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ACKNOWLEDGMENTS

The authors thank Dr. Walter Morozowich for valuable discussion on the synthesis of phosphate esters in general.

Dose-Dependent Pharmacokinetics of the Antihypertensive 2,3,4,4a-Tetrahydro-1*H*-pyrazino[1,2a]quinoxalin-5-(6*H*)-one in Dogs and Rats

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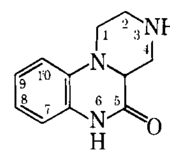
Received August 5, 1980, from the **Drug Metabolism Subdivision*, ‡*Biostatistics Section, Wyeth Laboratories, Inc., Radnor, PA 19087.* Accepted for publication July 22, 1981.

Abstract □ A sensitive and reproducible GLC assay was developed for determining 2,3,4,4a-tetrahydro-1*H*-pyrazino[1,2a]quinoxalin-5(6*H*)-one (I) in biological fluids, utilizing the electron-capturing capability of the heptafluorobutyryl derivative. After single 2.5- and 10-mg/kg oral and intravenous doses to three dogs, plasma concentration–time data for I were fitted to a biexponential equation and pharmacokinetic parameters were calculated. A dose-dependency for certain parameters, most notably total body clearance (Cl_T), was indicated. The difference in Cl_T for the low and high dose was statistically significant. After single 5-, 25-, and 50-mg/kg intragastric doses were given to rats, the decline in plasma concentrations of I with time followed a monoexponential equation. As with dogs, there was a disproportionate change in kinetic parameters with increasing dose for rats. While simple Michaelis–Menten kinetics were not evident, nonlinearity in biotransformation (intrinsic clearance) appeared to be the cause for the dose-dependent pharmacokinetics.

Keyphrases □ 2,3,4,4a-Tetrahydro-1*H*-pyrazino[1,2a]quinoxalin-5(6*H*)-one—dose-dependent pharmacokinetics, dogs and rats □ Antihypertensive agents — 2,3,4,4a-tetrahydro-1*H*-pyrazino[1,2a]quinoxalin-5(6*H*)-one, dose-dependent pharmacokinetics, dogs and rats □ Pharmacokinetics—dose-dependent, 2,3,4,4a-tetrahydro-1*H*-pyrazino [1,2a]quinoxalin-5(6*H*)-one, dogs and rats

The compound 2,3,4,4a-tetrahydro-1*H*-pyrazino[1,2a]-quinoxalin-5(6*H*)-one (I) is an antihypertensive agent that is effective in lowering blood pressure in hypertensive



I

dogs, cats, and rats[1]. Its synthesis has been described previously (1).

For metabolic disposition studies, single intravenous and oral doses of 2.5 and 10 mg/kg were given to normotensive male dogs and intragastric doses of 5, 25, and 50 mg/kg to normotensive male rats. From the resulting data, estimates of pharmacokinetic parameters were determined and the effect of dose on pharmacokinetics was investigated.

EXPERIMENTAL

The hydrochloride salt and free base of I were used and 8-fluoro-2,3,4,4a-tetrahydro-1*H*-pyrazino[1,2a]quinoxalin-5(6*H*)-one hydro-

[1] Dr. R. L. Wendt, Wyeth Laboratories Inc., Radnor, Pa., unpublished results.

0022-3549/ 82/ 0400-0414$01.00/ 0
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chloride (II) was the internal standard in the GLC assay. Concentrations of I and II are expressed as free base. Solvents were obtained commercially[2] and were distilled in glass by the manufacturer. Methylene chloride was washed successively with 1 *N* HCl, 1 *N* NaOH, and three times with water before using. Heptafluorobutyrylimidazole[3] (III) was obtained in 1-g ampuls and was used without further purification.

Drug Administration and Sample Collection—Three normotensive, purebred, male beagle dogs (10–14 kg) were used for the study. The dogs were fasted 16 hr prior to each dosing, and feeding was resumed 6 hr after dosing. Compound I was given orally (2.5 or 10 mg/kg) in capsule form and intravenously (2.5 or 10 mg/kg) in saline. Plasma specimens from heparinized whole blood were obtained after each dose at 0.25, 0.50, 1, 2, 4, 6, 8, 12, 24, and 48 hr, and also at 0.08 hr after each intravenous dose. The animals were kept in individual metabolism cages and urine was collected at 0–4, 4–8, 8–12, 12–24, 24–36, 36–48, 48–72, and 72–96 hr after dosing. Plasma and urine were frozen until analysis. Intervals of at least 4 weeks separated each dose.

Male albino rats[4] (250–300 g) were randomly assigned to three treatment groups of 45 animals each, fasted for 16 hr, and given either 5, 25, or 50 mg/kg of I in sterile water by gastric intubation. The animals were fasted except for those to be sacrificed at 24 and 48 hr. At intervals of 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 hr, five animals from each dose group were anesthetized with ether, blood was drawn from the inferior vena cava into heparinized tubes, and the plasma separated by centrifugation. Urine was collected at 0–24 and 24–48 hr after dosing from the five animals who remained in metabolism cages until sacrifice at 48 hr. Plasma and urine were frozen until analysis.

Analytical Procedures—To disposable 16- × 125-mm glass culture tubes[5] (with plastic-lined screw cap) containing a plasma sample (1.0 ml) were added 100 ng of II in 0.05 ml of water and 0.025 ml 1 *N* NH_4OH. Plasma samples less than 1 ml were adjusted to 1 ml with drug-free dog plasma, and water was added to bring the total volume to 2 ml. Ethyl acetate (5 ml) was added to each tube, the tubes were shaken for 5 min on a mechanical rocker-type shaker[6], and centrifuged for 5 min. The organic layer was transferred to another 16- × 125-mm culture tube, and the extraction of the aqueous phase was repeated with 4 ml of ethyl acetate. The combined extracts were mixed[7] with 1 ml of 0.1 *N* H_2SO_4 for 30 sec and centrifuged.

The organic layer was aspirated, and the aqueous phase was washed with 1.5 ml of methylene chloride. Methylene chloride (5 ml) and 1 *N* NH_4OH (0.3 ml) were added to the aqueous phase, and the contents were mixed for 30 sec and centrifuged. The aqueous phase was aspirated, and the organic phase was evaporated to dryness in a 37° water bath under a nitrogen stream. The residue was dissolved in 0.7 ml of toluene, and 50 μl of III was added. The contents were heated at 55° for 15 min and then washed with successive 1 ml portions of 1 *N* NH_4OH, 0.2 *N* H_2SO_4, and water. The washed toluene phase was transferred to a 12- × 75-mm test tube and stored at −20° until analysis by GLC.

A 1 μl volume was injected into the chromatograph. For each analysis, calibration standards were prepared by adding varying amounts (25–400 ng) of I in 0.05–0.1 ml of water to a series of tubes containing 1 ml of drug-free dog plasma. Internal standard, alkali, and water were added to these tubes in the same way as for the plasma samples of unknown concentration.

Aliquots of urine were diluted to 1 ml with water and processed in a manner similar to that for the plasma samples. The calibration standards contained 100–800 ng of I and 200 ng of internal standard in 0.1 ml of control urine. Additional water was added to adjust the total volume to 1 ml.

The samples containing the heptafluorobutyryl derivatives of I and II were chromatographed on a gas chromatograph[8] equipped with a nickel 63 electron-capture detector and a 122- × 0.2-cm glass column packed with 1% Silar 10CP on 100/20 mesh chromosorb W-HP[9]. The column temperature was 240°, the injection port was 250°, and the detector was 350°; the flow of carrier gas (95% argon, 5% methane) was maintained at 26 ml/min.

A calibration curve was constructed daily by plotting the ratio of peak heights for derivatized I to derivatized internal standard *versus* the concentration of the I standard. Unknown concentrations of I were determined from the calibration curve.

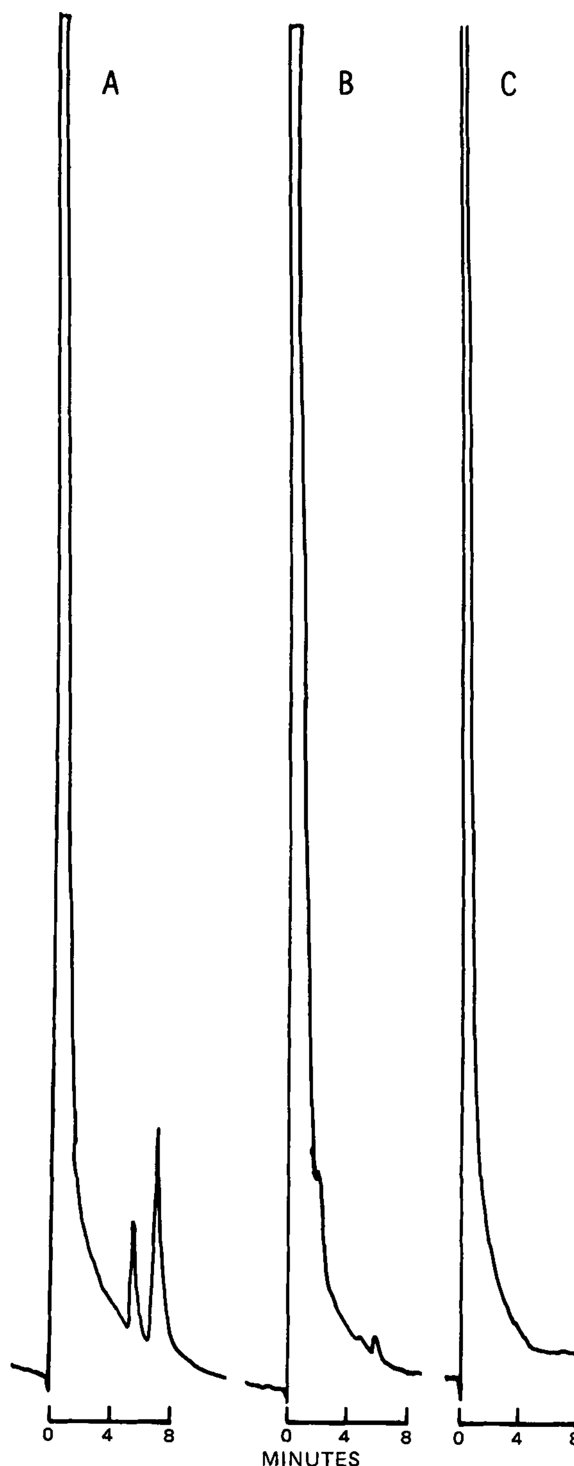

Figure 1—*Gas–liquid chromatograms. Key: A, 50 ng of I (as derivative, t_r = 5.5 min) and 100 ng of II (as derivative, t_r = 7.2 min) in dog plasma extract; B, blank dog plasma extract, 1 ml; C, blank dog urine extract (1 ml).*

Pharmacokinetic Analysis—A two-compartment model was applied to fit simultaneously the intravenous and oral plasma data from each dog at a given dose level according to the equations:

$$C_{iv} = \frac{\text{dose}}{V_c}\left(\frac{k_{21}-\alpha}{\beta-\alpha}e^{-\alpha t} + \frac{k_{21}-\beta}{\alpha-\beta}e^{-\beta t}\right) \quad \text{(Eq. 1)}$$

$$C_{po} = \frac{F\,\text{dose}\,k_a}{V_c}\left[\frac{k_{21}-k_a}{(\beta-k_a)(\alpha-k_a)}e^{-k_a t} + \frac{k_{21}-\alpha}{(k_a-\alpha)(\beta-\alpha)}e^{-\alpha t} + \frac{k_{21}-\beta}{(\alpha-\beta)(k_a-\beta)}e^{-\beta t}\right] \quad \text{(Eq. 2)}$$

[2] Burdick & Jackson Laboratories, Muskegon, Mich.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Charles River CD-1, Strain COBS.
[5] Corning 99449.
[6] Buchler Instruments, Fort Lee, N.J.
[7] Vortex Genie, Scientific Industries.
[8] Model 5710A, Hewlett-Packard.
[9] Supelco, Inc.

Table I—Precision of the GLC Assay of I in Dog Plasma

Concentration of I (Free Base), ng/ml	*n*	Mean Ratio ± *SD*	*CV*, %[a]
		Experiment 1	
25	5	0.234 ± 0.009	4.0
50	5	0.486 ± 0.011	2.3
100	5	0.957 ± 0.010	1.1
200	5	1.916 ± 0.042	2.2
400	5	3.866 ± 0.050	1.3
		Experiment 2	
25	5	0.258 ± 0.018	7.0
50	5	0.503 ± 0.018	3.5
100	5	0.989 ± 0.041	4.1
200	5	2.097 ± 0.050	2.4
400	5	4.194 ± 0.034	0.8

[a] Coefficient of variation.

where C is the plasma concentration at time t, k_a, α, and β are the first-order absorption, distribution, and elimination rate constants, respectively, V_c is the distribution volume of the central compartment, F is the systemic availability of the oral dose, and k_{21} is the intercompartmental distribution rate constant (2).

The initial values of model parameters were estimated using the AUTOAN computer program (3). F was estimated by:

$$AUC_{po} \times \text{body weight}_{iv}/AUC_{iv} \times \text{body weight}_{po} \quad \text{(Eq. 3)}$$

where AUC is the area under the plasma concentration–time curve between zero and infinity. Equations 1 and 2 were then applied to fit the intravenous and oral plasma data from each dog simultaneously with the aid of the NONLIN computer program (4). The statistical weighting factor in the least-squares procedure was the inverse of the observed plasma concentration and the overall goodness of fit was assessed by the coefficient of determination (r^2). Other parameters calculated were the total body clearance (Cl_T), the volume of distribution at steady state (Vd_{ss}), and after distribution equilibrium (Vd_β), the renal clearance (Cl_R), and the intercepts A and B (2). The fraction of the intravenous dose eliminated as unchanged I in urine was designated as f_{iv}.

The plasma concentration–time data from intragastrically dosed rats were fitted by a one-compartment model according to the equation:

$$C = \frac{FDk_a}{V(k_a - K)}(e^{-Kt} - e^{-k_a t}) \quad \text{(Eq. 4)}$$

Synthesis of Heptafluorobutyramides—Compound I as free base (200 mg) in 2 ml of ethyl acetate was reacted with 0.5 ml of III at 55°. After completion of the reaction (0.5 hr), the reaction mixture was washed once with 2 ml of 0.2 *N* H_2SO_4, twice with 2 ml of water, and evaporated to dryness at 55° under a nitrogen stream. The residue was recrystallized from ethanol and gave 150 mg of 3-(2′,2′,3′,3′,4′,4′,4′-heptafluorobutyryl)-2,4,4a-trihydro-1*H*-pyrazino[1,2a]quinoxalin-5-(6H)-one (IV), mp 188°.

Anal.—Calc. for $C_{15}H_{12}N_3O_2F_7$: C, 45.12; H, 3.03; N, 10.53. Found: C, 45.23; H, 3.14; N, 10.22.

The free base of II was extracted with ethyl acetate from an alkalinized solution of the hydrochloride and then derivatized and crystallized by the described method to give 8-fluoro-3-(2′,2′,3′,3′,4′,4′,4′-heptafluorobutyryl)-2,4,4a-trihydro-1*H*-pyrazino[1,2 a]quinoxalin-5(6H)-one(V), mp 190°.

Anal.—Calc. for $C_{15}H_{11}N_3O_2F_8$: C, 43.18; H, 2.66; N, 10.07. Found: C, 43.13; H, 2.69; N, 10.07.

Mass Spectrometry—Samples were examined by direct introduction with the mass spectrometer[10] in the electron-impact mode. Source temperature was 150–200° and ionizing potential 70 ev.

Differential Scanning Calorimetry (DSC)—DSC curves were obtained using a differential scanning calorimeter[11]. Samples (1 mg) were heated at the rate of 10°/min in a nitrogen atmosphere.

RESULTS

Analytical—The elemental analysis of IV indicated that a monoheptafluorobutyryl derivative of I was formed. Consistent with the elemental analysis, the mass spectrum of IV exhibited a molecular ion at *m/z* 399 (base peak) and a major ion at *m/z* 202 from the loss of the acyl group (C_4F_7O). The thermogram for IV from the differential scanning calorimeter (DSC) showed a single sharp peak at the melting point (188°) of the derivative and no decomposition up to 260°. The DSC curve confirmed the purity of the product and demonstrated the stability of the derivative at the GLC operating conditions.

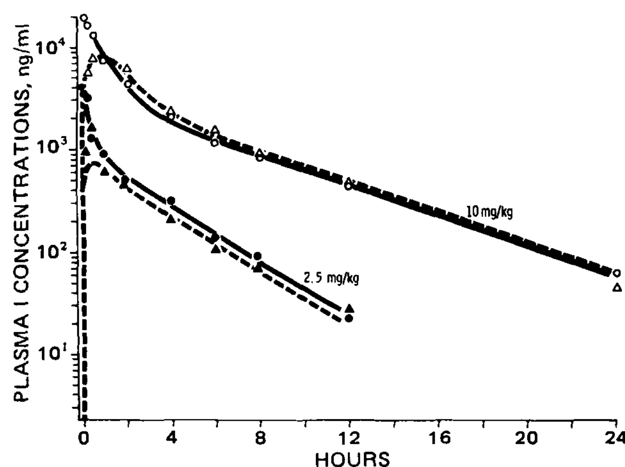

Figure 2—*Mean concentrations of I in the plasma of dogs given intravenous (O) and oral (Δ) doses of 2.5 and 10 mg/kg.*

The retention times (t_r) of IV and V were 5.5 and 7.2 min, respectively. A representative chromatogram from an analysis of 50 ng of I and 100 ng of II in 1 ml of dog plasma is illustrated in Fig. 1A. Chromatograms of blank dog plasma and urine (1 ml each) carried through the GLC assay are shown in Figs. 1B and 1C, respectively. No metabolites of I interfered with the chromatographic detection of IV and V. To determine reproducibility, the mean ratio of the peak heights of IV to V (n = 5) and its coefficient of variation (CV) at concentrations ranging from 25 to 400 ng/ml were determined during two analyses on separate days (Table I). The peak height ratio varied with a CV <7%. The mean ratio was linearly related to I concentration with a correlation coefficient of 0.999 between these two analyses. The recoveries of the drug at each concentration varied from 37 to 39% (CV = 2–13%). A minimum of 12 ng/ml could be quantified using a 2-ml plasma sample.

Pharmacokinetics of I in Dogs—Concentrations of I in dog plasma after 2.5 and 10 mg/kg iv and oral doses are illustrated in Fig. 2 and the derived pharmacokinetic parameters are listed in Table II. The decline in plasma concentrations followed the biexponential equation for a two-compartment model. After oral administration, observed peak plasma concentrations and standard deviations (C_{max}) were 1667 ± 651 and 7710 ± 6858 ng/ml for the 2.5- and 10-mg/kg doses, respectively, and occurred at 0.5 hr for each dose.

The mean drug recoveries in urine and their standard deviations after

Table II—Pharmacokinetic Parameters of I in Dogs following Single Intravenous and Oral Doses [a]

Parameter	Dose: 2.5 mg/kg, Mean ± *SD*	Dose: 10 mg/kg, Mean ± *SD*	Statistical Difference[b], *t*(*p*)
A, μg/ml	3.44 ± 0.91	19.5 ± 2.4	1.74[c] (*NS*)
B, μg/ml	0.65 ± 0.98	3.57 ± 0.56	2.81[c] (*NS*)
Cl_T, ml/min/kg	9.3 ± 0.70	4.70 ± 0.33	8.01 (<0.05)
β, hr⁻¹	0.24 ± 0.03	0.17 ± 0.01	3.35 (*NS*)
Vd_β, liters/kg	2.3 ± 0.2	1.70 ± 0.1	7.54 (<0.05)
AUC_{iv}, hr ng/ml	4514 ± 331	35620 ± 2584	7.9[c] (<0.05)
AUC_{po}, hr ng/ml	3075 ± 323	33678 ± 6327	5.02[c] (<0.05)
F	0.73 ± 0.05	0.95 ± 0.16	2.42 (*NS*)
k_{21}, hr⁻¹	0.58 ± 0.19	0.36 ± 0.05	1.92 (*NS*)
$t_{1/2\beta}$, hr	2.9 ± 0.40	4.10 ± 0.20	3.14 (*NS*)
k_a, hr⁻¹	3.0 ± 0.80	1.94 ± 2.05	1.57 (*NS*)
α, hr⁻¹	2.4 ± 1.0	1.36 ± 0.04	1.67 (*NS*)
$t_{1/2\alpha}$, hr	0.33 ± 0.13	0.51 ± 0.02	1.73 (*NS*)
V_c, liters/kg	0.61 ± 0.19	0.44 ± 0.04	1.34 (*NS*)
Vd_{ss}, liters/kg	1.6 ± 0.2	1.08 ± 0.06	3.67 (*NS*)
Cl_R, ml/min/kg	0.46 ± 0.02	0.62 ± 0.10	1.87 (*NS*)
f_{iv}, fraction of dose	0.05 ± 0.003	0.13 ± 0.04	3.22 (*NS*)

[a] The overall goodness of fit of measured plasma data to the generated curves (Fig. 2) was r^2 = 0.93. [b] Paired t test; degrees of freedom = 2. [c] For statistical analysis, these parameters were normalized by dose.

[10] AEI-MS902 equipped with a Data General Nova 3 computer and DS-50s software.
[11] Model 2, Perkin-Elmer Corp.

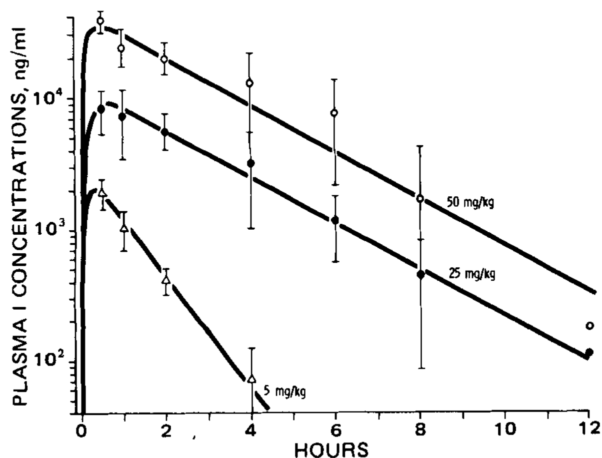

Figure 3—*Mean concentrations of I (±SD) in the plasma of rats given intragastric doses of 5 mg/kg (△), 25 mg/kg (○), and 50 mg/kg (●).*

the 2.5-mg/kg iv and oral doses were 5.0 ± 0.3 and 1.8 ± 0.8% of the administered dose, respectively. After the 10-mg/kg iv and oral doses 13.4 ± 4.4 and 5.4 ± 0.5% of the administered doses were recovered, respectively.

Pharmacokinetics of I in Rats—Concentrations of I in rat plasma after intragastric administration of 5, 25, and 50 mg/kg are shown in Fig. 3; the estimated pharmacokinetic parameters are listed in Table III. The decline in plasma concentrations after 5, 25, and 50 mg/kg was apparently monoexponential. In the order of increasing dose, the observed peak plasma concentrations ($C_{max} \pm SD$) were 1966 ± 529, 8609 ± 3176, and 38,999 ± 5896 ng/ml and occurred at 0.5 hr for each dose. The mean drug recoveries in urine after the 5, 25, and 50 mg/kg doses were 1.3 ± 0.4, 4.0 ± 2.0, and 6.9 ± 3.7% of the administered dose, respectively.

DISCUSSION

The described GLC assay is highly sensitive and reproducible. The minimum quantifiable concentration was 25 ng/ml for a 1-ml specimen, while a minimum of 12 ng/ml could be quantified in plasma using 2-ml samples.

In dogs, I was quickly absorbed with a Vd_{ss} (1.6 liter/kg) which is 2–3 times larger than the reported total body water of the dog [0.5–0.8 liter/kg (5)]. The calculated availability (F) was high (73% for a 2.5-mg/kg dose and 95% for a 10-mg/kg dose) and increased with increasing dose (Table II). The compound was eliminated rapidly from plasma and was excreted to a small extent into urine, but appears to be eliminated mainly by metabolism. The elimination half-lives in the rat (Table III) were less than those calculated for the dog (Table II), and similar plasma levels of the compound in rats and dogs were observed only when rats received higher doses. This species difference in disposition may account for a longer duration of the compound's hypotensive effect in the dog.[1]

Although linear pharmacokinetic models were used to fit the plasma data for both dogs and rats, the disproportionate increases in AUC with increasing dose indicate that dose-dependent kinetics are in effect. For example, total body clearance in dogs is almost reduced by one-half at the higher dose. However, the elimination of I from plasma does not appear to follow simple Michaelis–Menten kinetics since the elimination is slower after the higher doses than the lower doses in the same concentration range. This pattern suggests product inhibition as pointed out by Perrier *et al.* (6). Thus, the model employed to fit the data yields dose-average pharmacokinetic parameters at each dosage level which may not be operative as a function of specific plasma concentrations. Although the differences in β, $t_{1/2\beta}$, and F at the two doses were below statistical significance (Table II) these differences were assumed to be connected with the statistically significant change in Cl_T. The lack of visible nonlinearity and Michaelis–Menten characteristics precluded fitting the data with a specific nonlinear function. However, the NONLIN-fitted least-square value of Cl_T agreed well with the time-average value obtained from the quotient Dose/AUC_{iv}.

The factor responsible for the nonlinear disposition of I can be assessed further by considering first-pass concepts (7). If complete availability from the dosage form is assumed, then the apparent intrinsic clearance of I can be estimated as:

$$Cl'_{int} = \frac{(1 - f_{iv})_{Dose}}{AUC_{po}} \qquad \text{(Eq. 5)}$$

Table III—Pharmacokinetic Parameters of I in Rats following Single Intragastric Doses

Parameter	Dose, mg/kg 5	25	50
C_{max}, µg/ml	2.0	8.6	39.0
t_{max}, hr	0.5	0.5	0.5
K, hr^{-1}	0.97	0.41	0.41
$t_{1/2}$, hr	0.72	1.7	1.7
V/F, liters/kg	2.0	2.1	1.1
AUC, hr µg/ml	2.6	29.3	116
Cl_T/F, ml/min/kg	32	14	7
Cl_R, ml/min/kg	0.43	0.56	0.49

Table IV—Comparison of Apparent Intrinsic Clearance (Cl'_{int}) and Systemic Availability (F) of I after Oral and Intravenous Doses in Dogs

Dose, mg/kg	Oral Cl'_{int}, ml/min/kg	Oral F^a	Intravenous Cl'_{int}, ml/min/kg	Intravenous F^a
2.5	13.0 ± 1.4	0.76 ± 0.02	11.3 ± 1.1	0.78 ± 0.02
10.0	4.4 ± 1.2	0.90 ± 0.02	4.5 ± 0.6	0.90 ± 0.01

[a] Calculated from Cl'_{int} and Q_H as discussed in text.

where f_{iv} is the fraction of the intravenous dose excreted as unchanged I. Protein binding was not measured so these equations do not yield the true $Cl_{int} = V_{max}/K_m$. The intravenous doses give the body clearance (Cl_T):

$$Cl_T(1 - f_{iv}) = \frac{Q_H\, Cl'_{int}}{Q_H + Cl'_{int}} \qquad \text{(Eq. 6)}$$

where Q_H is hepatic blood flow.

Equation 6 can be rearranged to:

$$Cl'_{int} = \frac{Cl_T(l - f_{iv})Q_H}{Q_H - Cl_T(1 - f_{iv})}$$

Table IV shows calculated values of Cl'_{int} obtained by both methods. Hepatic blood flow in the dog was assumed to be 40 ml/min/kg (8). The good agreement and diminishment with dose indicates that nonlinearity in I disposition is probably due to a dose-dependent decrease in biotransformation rate. The Cl'_{int} values can be used to estimate values of F, as shown in Table IV, since:

$$F = \frac{Q_H}{Q_H + Cl'_{int}} \qquad \text{(Eq. 7)}$$

The larger F with increasing dose is consistent with the nonlinear Cl'_{int}. The significant decrease in Vd_β with increasing dose could result from changes in elimination (9).

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ACKNOWLEDGMENTS

The authors thank Mr. M. Wassermann for the GLC analyses, Mr. L. Sivieri for the DSC curves, Dr. John Sellstedt for the mass spectra, Dr. William J. Jusko for helpful discussions on the pharmacokinetics, and Mr. Frazer Hadley for reviewing the manuscript. The hydrochloride salts of I and II were obtained from Dr. M. Freed. The free base of I was obtained from Dr. C. Robinson.