between the concentration in red blood cells, $C_{RBC}$, and plasma water, $C_{PW}$, would be 2.08, indicating an erythrocyte binding in addition to volume partitioning. Since:

$$D' = C_{RBC}/C_p = \frac{C_{RBC}}{C_{PB} + C_{PW}} \qquad \text{(Eq. 7)}$$

where $C_{PB}$ is the concentration of II bound to plasma protein, and:

$$1 - f_B = \frac{C_{PW}}{C_{PB} + C_{PW}} \qquad \text{(Eq. 8)}$$

thus:

$$D = \frac{C_{RBC}}{C_{PW}} = D'/(1 - f_b) = 0.49/0.236 = 2.08 \qquad \text{(Eq. 9)}$$

When the blood samples were heparinized, there was an apparent inhibition of the partitioning of II into the red blood cells from plasma.

**Plasma Pharmacokinetics and Possible Dose Dependencies**— Plasma level–time data for nafronyl (I) were fitted to a sum of exponentials. A typical

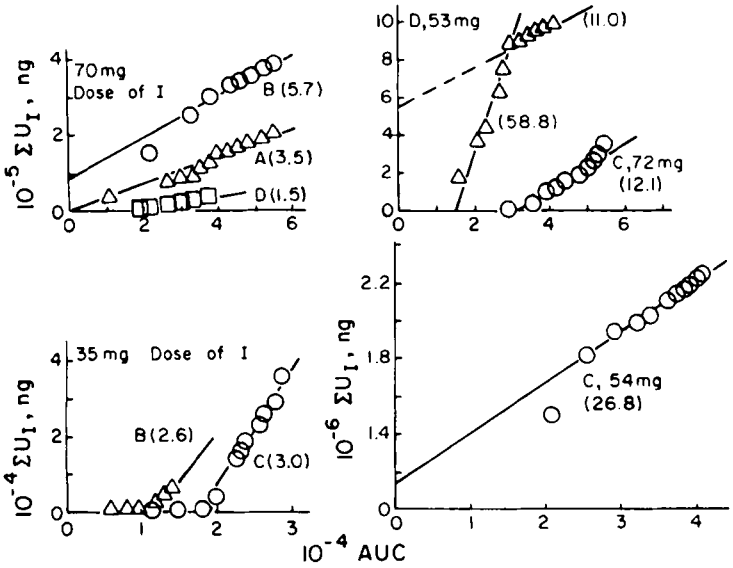


**Figure 3**—*Renal clearance plots for I in accordance with Eq. 11, where $AUC_0^I$ (ng·min/mL) is the area under the plasma level–time curve from the intercept of the linear plots. The plots are labeled with the dog identification, nafronyl dose, and the estimated $CL^I_{ren}$ values in mL/min (in parentheses).*

example is given in Fig. 1, and the parameters of best fit in accordance with:

$$C(\text{ng/mL}) = A'e^{-\alpha' t} + Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 10)}$$

are listed in Table III for the various studies. In most cases, only two exponentials were needed.

The plasma level–time data for I and its derived acid (II) as ng/mL/mg of the dose of I are plotted semilogarithmically against time for the studies at various doses in dogs B, C, and D (Fig. 2). Such plots of I and II for three different doses were superimposable for dog C, indicating no dose dependency of nafronyl pharmacokinetics. Whereas nafronyl (I) and the acid (II) plasma levels/mg of dose were higher at the 70-mg dose than at the 35-mg dose in dog B, the reverse was observed in dog D. Thus, dose-dependent pharmacokinetics of nafronyl (I) and its derived acid (II) cannot be concluded.

The apparent parallelism, in general, of these semilogarithmic plots of plasma levels against time for the various doses (Fig. 2) indicates that there are insignificant differences in the fitted rate constants ($\alpha$ and $\beta$) within a dog, notwithstanding the apparent differences in the best-fit parameters (Table III). The terminal plasma levels at the lower doses have the expected higher errors in their estimation since their levels approach the limits of valid detection. The half-lives of the biphasic curve for nafronyl averaged 12 ± 12 (*SEM*) min for $\alpha$ and 100 ± 18 min for $\beta$.

The apparent volumes of distribution references to total plasma concentration of nafronyl averaged 39.8 ± 5.1 (*SEM*) and 163 ± 24 (*SEM*) L for the central compartment and total body, respectively (Table III). These values are in high excess of extracellular and total body water, respectively, indicating a high degree of sequestration in body tissues.

**Clearances**—Total body clearances of nafronyl ($CL^I_{tot}$ = Dose/$AUC_\infty$) averaged (excluding outlier dog B at the 35-mg dose) 1295 ± 65 (*SEM*) mL/min. The renal clearances of nafronyl ($CL^I_{ren}$), estimated from both the slopes of plots of the rates of urinary excretion ($\Delta U^I/\Delta t$) against plasma levels at the midpoint of the urinary collection interval ($p_{t\,mid}$) and the slopes of plots of cumulative amounts excreted ($\Sigma U^I$) against area under plasma level–time curves ($AUC^I$), were not significantly different and averaged 8.3 ± 3.0 mL/min, which would indicate a high protein binding if unbound drug was excreted solely by glomerular filtration. These latter plots (Fig. 3) frequently did not go through the origin and could be best characterized by a straight line conforming to the equation:

$$\Sigma U = CL_{ren}(\text{AUC} - \text{AUC}_0) \qquad \text{(Eq. 11)}$$

where $AUC_0$ is the area under the plasma level–time curve at $\Sigma U = 0$ as estimated from the extrapolation of the best linear plot of $\Sigma U$ *versus* AUC. The plots for $\Delta U/\Delta t$ *versus* $p_{t\,mid}$ which gave the same estimates of renal clearance, were linear for the lower plasma concentrations and did go through the origin. There were no significant urinary pH changes within a study and no statistical dependence of apparent renal clearance $(\Delta U/\Delta t)/p_{t\,mid}$ on urine flow rate.

A rational explanation for the non-zero intercepts of the linear plots in

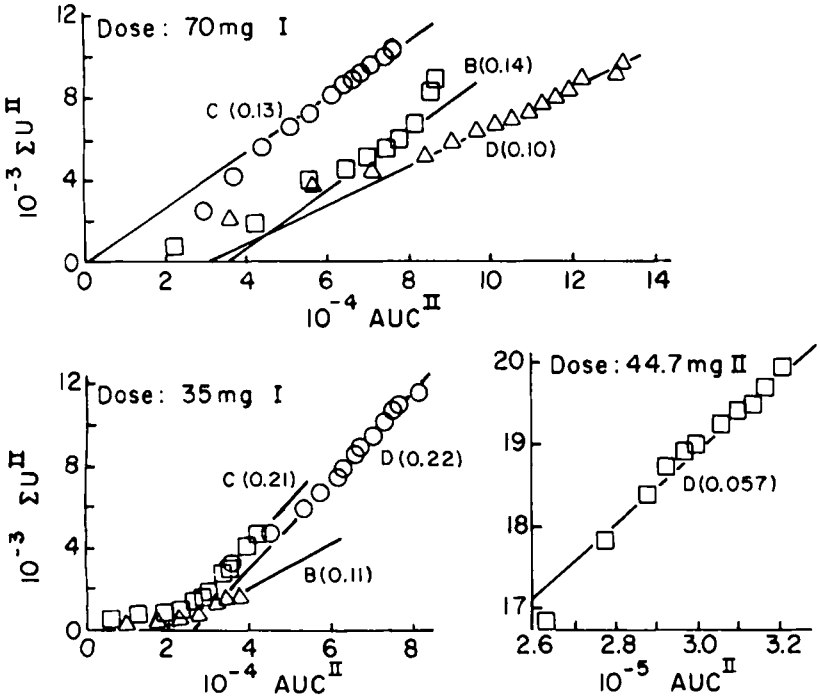


**Figure 4**—*Renal clearance plots for II (dose equivalent to 74.8 mg of nafronyl oxalate) in accordance with Eq. 11, where $AUC_0^{II}$ is as defined in Fig. 3. The plots are labeled with the dog identification, dose, and the estimated $CL^{II}_{ren}$ values in mL/min (in parentheses).*

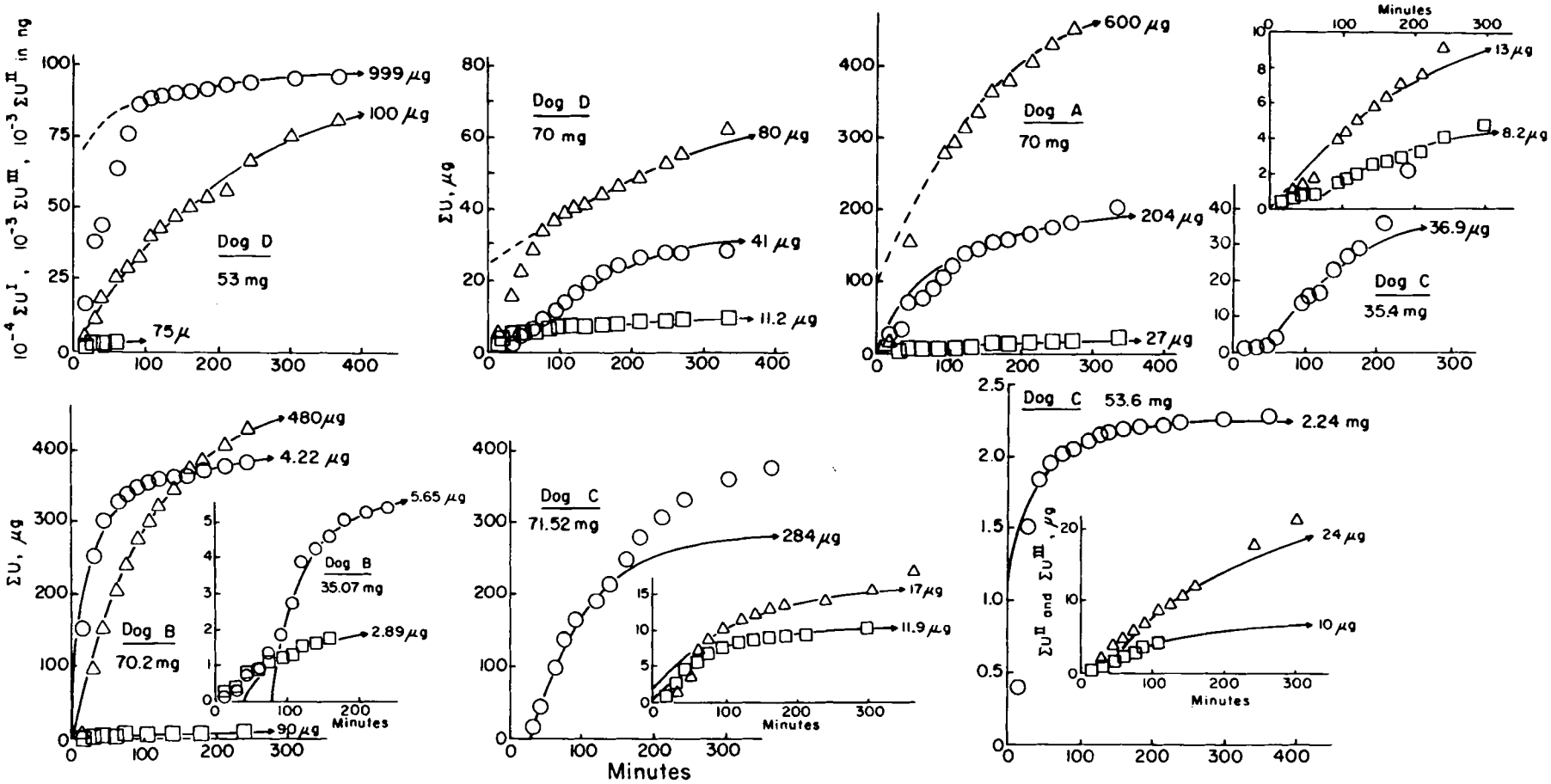


**Figure 5**—*Cumulative amounts of I (○), II (□) and the glucuronide III (△) excreted into the urine after intravenous administration of a labeled dose of I. The curves through the data for I and II were calculated from* $\Sigma U = CL_{ren}(AUC - AUC_0)$ *for the parameters in Table III. The curves through III were calculated from* $\Sigma U^{III} = \Sigma U_\infty - \Sigma U'^{III}_\infty e^{-k_{III}t}$ *using the parameters in Table III.*

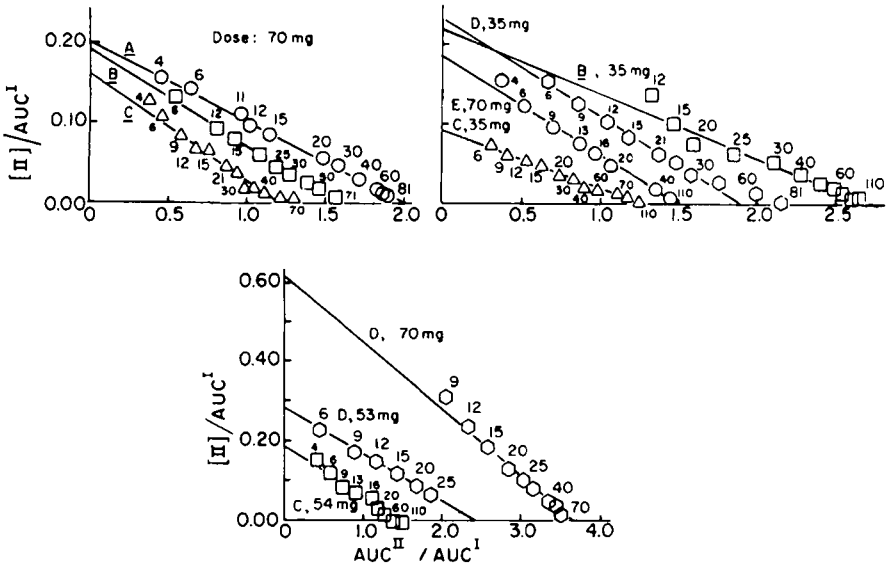


**Figure 6**—*Plots of the quotients of II plasma levels and the area under the plasma level-time curve of I against the ratios of the areas of II and I until that time in accordance with* $[II]/AUC^{I} = CL^{I\rightarrow II}/V_{II} - (CL^{II\rightarrow X}/V_{II})(AUC^{II}/AUC^{I})$ *where* $CL^{I\rightarrow II}$ *and* $CL^{II\rightarrow X}$ *are the clearances of I to II and of II, respectively, and where* $V_{II}$ *is the apparent volume of distribution of II. The curves are labeled with the intravenous dose of I and the dog identification; each datum point is labeled with the time after administration.*

accordance with Eq. 11 is that monitored plasma levels did not reflect those at the sites of renal clearance at early time periods when pseudo-steady-state conditions had not been achieved for the drug. The hypothesis of decreasing concentration gradient from the plasma monitoring site to the glomerulus is a reasonable explanation for this renal lag. This is also consistent with the possible extrahepatic clearance of nafronyl, supported by its 3.2-h half-life in plasma *in vitro* (1). In one dog study (dog D, 53.0-mg dose) the assays of initial plasma levels of I from the femoral vein were consistently less than those taken simultaneously from the jugular [time(min), femoral, jugular (ng/mL)]: 4, 595, 1318; 6, 477, 950; and 15, 185, 536. This has been observed previously with other highly metabolized and quickly cleared drugs. An example is sulmazole (10) where the initial early plasma levels from the femoral and jugular veins did not agree. Drugs that are not highly metabolized and are more slowly cleared do not show significant differences between levels from jugular and femoral plasma. An example is the immunomodulatory 1,2-*O*-isopropylidene-3-*O*-3′-(*N*′,*N*′-dimethylamino-*n*-propyl)-D-glucofuranose hydrochloride (11).

The hepatic flow in the dog is in the range of 30–45 mL/min/kg [750–1125 mL/min for a 25-kg dog (12)]. If there is no biliary excretion of unchanged nafronyl, its metabolic clearance, $CL^{I}_{tot} - CL^{I}_{ren}$, referenced to total plasma

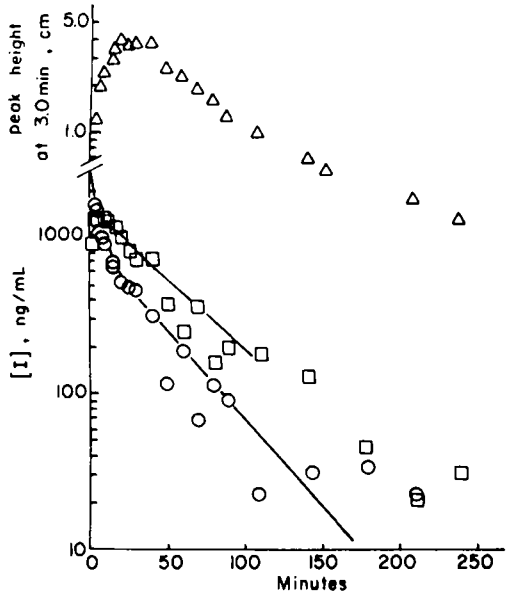


**Figure 7**—*Plasma concentrations of I (○) and II (□) in equivalents of nafronyl oxalate against time for bile-cannulated dog E after a 70.5-mg iv bolus dose of I. The curve through I was calculated from the sum of exponentials (Eq. 10); the curve through II was calculated from Eq. 15. The peak height at retention time 3.00 min of IV, which differed from I (10.9 min), II (3.90 min), and the internal standard (6.15 min), is also plotted against time (△).*

concentration is 1287 mL/min. Since this value exceeds even the highest expected extreme of maximal hepatic flow rate, which is the maximal hepatic clearnace possible, extrahepatic metabolism of nafronyl is indicated.

The renal clearances of the derived acid ($CL^{II}_{ren}$) were obtained by the methods described for nafronyl and averaged even lower, 0.18 ± 0.04 mL/min. This would imply a renal clearance of unbound II of 0.8 mL/min (0.18/fraction unbound), indicative of a high excess of tubular reabsorption. Generally, an $AUC_0$ was observed in accordance with Eq. 11 (Fig. 4). The assay of initial plasma levels of II from the femoral vein were consistently less than those taken simultaneously from the jugular [time(min), femoral, jugular (ng/mL in nafronyl equivalents)]: 4, 807, 1930; 6, 1116, 1930; and 15, 1324, 1789.

**Urinary Excretions**—The percentage of the dose renally eliminated at 330 min and the estimated total eliminated at infinite time are given in Table III. The latter values for I and II were estimated from Eq. 11 for $AUC = AUC_\infty$ using the estimates of $CL_{ren}$ and $AUC_0$ given in Table III. Extremely small percentages of the dose are eliminated by this route; averaging 0.48 ± 0.25% for nafronyl (I) and 0.021 ± 0.004% for the derived acid (II). Plots of the cumulative urinary excretion of I and II with time are given in Fig. 5 for the various studies. The curves through the data were drawn in accordance with Eq. 11 for the particular $AUC_0$ and $CL_{ren}$ values given in Table III.

Semilogarithmic plots of amount not-yet-excreted of the conjugate of II, (*i.e.*, III), assayed as II for solvolysis, against time ($\Sigma U^{III}_\infty - \Sigma U^{III}$) were linear after the first 10–70 min, for a chosen $\Sigma U^{III}_\infty$ in accordance with:

$$\ln (\Sigma U^{III}_\infty - \Sigma U^{III}) = \ln \Sigma U^{III}_\infty - k_{III}t \qquad \text{(Eq. 12)}$$

where $\Sigma U^{III}_\infty$ is obtained from the intercept of the extrapolated linear terminal data. The estimated $\Sigma U^{III}_\infty$, $\Sigma U'^{III}_\infty$, and $k_{III}$ values of urinary excretion of the III are given in Table III. The cumulative amounts of renally excreted conjugates are plotted against time in Fig. 5. The curve through the data is plotted in accordance with the nonlogarithmic transformation of Eq. 12:

$$\Sigma U^{III} = \Sigma U_\infty - \Sigma U'_\infty e^{-k_{III}t} \qquad \text{(Eq. 13)}$$

for the specific parameters listed in Table III and, as expected, gave excellent fits. The terminal half-lives of urinary excretion of III derived from $k_{III}$ averaged 145 ± 18 min. The percentage of the dose renally eliminated as III (the conjugates of II) were the $\Sigma U^{III}_\infty$ values and averaged 0.32 ± 0.12%.

Since the total amounts of these three compounds (I, II, and III) were only 0.82% of the total administered nafronyl dose, it is apparent that >99% of the administered dose is eliminated by processes other than renal and/or as other metabolites in the dog.

**Relationships Between Metabolic Clearances of Nafronyl (I) and the Total Clearance of Its Acid Metabolite (II), and the Fitting of II Plasma Levels with Time**—If there are no dose-dependent pharmacokinetics of I and its produced acid metabolite, a relation can be constructed (13, 14) from the constant metabolic clearance of nafronyl to the metabolite, $CL^{I\rightarrow II}$, and for the constant total clearance of the metabolite, $CL^{II\rightarrow X}$, as:

$$II = CL^{I\rightarrow II}AUC^{I} - CL^{II\rightarrow X}AUC^{II} \qquad \text{(Eq. 14)}$$

to calculate the amount of metabolite II in the body at any time, where $AUC^{I}$ and $AUC^{II}$ are the areas under the plasma level-time curves of I and II at that time. The amount of II can be formulated as $V_{II}[II]$ on the presumption that

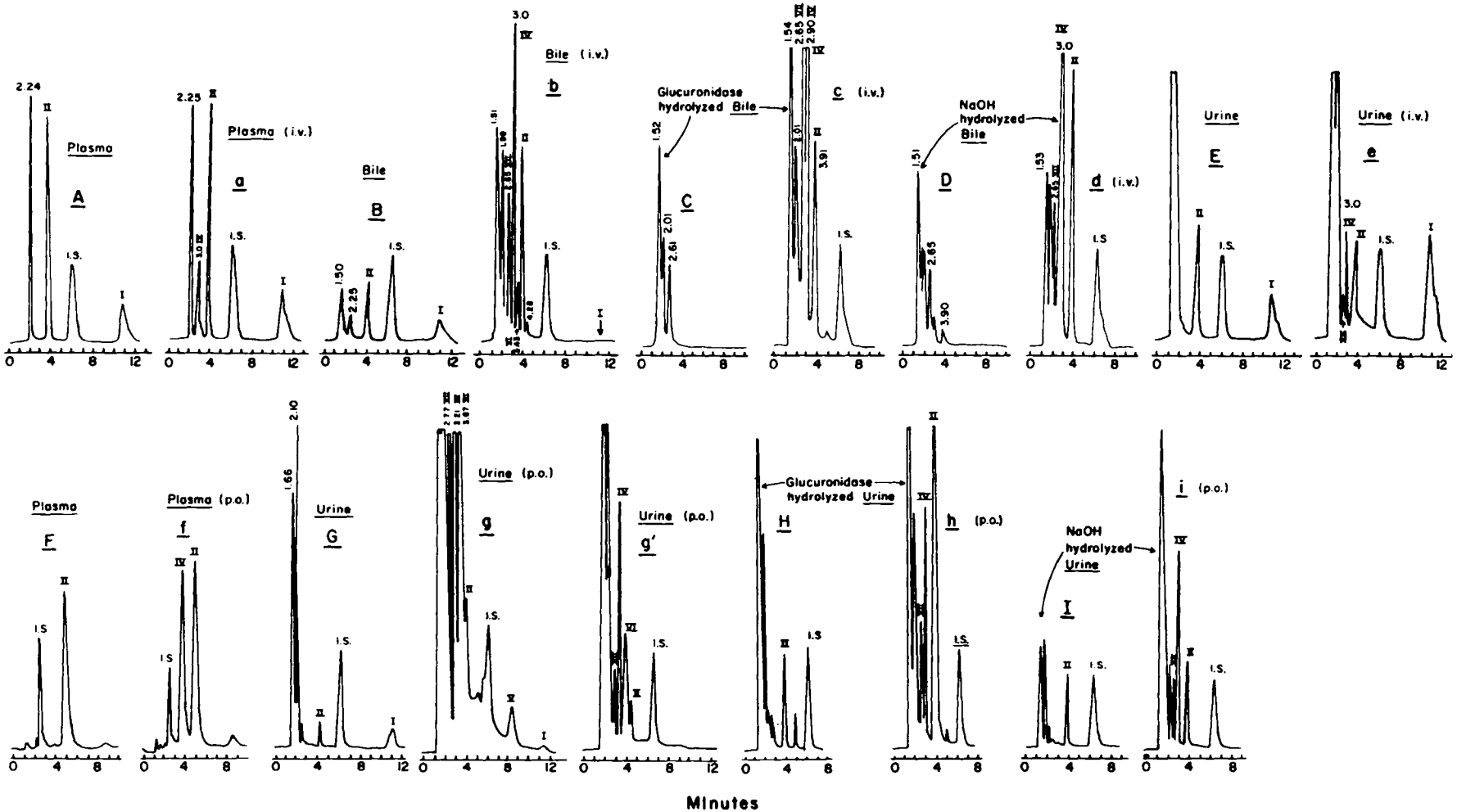


**Figure 8**—*Chromatograms (method A2) of organic extracts of plasma, urine, and bile, alone or spiked with I and II, from normal dogs (A–E) and from bile-cannulated dog E (a–e) after a 70.46-mg iv bolus dose of I. The internal standard (I.S.) was butacaine. Numbers at peaks represent retention times. Key: (A) plasma spiked with 325 ng/mL of I and 328 ng/mL of II; (a) plasma sample at 40 min; (B) nonhydrolyzed bile spiked with 250 ng/mL of I and 101 ng/mL of II; (b) nonhydrolyzed bile sample at 200 min; (C) blank bile treated with β-glucuronidase; (c) β-glucuronidase-treated bile sample at 214 min; (D) Blank bile treated with 0.2 M NaOH; (d) 0.2 M NaOH-treated bile sample at 214 min; (E) urine spiked with 752 ng/mL of I and 303 ng/mL of II; (e) urine sample at 190 min. Comparisons of chromatograms of organic extracts of plasma and urine spiked with I and II (identified (F–I) with samples taken from a dog orally administered 250 mg of I (f–i) are also presented. Key: (F) plasma spiked with procaine (internal standard) and 530 ng/mL of II using method A1; (f) plasma sample of dog C at 140 min; (G) urine spiked with butacaine (internal standard), 103 ng/mL of I, and 84 ng/mL of II using method A2; (g) urine sample of dog D at 201 min; (g') urine sample at 482 min; (H) urine spiked with butacaine and 303 ng/mL of II, treated with β-glucuronidase using method A2; (h) β-Glucuronidase-treated 160-min urine samples from dog D; (I) urine spiked with butacaine and 788 ng/mL of II, treated with 0.2 M NaOH using method A2; (i) 0.2 M NaOH-treated 270-min urine sample from dog D.*

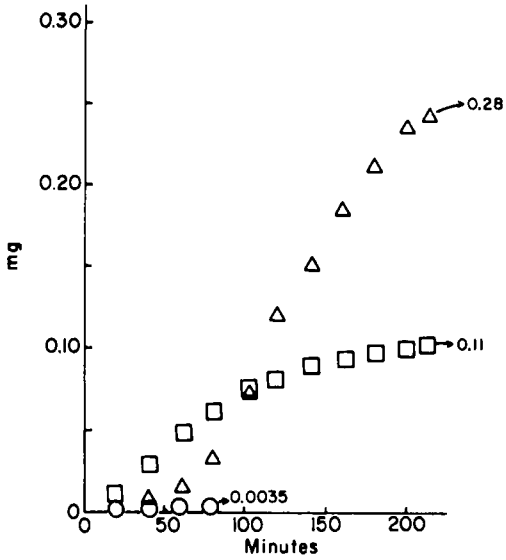


**Figure 9**—*Plots of cumulative amounts of I (○), II (□), and III (△) excreted into bile with time in the study of bile-cannulated dog E. The curves are labeled with the estimated total amounts eliminated in bile.*

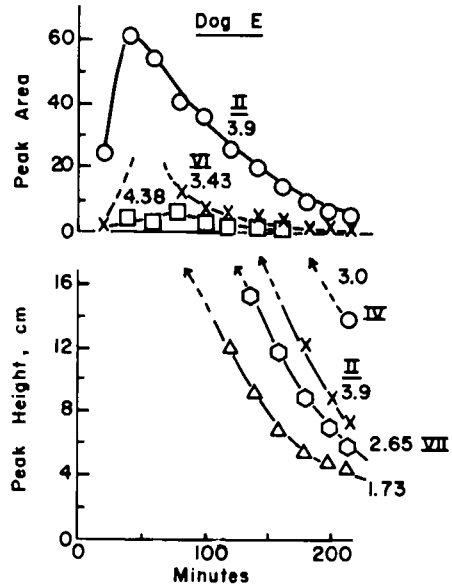


**Figure 10**—*Time course of peak heights and areas of peaks, labeled with retention times, appearing in chromatograms (method A2) of nonhydrolyzed bile taken from dog E (administered 70.46 mg iv of I) that did not appear in bile from the control dog. The dashed lines indicate higher values that could not be estimated. Values for II (3.9-min retention time) are given for comparison.*

the formed II is rapidly equilibrated in a one-compartment body model of volume $V_{II}$ and plasma concentration [II]. Thus, Eq. 14 can be rearranged to:

$$[II] = \frac{CL^{I\to II}}{V_{II}} \cdot AUC^{I} - \frac{CL^{II\to X}}{V_{II}} \cdot AUC^{II} \qquad \text{(Eq. 15)}$$

or:

$$\frac{[II]}{AUC^{I}} = \frac{CL^{I\to II}}{V_{II}} - \frac{CL^{II\to X}}{V_{II}} \cdot \frac{AUC^{II}}{AUC^{I}} \qquad \text{(Eq. 16)}$$

Thus, if the premises are valid, plots of the quotient of acid metabolite plasma concentrations and the area under the plasma level–time curve of nafronyl ($[II]/AUC^{I}$) against the ratios of the respective areas of II and I ($AUC^{II}/AUC^{I}$) should give a straight line where $CL^{I\to II}/V_{II}$ and $CL^{II\to X}/V_{II}$ can be obtained from the intercept and slope, respectively.

Such plots for the various studies in the dogs are given in Fig. 6, and their linearities are consistent with the premises. The derived clearance/volume ratios are listed in Table III. The $CL^{II\to X}/V_{II}$ ($= 0.693/t_{1/2}$) ratio also has the significance of an elimination rate constant for II. The average of the half-lives (Table III) for this II elimination was 6.4 ± 0.5 min.

The total areas under the curves for II (obtained by the trapezoidal rule plus terminal plasma divided by terminal rate constant for II) per 70-mg dose were consistent for dogs A, B, and C at all doses and averaged 9.83 ± 0.57 × $10^4$ ng·min/mL for a 70-mg dose of I. Dog D showed a significantly higher total area under the curve for II, which averaged 26.4 ± 3.4 × $10^4$ ng·min/mL for a 70-mg dose of I. This implies that dog D has a greater metabolic formation of II from I and/or a smaller apparent volume of distribution, $V_{II}$, for the acid metabolite.

The validity of this development was challenged by calculating plasma levels of II from Eq. 15 using the obtained parameters given in Table III. The curves drawn through the plasma level [II] against time in Figs. 1, 2, and 7 are based on these calculations and are consistent with the actual data for a significant portion of their time course (*i.e.*, up to 110 min), but not for all. This can be reconciled by postulating that $V_{II}$ is the volume of the central compartment for the acid metabolite, II, and $V_{II}$ is not representative of the apparent overall volume of distribution of II when the slow equilibration of II among other tissues becomes significant.

**Pharmacokinetic Study in Bile-Cannulated Dog E**—In the pharmacokinetic study of bile-cannulated dog E, a single injection method (method A2) was used for the simultaneous assay of both II (retention time, 3.90 min) and I (10.9 min) with butacaine as internal standard (6.15 min). Plasma (Fig. 7) and bile were assayed for both I and II. An additional peak (IV) at 3.00-min retention time was observed in plasma samples (compare Fig. 8 A and a). The maximum concentration of IV (Fig. 7) in the plasma was observed at 25 min, in comparison with the 9-min maximum of II in plasma, with a peak height equivalent to IV to that of 350 ng/mL of II at retention time 3.90 min in the plasma. It is apparent that an unknown metabolite (IV) can be observed in plasma that lags after II. This could be a metabolite derived from II.

The cumulative amount of II excreted in the bile can be estimated as 0.16% of the dose (0.14% at 214 min). The low amount of I was 0.005% (0.0043% at 80 min) (Fig. 9). Additional HPLC peaks were observed on bile assay after drug administration (Fig. 8b). These peaks did not appear in the bile assay when drugs were not administered (Fig. 8B). The chromatograms of the extracts (before hydrolysis) gave peaks at 1.5, 1.73, 1.99, 2.65 (VII), 3.0 (IV), 3.43 (VI), and 4.38 min, as well as the very small amounts of I (10.9 min) and II (3.9 min). The time courses of the concentrations of these compounds of different retention times, in terms of peak height or peak area, are shown in Fig. 10 at the times they could be evaluated. Since bile at each time interval was very constant (3.4 ± 0.2 mL/20 min), these peak heights and areas are good estimates of the relative rates of elimination. The dashed lines in Fig. 10 indicate peak heights or areas that were so out of scale that they could not be estimated. For purposes of comparison, the peak heights and areas of II from the bile samples are also given. It is apparent that there were large amounts of the same 3.0-min retention time material (IV) that could be detected in plasma (Figs. 7 and 8a). It must be concluded that multiple organic-extractable metabolites derived from nafronyl are excreted into the bile in significant quantities and have the potential of being quantified by HPLC.

The extracted bile samples were subjected to alkaline (0.2 M NaOH) and β-glucuronidase (*E. coli*) hydrolysis and again extracted with methylene chloride. These extracts were assayed and showed significant peaks at 3.0 (IV), 3.6 (VI), and 3.9 (II) min for β-glucuronidase hydrolysis (Fig. 8, C and c) and at 3.0 (IV) and 3.9 (II) min for alkaline hydrolysis. There may be others, but the background from similarly treated bile samples from the nondosed animal makes it difficult to determine conclusively if the metabolites were present in the extracts of hydrolyzed bile. The presence of II in these hydrolyzed bile extracts is conclusive proof of conjugates of II, *i.e.*, III, excreted into bile; this can be estimated as 0.40% (0.34% at 214 min) of the administered nafronyl dose (Fig. 9). This estimated yield of III was based on the assay of the extract of alkaline-hydrolyzed bile, preextracted for II since the HPLC base line at 3.90 min (Fig. 8d) was so much better than that of the extract of β-glucuronidase-treated bile (Fig. 8c). The question is yet unanswered as to whether the presence of IV is due to its release from a conjugate or due to its incomplete extraction from the bile prior to the hydrolysis. The total areas under the respective peaks up to 214 min after drug administration were 215 (IV, 3.0 min) as compared with 149 (II, 3.9 min) after β-glucuronidase treatment. The 3.6-min (VI) peak total area was 36.5. The total areas up to 214 min were 248 (IV, 3.0 min) as compared with 278 (II, 3.9 min) after alkaline hydrolysis of the bile. The 2.65-min (VII) peak total area was 118.

In general,the dichloromethane extracts of β-glucuronidase-treated samples showed large backgrounds; the 3.0-, 3.4-, and 3.9-min peaks did not arise from a constant baseline, so peak area quantification of these peaks may not be accurate. This may possibly explain, in part, the difference in the estimates of these peak areas from the two methods of hydrolysis (Fig. 11).

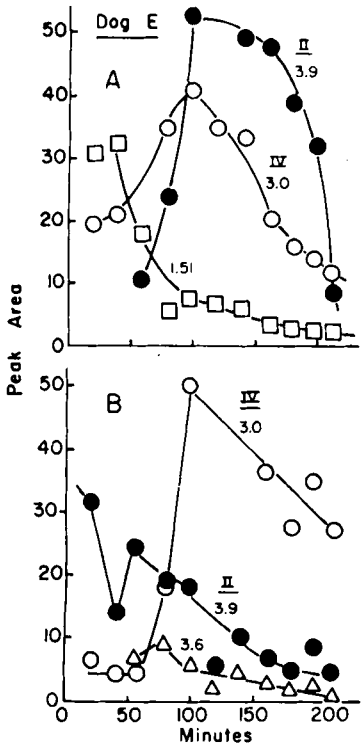


**Figure 11**—*Time course of peak areas, labeled with retention time, appearing in chromatograms (method A2) of hydrolyzed bile taken from dog E (administered 70.46 mg iv of I) that did not appear in hydrolyzed bile from the control dog. Values for II are given for comparison. Key: (A) β-glucuronidase-treated bile; (B) 0.2 M NaOH-treated bile.*

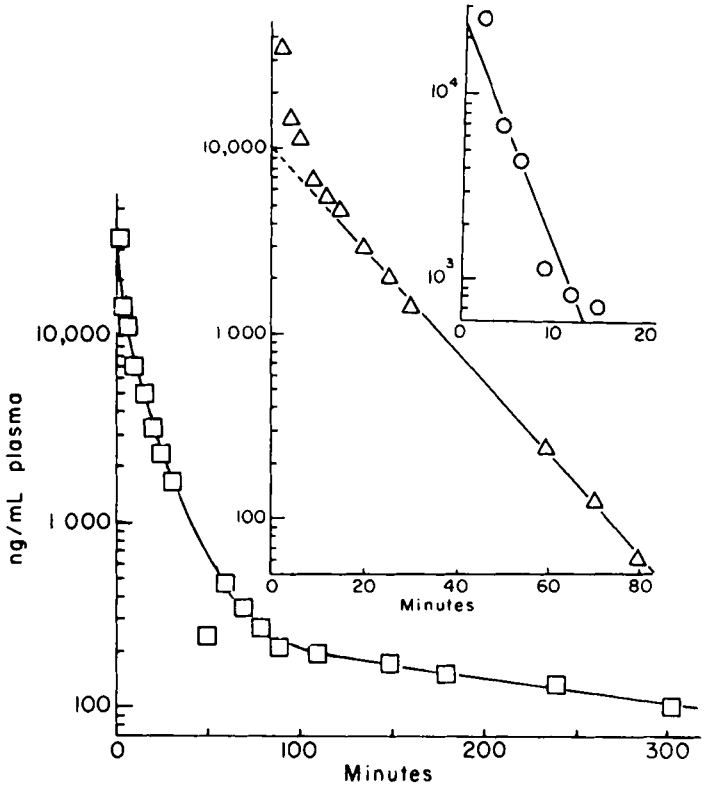


**Figure 12**—*Plasma levels of II, in equivalents of nafronyl oxalate, against time for a 44.7-mg iv (74.8-mg equivalent) bolus dose of II given to dog D. The curve through the data points was calculated from the sum of exponentials (see text). The insets show the successive feathering necessary to obtain the parameters of the three exponentials.*

Unfortunately the urinary catheterization of female dog E did not permit the collection of urine at different intervals of time. Nevertheless, assay of the organic extracts of the few urine samples obtained show a compound (IV) at a retention time of 3.0 in addition to those peaks assignable to I (10.9 min) and II (3.9 min); this peak did not exist in spiked urine from the same dog (compare E and e, Fig. 8). The 2.66-min peak (VII) observed in extract of nonhydrolyzed and alkaline-hydrolyzed bile was also observed.

**Pharmacokinetics of an Intravenously Administered Bolus of II**—The plasma levels of II on administration of 44.7 mg iv (74.8 mg as equivalents of nafronyl oxalate, I) of II are plotted against time in Fig. 12. The data were fitted to the sum of three exponentials for time $t$, in min, to calculate plasma levels in ng/mL as equivalents of nafronyl oxalate by:

$$[\mathrm{II}],\ \mathrm{ng/mL} = 24{,}500e^{-0.280t} + 10{,}000e^{-6.34\times10^{-2}t} + 270e^{-3.08\times10^{-3}t} \qquad \text{(Eq. 17)}$$

to demonstrate half-lives of the three respective phases as 2.47, 10.91, and 225 min. The total clearance of II is 225 mL/min. The area under the curve per milligram of nafronyl equivalent dose is 4451 ng·min/mL per milligram.

The renal clearance of II from a plot (Fig. 4) in accordance with Eq. 11 was 0.057 mL/min, the same value obtained from the slope of the linear plot with zero intercept of the rate of urinary excretion against the plasma level at the midpoint of the urine collection interval. The estimated total urinary excretions were 0.027 and 0.441% of the dose, unchanged and as the glucuronide, respectively. The terminal rate constant of renal elimination of the glucuronide, $k_{III}$, was $9.59 \times 10^{-3}$ min$^{-1}$ for a half-life of 72 min. In addition to [II] in the urine (retention time, 4.0 min), extremely large peaks due to other compounds were observed at ~1- and 2-min retention times.

The apparent volumes of distribution of the central and pseudo-steady-state volumes of distribution referenced to total plasma concentration are 2.15 and 73.0 L, respectively. The respective values for a 0.236 fraction unbound to plasma proteins are 9.1 and 310 L, respectively. The former value implies relatively instantaneous distribution in the total body water; the latter implies a high degree of sequestration in the relatively slowly equilibrated total body tissues.

**Estimation of the Extent of Nafronyl Metabolic Transformation through Its Acid Metabolite**—There are several methods to estimate the fraction of nafronyl generating its acid metabolite (II). The relative area under the plasma level-time curve of II for administered nafronyl (I) to administered acid (II) can estimate the fraction being metabolized by this route:

$$f_{\mathrm{I}\to\mathrm{II}} = \mathrm{AUC}^{\mathrm{II}}_{\mathrm{II\ admin}}/\mathrm{AUC}^{\mathrm{II}}_{\mathrm{I\ admin}} = 1404/4451 = 0.32 \qquad \text{(Eq. 18)}$$

where both the numerator and denominator are given per milligram of the administered dose of I and the latter is based on the $\mathrm{AUC}^{\mathrm{II}}_{\infty}$ average per 70-mg dose given in Table III.

The average of the ratio of total clearance of II to its apparent volume of distribution ($CL^{\mathrm{II}\to\mathrm{X}}/V_{\mathrm{II}}$), as based on application of Eq. 16 to the plasma concentration data obtained from the pharmacokinetic studies of intravenously administered I (Fig. 6), was $0.120 \pm 0.009$ min$^{-1}$. This value can also represent the apparent rate constant ($t_{1/2} = 6.4 \pm 0.5$ min) for the elimination of II and is of the same magnitude as the 10.9 min half-life observed for intravenously administered II (Fig. 12) during the significant portion of the time courses of I and II (Figs. 1, 2, 7) on which the estimations of $CL^{\mathrm{II}\to\mathrm{X}}/V_{\mathrm{II}}$ were based (Eq. 16). If $CL^{\mathrm{II}\to\mathrm{X}}$ is equated to the 225 mL/min observed in the pharmacokinetic study of II (Fig. 12), the apparent volume of distribution, $V_{\mathrm{II}}$, is estimated as $(0.120 \pm 0.009) \times 225 = 1875 \pm 245$ mL, which is not significantly different from the estimated apparent volume of distribution of the central compartment of II (Fig. 12) of 2151 mL. These considerations support the premise that the greater time course of II formation from I, with its concomitant elimination, occurs largely in the central compartment before significant equilibration of II among the tissues has been effected. The average of the ratio of the clearance of I *via* II to the apparent volume of distribution, $V_{\mathrm{II}}$, is $CL^{\mathrm{I}\to\mathrm{II}}/V_{\mathrm{II}} = 0.246 \pm 0.044$ min$^{-1}$ (Table III, Eq. 16, Fig. 6). Thus, if $V_{\mathrm{II}}$ is $1875 \pm 245$ mL, the estimated clearance ($CL^{\mathrm{I}\to\mathrm{II}}$) is $0.246 \times 1875 = 461$ mL/min. Since the average total clearance of I less its renal clearance is $CL^{\mathrm{I}}_{\mathrm{met}} = 1287$ mL/min, the fraction of I cleared to II is:

$$f_{\mathrm{I}\to\mathrm{II}} = CL^{\mathrm{I}\to\mathrm{II}}/CL^{\mathrm{I}}_{\mathrm{met}} = 461/1287 = 0.36 \qquad \text{(Eq. 19)}$$

This latter value is in reasonable agreement with the 0.32 value estimated from Eq. 18, implying that not all of the I is metabolized through systemically appearing II, that 60–70% of nafronyl has primary metabolites other than II, and/or that I and its derived metabolites are excreted in bile so as not to appear in the systemic circulation. The high metabolic clearance of I, in excess of the hepatic flow, could permit sequential metabolism within the first pass through the liver.

**Pharmacokinetics and Bioavailabilities of Orally Administered Nafronyl**—Extremely small concentrations of orally administered nafronyl (I) were observed in the plasma of dogs (Fig. 13). The apparent terminal half-lives

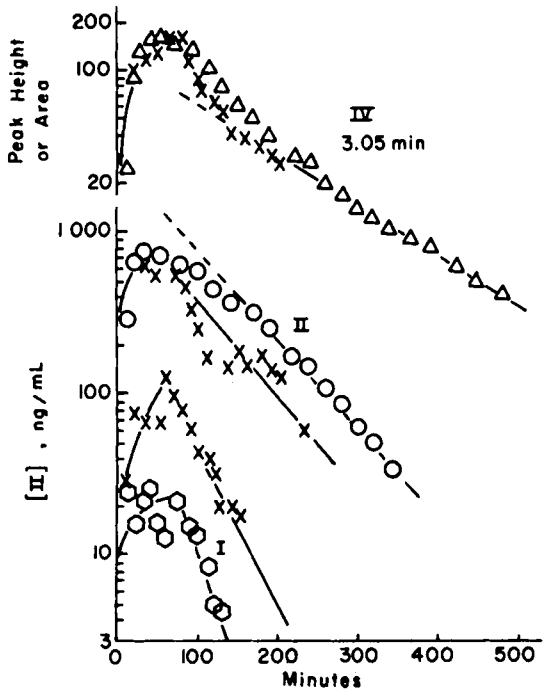


**Figure 13**—*Time courses of I, II (plotted as I equivalents), and IV in plasma after oral administration of 250 mg of I to dogs D (○, △, ○) and C (×), as assayed by the single-injection method A2 or its modification. The peak heights of IV at 3.05 min are plotted for dog D; the peak areas of IV at 2.48 min are plotted for dog C.*

(20–30 min) of I on oral administration were between those of the $\alpha$ (9 min) and $\beta$ (100 min) phases obtained on intravenous administration. The estimated bioavailabilities of I, determined from the ratios of the $AUC^{I}$/mg values (oral to intravenous), were 0.3–2.7% of the 250-mg oral dose in three studies (Table IV). When dog D was administered 502 mg, the dog regurgitated the material 25 min after administration.

The apparent terminal plasma half-lives of II after oral administration of 250 mg of I were 55–110 min (Figs. 13 and 14). The estimated bioavailabilities of II, determined from the ratios of the $AUC^{II}$/mg values obtained after oral administration of I to those obtained after intravenous administration of II, were 9.7–16.7%. It is apparent that there is a definite presystemic and/or first-pass metabolism of both I and II on oral administration. In one of the 250-mg po studies of dog C, a time lag of 100 min was observed before significant appearance of II in the plasma (Fig. 14).

In all dogs, a peak assignable to a compound other than I or II was observed in the plasma, but was not present in control plasma. This peak, IV, was observed with all HPLC methods. The chromatograms of extracted plasma for dog D, 250 mg po, were similar to those given in Fig. 8a. Chromatograms for dog D in the second study are given in Fig. 8F,f. The retention times of I, II, and IV from plasma extracts in other mobile phases and HPLC systems are listed in Table IV. The time course of IV in the plasma generally paralleled that of II (Figs. 13 and 14). Peak heights and areas of IV and II were very similar at a given time (Fig. 14). For purposes of comparison, the maximum peak height of IV for dog D, 250 mg (Fig. 13) at 3.05-min retention time, corresponded to a concentration of II (retention time, 4.03 min) of 678 ng/mL equivalents of I.

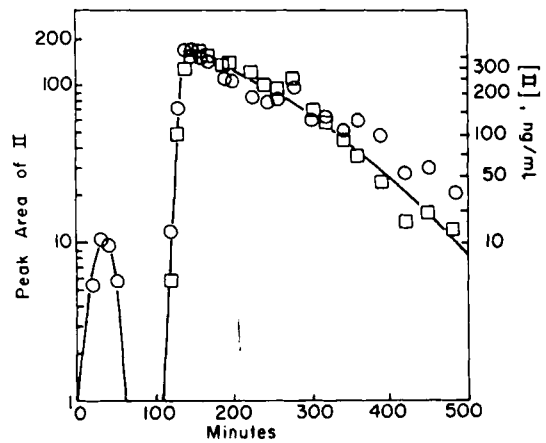


**Figure 14**—*Time course of peak areas of II (○) and IV (□) in extracts of plasma after oral administration of 250 mg of I to dog C. In this system the retention times of II and IV were 4.97 and 3.74 min, respectively. A 100-min lag period existed before significant absorption of I and II was observed.*

**Table IV—Parameters and Bioavailabilities of Orally Administered Nafronyl (I)**

| Dog (Weight, kg) | D(26.0) | C(23.8) | C(21.8) | D(25.0) |
|---|---|---|---|---|
| Dose, mg | 250 | 251 | 251 | 502 |
| $AUC^{I}_{\infty}$/mg dose[a] | 8.39 | 35.1 | 3.54 | —[f] |
| $AUC^{II}_{\infty}$/mg dose[a] | 744 | 470 | 430 | 440 |
| Bioavailability, % | | | | |
| I[b] | 0.65 | 2.71 | 0.27 | —[f] |
| II[c] | 16.7 | 10.6 | 9.7 | 9.9 |
| Dose recovery in urine, %[d] | | | | |
| I | 0.003 | 0.061 | —[g] | —[g] |
| II | 0.030 | 0.42 | —[g] | —[g] |
| Glucuronidase III | 0.28 | 0.07 | —[g] | —[g] |
| NaOH | 0.22 | 0.13 | —[g] | —[g] |
| Plasma terminal half-lives, min | | | | |
| I | 20 | 30 | —[h] | —[g] |
| II | 55 | 58 | 110 | 74 |
| IV | 105 | 105 | 78 | 47 |
| Assay Method | A2 | Modified A2[e] | A1 | A1 |
| Plasma retention times, min | | | | |
| I | 10.33 | 7.69 | —[i] | —[i] |
| II | 4.03 | 2.78 | 4.97 | 4.80 |
| IV | 3.05 | 2.48 | 3.74 | 3.57 |
| Urine retention times, min | | | | |
| I | 11.6 | 9.26 | —[i] | —[i] |
| V | 8.6 (small) | 7.81 | — | — |
| II | 4.26 | 2.96 | 4.92 | —[g] |
| VI | 3.7 | — | 3.76 | —[g] |
| IV | 3.20 | 2.51 | 5.20 | —[g] |
| VII | 2.78 | <2.08 | <2.8 | —[g] |
| Hydrolyzed urine retention times, min | | | | |
| II | 3.95 | 3.92 | —[j] | —[j] |
| IV | 3.00 | 2.98 | —[j] | —[j] |
| VII | 2.62 | 2.62 | —[j] | —[j] |

[a] Estimated total area in nafronyl equivalents under plasma level-time curve per mg of oral dose calculated from trapezoidal rule plus the quotient of terminal plasma level and the rate constant for the terminal loss of plasma concentration. [b] Calculated from 100-$AUC^{I}_{\infty}$/mg dose po ÷ $AUC^{I}_{\infty}$/mg dose iv, where the latter denominator is the total clearance, 1295 mL/min. [c] Bioavailability of II from the administered oral dose of I, calculated from 1.666-$AUC^{II}_{\infty}$/mg dose po ÷ $AUC^{II}_{\infty}$/mg dose iv, where the latter value is 4451/mg dose of II (as I equivalents) taken from the intravenous study. [d] Based on total amounts excreted in urine at 430 min in nafronyl equivalents; III was assayed as organic-extractable II after hydrolysis of previously extracted urine by either $\beta$ glucuronidase or 0.2 M NaOH. [e] Mobile phase composition and column differed, but single-injection method was used. [f] Peak for I merged with the peak for the internal standard and could not be readily quantified as to peak height or area. Also, dog regurgitated at 23 min after oral administration. [g] Too many interferences with the peaks for I and II to permit their quantification and/or identification in urine. [h] Plasma level data for I were too scattered to estimate. [i] The assay for I used a different mobile phase than the assay for II where IV was observed. Retention times for I were 4.2 min for dog C and 6.62 min for dog D. The urine studies for these dogs showed peaks in addition to those in blank urine for the mobile phase used to assay I, with decreasing peak height magnitudes at 1.85 > 3.86 > 2.63 > 3.55 > 4.20 (I) min for dog C and at 4.15 and 3.00 min for dog D. [j] Not studied.

The cumulative amounts of I and II excreted unchanged in the urine after oral administration of 250 mg of I were in the ranges of 0.003–0.061 and 0.030–0.42%, respectively. In addition to these compounds, extractable from nonhydrolyzed urine, IV, V, VI, and VII were identifiable at separate retention times in the urine extracts (Fig. 8G, g, and g′). Retention times in various mobile phases and systems are given in Table IV. The time courses of peak heights in sampled urine at these various retention times are given in Fig. 15 and can be compared with that for II. Some of these peaks were well out of the recording range for many of the collection intervals. Corresponding peaks for IV and VII had been observed previously in organic extracts of nonhydrolyzed urine samples obtained from a dog administered I intravenously (Fig. 8e). Corresponding peaks for IV, VI, and VII have been observed previously in organic extracts of bile from this dog (Figs. 8b, c, d).

$\beta$-Glucuronidase and NaOH treatment, after prior extraction of the urine, gave chromatograms of subsequent organic extracts (compare H and h and I and i in Fig. 8) that indicated significant amounts of II (3.95 min) derived from its glucuronide (*i.e.*, III) and additional higher peaks with retention times

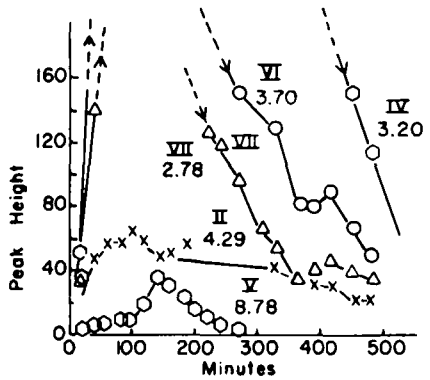


**Figure 15**—*Plots of peak heights against time of various chromatographic peaks obtained on assay (method A2) of urine extracts of dog D after oral administration of 250 mg of I.*

of 3.00 min (IV) and 2.62 min (VII). It is not possible to state if IV or VII were derived from glucuronides or if they were just not as completely removed as II in the extraction prior to hydrolysis. The cumulative amount of III in the urine (estimated as II after treatment) was 0.07–0.28% of the dose (Table IV).

**Summary of Pharmacokinetics of Nafronyl in the Dog**—Intravenously administered nafronyl (I) is rapidly cleared, with a total body clearance referenced to total plasma concentration of 1295 mL/min. Dose-dependent plasma pharmacokinetics of I and derived II could not be concluded in the 35–70-mg dose range. The plasma level–time data of I were usually well characterized by a sum of two exponentials with respective half-lives of 12 and 100 min. The latter was similar to the 90 min suggested for humans (7). The initial phase accounted for the major fraction (0.85) of the loss of I in plasma. The time course of derived II from I in the plasma could be quantified by a systemic metabolic clearance of I to II of 461 mL/min in an apparent volume of distribution, $V_{II}$, of the central compartment of II of 1.88 L, with a simultaneous total body clearance of II of 225 mL/min.

The estimated renal clearance of nafronyl was 8.3 mL/min with a renal elimination of only 0.5% of unchanged drug. This indicated a net metabolic clearance of I in excess of hepatic blood flow, favoring extrahepatic metabolism. Although the derived acid (II) was a major plasma metabolite of nafronyl, it could only account for 30–40% of the intravenously administered drug in the plasma and 0.02% in the urine, with a renal clearance of II of 0.18 mL/min. Thus, other metabolic transformations of nafronyl must exist that do not give direct formation of systemically appearing II.

The glucuronide (III) of II in the urine, liberated by β-glucuronidase or alkali, only accounted for 0.32% of the intravenous dose of I. The bile accounted for 0.005% of the dose as I, 0.16% of the dose as II, and 0.40% of the dose as III. Thus, >98.5% of the intravenously administered nafronyl dose is not eliminated as I, II, or III in bile and urine, and these compounds do not account for the principal fates for nafronyl disposition in the dog.

Considerable amounts of a chromatographically identifiable compound (IV) also extractable into dichloromethane, appeared in plasma after intravenous and oral nafronyl administration. It also appeared in normal and hydrolyzed preextracted bile and urine in considerable quantities and was consistent with the naphthol-containing carboxylic acid suggested previously to be a metabolite (4). Chromatographically identifiable, but as yet uncharacterized, compounds other than IV that were also extractable into dichloromethane appeared in normal (V, VI, and VII) and hydrolyzed preextracted (VII) urine and in normal (VI and VII) and hydrolyzed preextracted (VII) bile in apparently large quantities. Undoubtedly, these compounds can account for the major fates of nafronyl (I) and II in the dog. Other possible unextractable compounds could exist.

A study of the pharmacokinetics of intravenously administered II demonstrated a triphasic plasma level–time curve with respective half-lives of 2.5, 10.9, and 225 min. The initial two phases accounted for 99.2% of the loss of II in plasma. The total body and renal clearances were 225 and 0.057 mL/min, respectively. The percentages of the dose renally excreted and as glucuronide were 0.027 and 0.44%, respectively. Thus, II is also metabolized by routes other than glucuronidation.

The bioavailability of I after oral administration of 250 mg of nafronyl to the dog is only 0.3–2.7%. The percentage of potentially systemically available II was only 10–17%. These facts clearly demonstrate presystemic and/or first-pass metabolisms of both I and II.

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