Prediction of Stability in Pharmaceutical Preparations XVIII: Application of High-Pressure Liquid Chromatographic Assays to Study of Nafronyl Stability and Bioanalysis

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Abstract □ Specific, sensitive, reversed-phase high-pressure liquid chromatographic assays of nafronyl (I) and its acidic metabolite and hydrolysis product (II) were developed in aqueous solutions and in biological fluids with sensitivities of 100 ng/ml using butacaine as the internal standard and spectrophotometric detection at 224 nm. Heparinized plasma can be analyzed easily in the organic phase immediately after acetonitrile denaturation. Both I and II can be extracted with haloalkane solvents, and the extracts are evaporated, reconstituted, and assayed in a minimal amount of acetonitrile. Conditions are presented that minimize the interference of II and extracted plasma components. The assay was used to determine the stability of nafronyl in aqueous solutions, to establish its log k–pH profiles at various temperatures, and to evaluate the Arrhenius parameters. Nafronyl is hydrolyzed by specific hydrogen-ion (15.2 kcal/mole) and hydroxide-ion (7.72 kcal/mole) catalysis of the neutral species and specific hydroxide-ion catalysis (5.91 kcal/mole) of the protonated species. The pH of maximum stability is 3.0, and pH 5.4 is the maximum that can be tolerated at 30°, with a 10% solvolysis in 3 years. The half-life of nafronyl at 30° was 7 days at pH 7, 12 hr at pH 10, and 21 min in 0.5 N NaOH. Since nafronyl has a half-life of 3.2 hr in heparinized dog plasma at 25°, blood samples for pharmacokinetic studies of nafronyl must be assayed immediately after sampling. The partition coefficients of I and II determined as functions of pH permit the extraction of both compounds at pH 4.5, but only I can be extracted at pH values above 9.5.

Keyphrases □ Nafronyl oxalate—high-pressure liquid chromatographic analysis in stability studies □ High-pressure liquid chromatography—analysis, nafronyl oxalate, application to stability studies □ Vasodilators—nafronyl oxalate, high-pressure liquid chromatographic analysis in stability studies

When this series of stability studies was initiated 25 years ago (1), its purpose was to demonstrate the use of basic physical–chemical relations in predicting the stability of drugs over long intervals under conditions that could be present in pharmaceutical formulations. The validity of these procedures has been demonstrated fully (2, 3). Short-term studies under high temperature, high acidity and alkalinity, and other excessive stresses have permitted such predictions and have given insight into the conditions that favor the stabilization of drugs in unit dosage forms.

The objective of these investigations has been expanded to encompass those systematic studies necessary to establish optimum and valid assays for drugs and their metabolites *in vitro* and *in vivo*. Many active agents and their metabolites must be extracted selectively from pH-adjusted solutions for the assay to be valid. Their stability and partition properties as a function of pH must be known to design optimum conditions.

BACKGROUND

The advent of the pharmacokinetic era has necessitated the determination of the time course of drugs and metabolites in living organisms for proper evaluation of effective dosage regimens and for determination of the bioavailability of the active agent from the dosage forms. Many published studies are suspect since uncritically instituted assay conditions may have transformed circulating drugs into pseudometabolites, and since the drug itself may have degraded in biological fluids and tissues stored and/or refrigerated prior to assay. A classical example, heroin, was considered recently (4), where its facile degradation in sampled plasma ($t_{1/2}$ 8 min) or upon extraction from moderately alkaline solutes could give erroneous estimates of the amounts of drugs and metabolites.

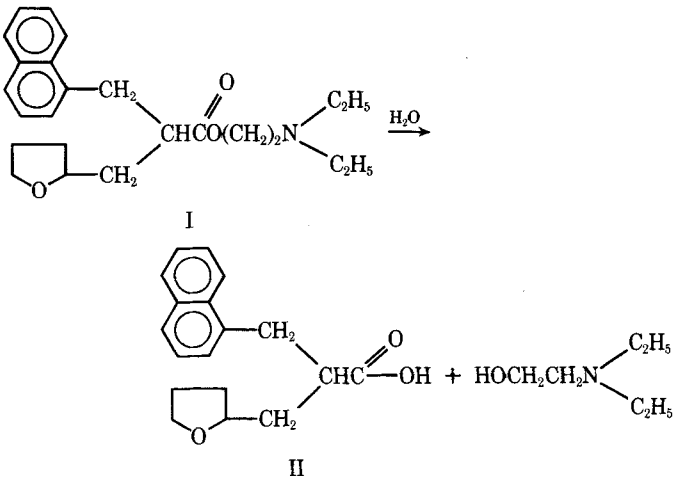
All compounds containing possibly readily hydrolyzable groups such as esters are susceptible to such erroneous analytical procedures. The development of proper methods demands a systematic study of stability as a function of pH and temperature.

Optimum conditions for extraction must be established that lead to minimal transformations during the analytical workup. The maintenance of the integrity of the drug and its metabolites must be assured for their projected storage times in sampled biological fluids and tissues.

The vasodilator and metabolic activator nafronyl [tetrahydro-α-(1-naphthalenylmethyl)-2-furanpropanoic acid 2-(diethylamino)ethyl ester, I], also known as naftidrofuryl (5, 6), is prepared as the acid oxalate. Its smooth muscle relaxation properties and its ability to increase peripheral and cerebral blood flow and cerebral adenosine triphosphate concentrations and glucose utilization may have value in the treatment of senile brain disease (5–7). Tetrahydro-α-(1-naphthalenylmethyl)-2-furanpropanoic acid (II), a hydrolysis product of I (Scheme I), was observed in the plasma and urine of dogs and humans (8) and rats (9) administered I. As explained by Fontaine *et al.* (8), the presumed low plasma levels of I were due to its hydrolysis by pseudocholinesterase in the plasma, with 46% of I disappearing from plasma (presumably *in vitro*) after 1 hr at 37°.

The assays in such studies were conducted after extraction with ether from biological fluids adjusted to 10% ammonia before and after hydrolysis with β-glucuronidase. The analytical method used to assay I (10) was based on ion-pair formation of I in dichloroethane with an acidic fluorescent dye in pH 5 buffer; the fluorescence of this salt due to the counteranion in dichloroethane provided estimates of the concentration of I. The sensitivity may be 0.2 μg of I/ml of dichloroethane for this nonspecific assay.

The development of proper and unique assays of I and II in biological fluids demands a thorough knowledge of the pH profile of degradation



Scheme I

0022-3549/81/0100-0039$01.00/0

of I to choose optimum pH values for nondegrading conditions of separation. The determination of the effective partition of I and II into appropriate organic solvents as a function of pH may permit the optimum choice of extraction conditions. The development of sensitive and specific assays of I and II in various solutions is necessary for performance of these tasks. This paper presents sensitive and specific high-pressure liquid chromatographic (HPLC) assays of I and II in buffer solutions and biological fluids. The assays are applied to the determination of the log k–pH stability profile of I and to the determination of the stability of I in plasma.

EXPERIMENTAL

Materials—The following analytical grade materials were used: dibasic and monobasic sodium phosphates[1], dibasic and monobasic potassium phosphates[1], sodium acetate[1], acetic acid[1], volumetric concentrates of sodium hydroxide and hydrochloric acid[2], and boric acid[3]. Methylene chloride[4] was ACS grade, and acetonitrile[5] and chloroform[5] were GLC, liquid chromatographic, and UV grade. Pure I and II reference standards were used as received[6].

Apparatus—A high-pressure liquid chromatograph[7] equipped with a variable-wavelength UV detector[7] was used with a reversed-phase μ-Bondapak alkylphenyl column[8]. Areas under the chromatogram peaks were measured by an integrator[9].

HPLC Procedures—Prepared solutions of sodium acetate of known molarity were adjusted to a measured pH with acetic acid. Solutions generally were 0.01–0.1 M, adjusted to pH values between 3.5 and 5.0. The mobile phase was a mixture of this buffer and acetonitrile (50:50 or 40:60). The flow rate of 2.5 ml/min gave a back pressure of ~2000 psi. The wavelength of the variable-wavelength detector was adjusted to 224 nm, which was the maximum absorbance of I and II; a weak absorbance at 282 nm and a negligible absorbance at 254 nm were noted. When an internal standard was used, a specified amount was dissolved in the acetonitrile used to dilute the buffer or plasma sample to be assayed. Generally, the sample was diluted to give the same percentage of acetonitrile as the mobile phase, and 25 μl of this mixture was injected. This procedure reduced the intensity of the solvent peak and its tailing.

Kinetics of Hydrolytic Degradation of Nafronyl (I)—*Acid Hydrolyses*—Nafronyl (I) acid oxalate (2.0 mg) was weighed accurately and dissolved in 10 ml of thermally preequilibrated 0.5, 0.4, 0.2, and 0.1 N HCl (4.223×10^{-4} M), prepared from a standardized concentrate and confirmed by titration against standardized sodium hydroxide. An aliquot (100 μl) was taken immediately after dissolution and transferred to a 3.0-ml reaction vial containing 20 μl of sodium hydroxide solution, having a molarity five times greater than that of the hydrochloric acid solution used, to effect neutralization of the strong acid. A mixture of acetate buffer (pH 4.0) and acetonitrile then was added (80 μl) to adjust the pH and composition of the solution to those of the mobile phase. An aliquot (25 μl) was injected into the chromatograph under the described analytical conditions at intervals.

Alkaline Hydrolyses—Nafronyl (I) acid oxalate (40.0 mg) was weighed accurately and dissolved in 2.0 ml of distilled water in a 4.0-ml reaction vial to prepare the stock solution for alkaline hydrolyses. Aliquots (50 μl) from this stock solution were taken with a 50-μl syringe and dissolved in 10.0 ml of 0.01, 0.02, 0.04, and 0.05 N NaOH and other solutions preequilibrated at 30° in a water bath. Similar studies were effected at other temperatures. Immediately, 100 μl of the 2.112×10^{-4} M I solution (0-hr sample) was transferred to a 2.0-ml reaction vial containing 20 μl of hydrochloric acid solution, which had a sufficient normality to neutralize the excess strong base. A mixture of 0.4 M sodium acetate buffer (pH 4.0) and acetonitrile (80 μl) then was added to adjust the pH of the solution to ~4.0. Samples taken from the sodium hydroxide solutions were quenched in this manner every 5 min for the first 30 min and then every 15 min until all I was degraded to II. An aliquot of the quenched solution (25 μl) was injected into the chromatograph for the described assay.

Studies at Intermediate pH Values—Acetate (pH 3.4–5.4), phosphate (pH 5.6–8.2), and borate (pH 8.0–10) buffers were prepared; the ionic strength was adjusted to 0.1 with potassium chloride. The buffer solutions (10.0 ml) were preequilibrated in an oil bath at the appropriate temperature before addition of 25.0 or 50.0 μl of a solution of nafronyl (I) acid oxalate containing 20 mg/ml. Aliquots (100 μl) of the 2.101×10^{-4} M I solution were removed at different time intervals, and the pH of the solution was adjusted to 4.0 by adding 100 μl of 0.4 M acetate buffer–acetonitrile. Then 25.0 μl of this solution was injected into the chromatograph.

Stability of I in 10% Ammonia Solution—A solution of 10% ammonia (10 ml) was preequilibrated at 30° in a water bath, and 25 μl of a solution of nafronyl (I) acid oxalate (20.0 mg/ml) was added. Aliquots of the 1.05×10^{-4} M solution of I were removed at different times. The reaction mixture was quenched to pH 4 with hydrochloric acid–acetate buffer and mixed with acetonitrile, and 25 μl was injected into the chromatograph.

Partition Studies as Functions of pH—An aliquot (25 μl) of a stock solution of I as the oxalate (20.00 mg/ml) was dissolved in 5.00 ml of various buffer solutions to prepare solutions of 2.101×10^{-4} M I. Chloroform (250 μl, V_{org}) was added to 1 ml (V_{aq}) of each buffer solution (99.5 μg/ml) in a 3.0-ml reaction vial. The tightly closed vial was vortexed for exactly 60 sec, and a 100-μl aliquot of the separated aqueous phase was mixed with 100 μl of a 50:50 mixture of 0.4 M, pH 4 acetate buffer and acetonitrile. An aliquot (25 μl) was injected into the chromatograph; an aliquot of the buffer solution prior to extraction was assayed similarly.

HPLC Analytical Methods for I in Plasma Using Acetonitrile as Deproteinizing Agent—*Method A*—Acetonitrile (250 μl) was added to 100 μl of heparinized human plasma spiked with I. The mixture was vortexed and centrifuged at 3000 rpm for 5 min, and then 25 μl of the acetonitrile supernate was injected into the chromatograph. The mobile phase was a mixture of 50% 0.01 M sodium acetate adjusted with acetic acid to pH 3.5 and 50% acetonitrile. The flow rate was 2.5 ml/min on a μBondapak alkylphenyl column.

The retention times for II and I were 2.76 and 7.98 min, respectively.

Method B—Acetonitrile (1.0 ml), containing butacaine as an internal standard, was added to 1.0 ml of heparinized human plasma spiked with I. The mixture was vortexed and centrifuged at 3000 rpm for 5 min. Then 0.8 ml of the supernate was transferred to a reaction vial and evaporated to dryness under nitrogen. The residue was reconstituted in 75 μl of acetonitrile and vortexed, and 25 μl of the solution was injected into the chromatograph.

HPLC Analytical Methods for I in Plasma Using Haloalkane Extraction—*Method C*—An aliquot of heparinized human plasma (2.0 ml) was extracted with 2.0 ml of chloroform, and the mixture was vortexed for 10 min and centrifuged for 5 min at 3000 rpm. The upper layer was aspirated, and 1.5 ml of the chloroform phase was evaporated to dryness. The residue was reconstituted in 100 μl of acetonitrile with vortexing, and 25 μl of the solution was injected into the chromatograph. The mobile phase consisted of 0.01 M acetic acid adjusted to pH 4.0 and acetonitrile (50:50). The retention time for I was 9.62 min.

Method D—Aliquots (2 ml) of freshly prepared heparinized dog plasma (within 1 hr after obtainment from the dog) were adjusted to pH 3.78 with 0.35 ml of 0.2 N HCl. The plasma was extracted with methylene chloride (3 ml), with 10 min of mild shaking and centrifuging for 5 min at 3300 rpm. The aqueous phase was aspirated, and 2 ml of the methylene chloride phase was evaporated to dryness under a nitrogen stream. The residue was reconstituted in 50 μl of acetonitrile containing 750 μg of butacaine sulfate/ml. The mobile phase consisted of 0.1 M acetate buffer adjusted to pH 4.0 and acetonitrile (45:55).

RESULTS AND DISCUSSION

Reversed-Phase HPLC Assay of I and II for Degradation and Partition Studies—The HPLC mobile phase of acetate buffer–acetonitrile (50:50) with a 224-nm UV detector and a flow rate of 2.5 ml/min provided a chromatogram (Fig. 1A) with a steady baseline and specific and sensitive quantitative analyses of I and its acid degradation product (II), with retention times of 6.66 and 3.26 min at pH 4.5 and of 8.00 and 2.79 min at pH 4.0, respectively. A series of potential internal standards was studied at pH 4.0 with the following retention times (in minutes): caffeine, 1.64; hippuric acid, 1.80; phenobarbital, 2.50; ephedrine, 2.64; codeine, 2.73; atropine, 2.92; amphetamine, 2.82; procaine, 2.51; lidocaine, 2.94; heroin, 2.90; tetracaine, 3.99; butacaine, 5.44; and papaverine, 3.60. Butacaine was selected because its retention time (5.44 min at pH 4 and 4.90 min at pH 4.5) is intermediate between those of I and II. Excellent calibration curves for assays in buffer solutions were obtained from the

[1] Mallinckrodt Chemical Works, St. Louis, MO 63160.
[2] Harleco, Philadelphia, PA 19143.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Mallinckrodt, Paris, KY 40361.
[5] Burdick & Jackson Laboratories, Muskegon, MI 49442.
[6] Lot 1956 (I) and lot 9751 (II), Lipha Chemicals, New York, NY 10022.
[7] Model 6000A solvent delivery system and model U6K injector equipped with a model 450 variable-wavelength UV detector, Waters Associates, Milford, Mass.
[8] Waters Associates, Milford Mass.
[9] Model 3380A, Hewlett-Packard, Avondale, Pa.

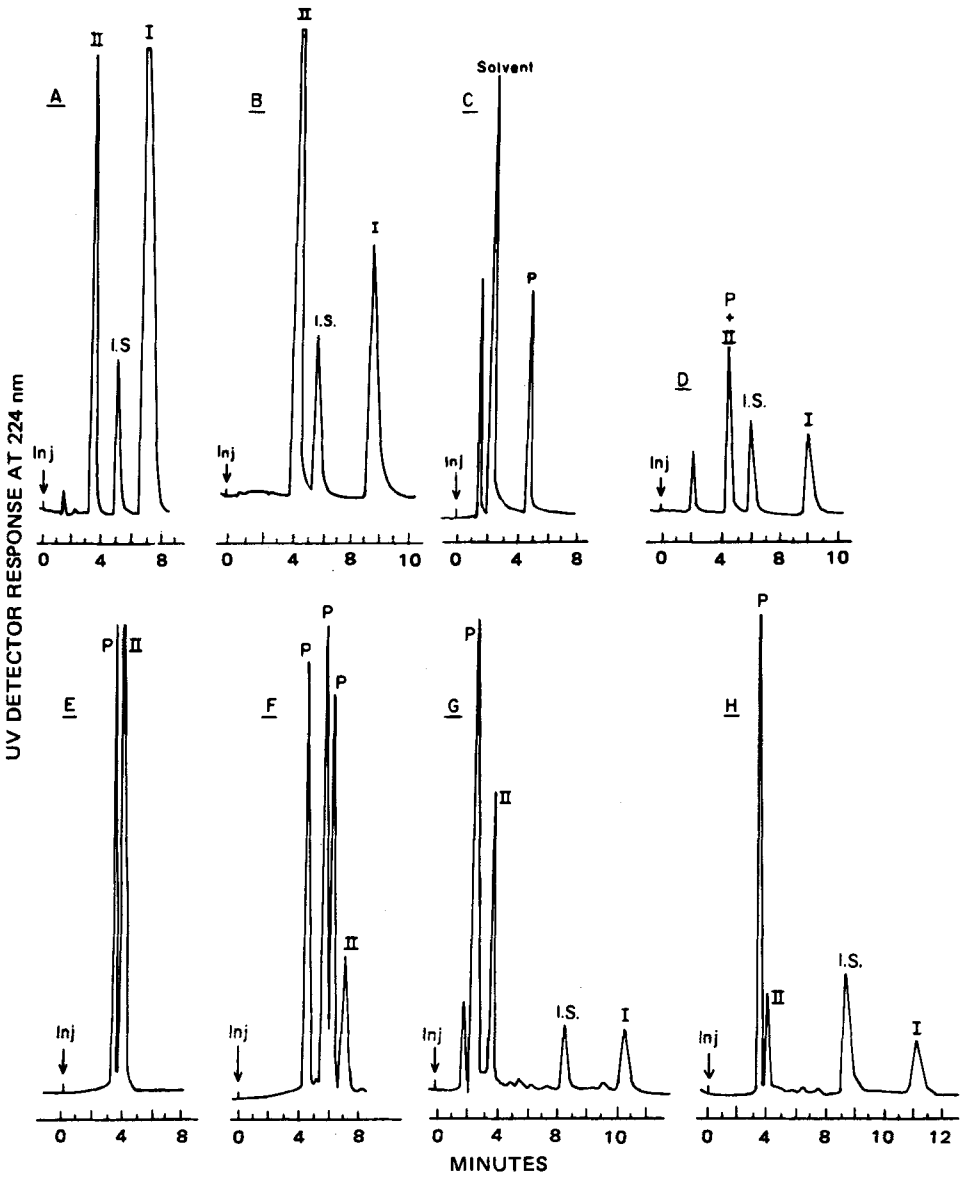


Figure 1—*Reversed-phase high-pressure liquid chromatograms with 224-nm detection of 25 μl of solutions of I (as acid oxalate) and its acid degradation product (II) with a butacaine sulfate internal standard (IS). Plasma interferences are labeled* P. *The flow rate was 2.2 ml/min. Key: A, aqueous solution containing 10 μg each of I, II, and the internal standard injected into a mobile phase of pH 4.5, 0.1* M *acetate buffer–acetonitrile (50:50); B, aqueous solution containing 2.5 μg each of I and II and 10 μg of the internal standard injected into the same mobile phase except in a 60:40 mixture; C, acetonitrile-reconstituted residues from Method C of plasma analysis without I and II; D, acetonitrile-reconstituted residues from Method C of plasma analysis with 5 μg of I and II/ml of plasma (the II and* P *peaks were not separated); E, separation of II and* P *peaks from acetonitrile-reconstituted residues from plasma with 2.5 μg of II/ml injected into a mobile phase of pH 3.5, 0.01* N *acetate buffer–acetonitrile (50:50) (flow rate 2.0 ml/min); F, separation of II and* P *peaks from acetonitrile-reconstituted residues from plasma with 0.5 μg of II/ml injected into a mobile phase of pH 5.0 buffer–acetonitrile (60:40) (flow rate 1.0 ml/min); and G and H, acetonitrile-reconstituted residues from plasma assays with 0.5 μg of I and II/ml of plasma injected into 0.1* N *acetate buffer–acetonitrile (55:45) (flow rate 1.5 ml/min) at pH 3.75 and 4.0, respectively.*

peak area alone (Fig. 2) so no internal standard was needed for the kinetic studies in aqueous solutions.

The standard errors of the estimates of the concentrations of I and II were 37 and 41 ng/ml, respectively, for the calibration curves prepared in the 0–2.5-μg/ml range and indicate analytical sensitivities of 100 ng/ml. The linear regression equations with standard errors of the estimates for nafronyl (I) acid oxalate are: 0–75 μg/ml, C_I (±0.155) μg/ml = −0.119 (±0.080) + 1.1755 (±0.0026)A, $n = 7$, $r^2 = 1.000$; and 0–2.5 μg/ml, C_I (±0.037) = −0.089 (±0.042) + 1.229 (±0.029)A, $n = 4$, $r^2 = 0.999$. For the degradation product (II), they are: 0–26 μg/ml, C_{II} (±0.142) μg/ml = −0.140 (±0.068) + 0.766 (±0.005)A, $n = 7$, $r^2 = 1.000$; and 0–2.6 μg/ml, C_{II} (±0.0408) = −0.103 (±0.034) + 0.741 (±0.017)A, $n = 4$, $r^2 = 0.999$, where A is the relative area and was 10^{-5} times the actual number of area units measured. The values in parentheses are the standard errors.

The reproducibility of the assay of 10 μg/ml within and among days was challenged by assaying a refrigerated sample 10 times and then on 10 separate days. The assay of I with its standard deviation was 10.03 ± 0.23 within a day and 9.94 ± 0.50 among days; for the derived acid (II), the reproducibility was 10.07 ± 0.20 within a day and 10.09 ± 0.43 among days.

Kinetics of I Solvolysis—At constant pH, I degrades by an apparent first-order process. Representative semilogarithmic plots of the HPLC relative peak area for I against time are given for hydrochloric acid solutions (Fig. 3), acetate buffers (Fig. 4), phosphate and borate buffers (Fig. 5), and sodium hydroxide solutions (Fig. 6), where the apparent first-order rate constants, k, were obtained from the slopes in accordance with:

$$\log A = -\frac{k}{2.303}t + \log A_0 \qquad \text{(Eq. 1)}$$

where A and A_0 are the relative peak areas at time t and time zero, respectively.

The pH values for the strongly acidic and alkaline solutions were calculated from data for γ and pKw in the literature (11). The concentrations of I in alkaline solution studies had to be lowered to 2×10^{-4} M so as not to exceed the solubility of the neutral compound. Higher concentrations showed rate plots that were initially zero order. The solubility of neutral I is estimated to be 0.03–0.05 mg/ml.

Log k–pH Profiles for Hydrolysis of I—For hydrochloric acid solutions, the pH values were calculated from:

$$\text{pH} = -\log \gamma[\text{HCl}] \qquad \text{(Eq. 2)}$$

where γ is the mean activity coefficient for the hydrochloric acid solutions (11). For sodium hydroxide solutions:

$$\text{pH} = \text{pKw} - \text{pOH} = \text{pKw} + \log \gamma[\text{NaOH}] \qquad \text{(Eq. 3)}$$

where γ is the mean activity coefficient for the sodium hydroxide solution. The values of pKw = $-\log K_w$ are listed in Table I, where K_w is the hydrolysis constant for water (11).

The log k–pH profiles for the solvolysis of I in aqueous solutions with ionic strengths (μ) of 0.1 in the buffer solutions are given in Fig. 7. They are fitted to the expression:

$$k = (k_{H^+}a_{H^+} + k_{OH}a_{OH^-})f_{(\equiv NH^+)} + k'_{OH}a_{OH^-}f_{(\equiv N)} \qquad \text{(Eq. 4)}$$

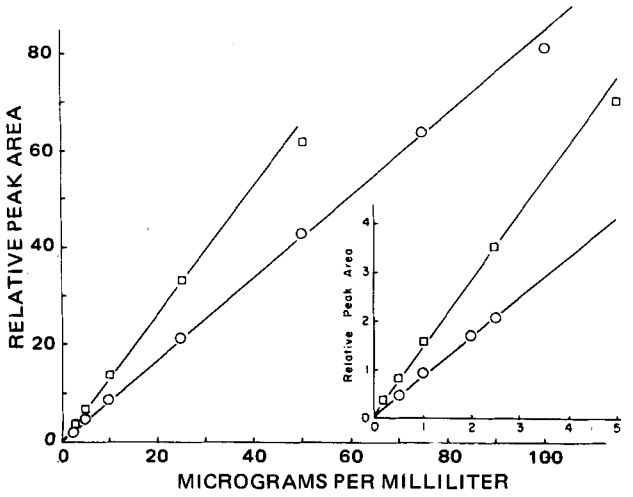


Figure 2—*HPLC calibration curves of relative peak area against concentration (micrograms per milliliter) for nafronyl (I) acid oxalate (○) and its derived acid (II) (□) in pH 4 acetate buffer–acetonitrile (50:50) at a flow rate of 2.5 ml/min.*

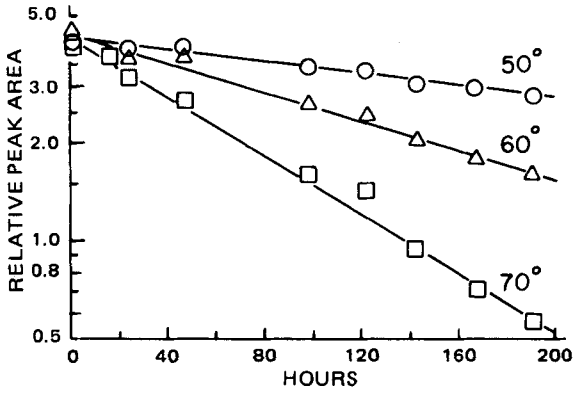


Figure 3—*First-order plots of HPLC relative peak area of I in 0.4* N *HCl against time. The curves are labeled with the temperature of the study.*

where the activities of the catalytic species are calculated from $a_{OH^-} = 10^{-pOH} = 10^{-(pKw-pH)}$ and $a_{H^+} = 10^{-pH}$. The fractions of protonated and nonprotonated drug are, respectively:

$$f_{(\equiv NH^+)} = a_{H^+}/(K'_a + a_{H^+}) \quad \text{(Eq. 5)}$$

and:

$$f_{(\equiv NH)} = K'_a/(K'_a + a_{H^+}) \quad \text{(Eq. 6)}$$

where K'_a is the apparent dissociation constant of protonated I. The parameters for the best fits of the log k–pH profiles (Fig. 7) are given in Table I. These values can be substituted into Eqs. 4–6 to permit calculation of the apparent first-order rate constant under any given pH

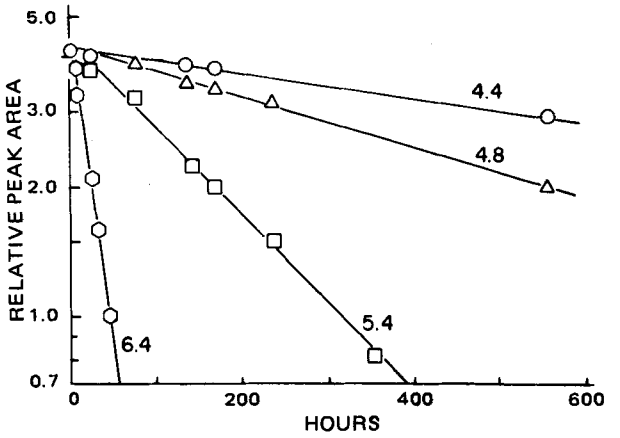


Figure 4—*First-order plots of HPLC relative peak area of I in acetate buffers of various pH values against time with $\mu = 0.1$ at 70.0°. The curves are labeled with the pH of the solutions.*

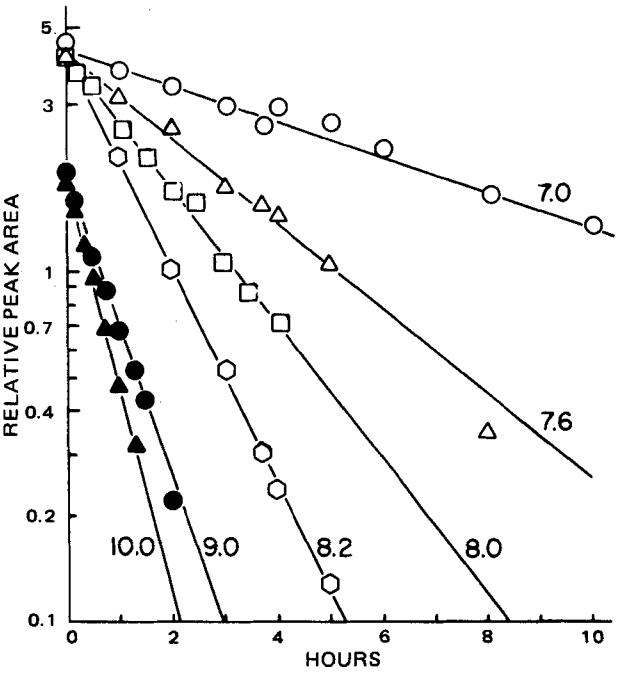


Figure 5—*First-order plots of HPLC relative peak area of I in phosphate and borate buffers of various pH values against time with $\mu = 0.1$ at 70.0°. The curves are labeled with the pH of the solutions.*

conditions. The temperature dependence of the rate constant for a given alkali or acid concentration or pH (Fig. 8 and Table II) also was considered in adjusting the fits for a temperature with fewer actual studies.

The log k–pH profile in conformity to Eq. 4 clearly showed that protonated I is hydrogen ion and hydroxide ion catalyzed, with maximum

Table I—Parameters of Fit [a] of Log k–pH Profiles for the Solvolysis of I

Parameter	70.0°	60.0°	50.0°	30.0°
log k_{OH}	3.132	3.041	2.923	2.636
k_{OH}, liters/mole/min	1357	1100	837	433
log k'_{OH}	0.180	0.018	−0.140	−0.572
k'_{OH}, liters/mole/min	1.510	1.042	0.724	0.268
log k_{H^+}	−3.260	−3.523	−3.928	−4.611
k_{H^+}, liters/mole/min	5.50×10^{-4}	3.00×10^{-4}	1.18×10^{-4}	2.45×10^{-5}
pKa′	7.90	7.90	7.90	8.20
K'_a	1.259×10^{-8}	1.259×10^{-8}	1.259×10^{-8}	6.310×10^{-9}
pKw	12.79	13.02	13.26	13.83
K_w	1.622×10^{-13}	9.550×10^{-14}	5.495×10^{-14}	1.479×10^{-14}

[a] Fitted to $k = (k_{H^+}a_{H^+} + k_{OH}a_{OH^-})f_{(\equiv NH^+)} + k'_{OH}a_{OH^-}f_{(\equiv N)}$, where the activities of the catalytic species are $a_{OH^-} = 10^{-pOH} = 10^{-(pKw-pH)}$ and $a_{H^+} = 10^{-pH}$, the fraction of drug that is protonated and nonprotonated, respectively, is $f_{(\equiv NH^+)} = a_{H^+}/(K'_a + a_{H^+})$ and $f_{(\equiv NH)} = K'_a/(K'_a + a_{H^+})$, and the pH values in the strongly acidic and alkaline ranges are calculated, respectively, from pH = −log γ[HCl] and pH = pKw − pOH = pKw + log γ[NaOH], where the pKw values and activity coefficients (γ) are obtained from the literature (11).

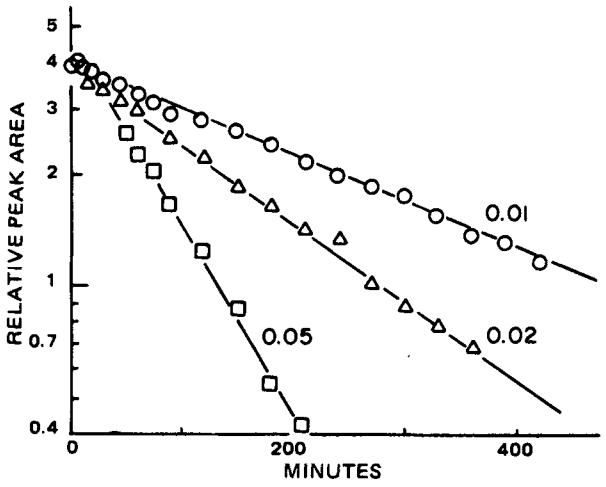


Figure 6—*First-order plots of HPLC relative peak area of I in sodium hydroxide solutions against time. The curves are labeled with the molarities of sodium hydroxide.*

stability at pH 3.0. Reasonable slopes of −1 and +1 were obtained for these acid and alkaline branches of the log k–pH curves. The plateau achieved in the pH 8–10 region indicates lessened catalytic efficiency of the hydroxide-ion attack on uncharged I with a kinetic pKa′ of ~8.0. The microscopic rate constants were determined from the intercepts of the linear segments with slopes of unity in accordance with:

$$\log k = \log k_{H^+} - \text{pH} \qquad \text{(Eq. 7)}$$

for the acid branch characterizing the hydrogen-ion attack on protonated I below pH 3, or in accordance with:

$$\log k = \log k_{OH} - \text{pH} + \text{pKw} \qquad \text{(Eq. 8)}$$

for the alkaline branch characterizing the hydroxide-ion attack on protonated I for pH 3–8, and in accordance with:

$$\log k = \log k'_{OH} - \text{pH} + \text{pKw} \qquad \text{(Eq. 9)}$$

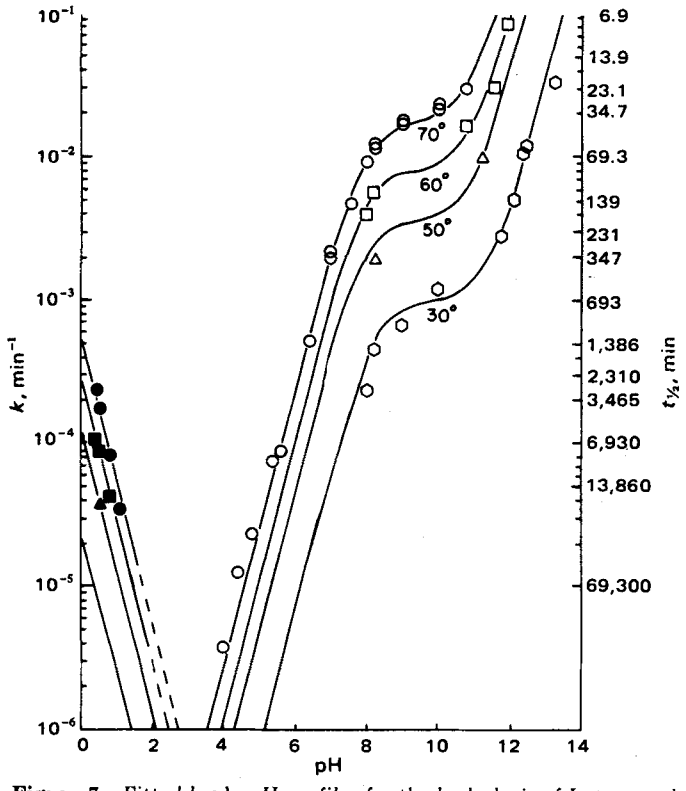


Figure 7—*Fitted log k–pH profiles for the hydrolysis of I at several temperatures.*

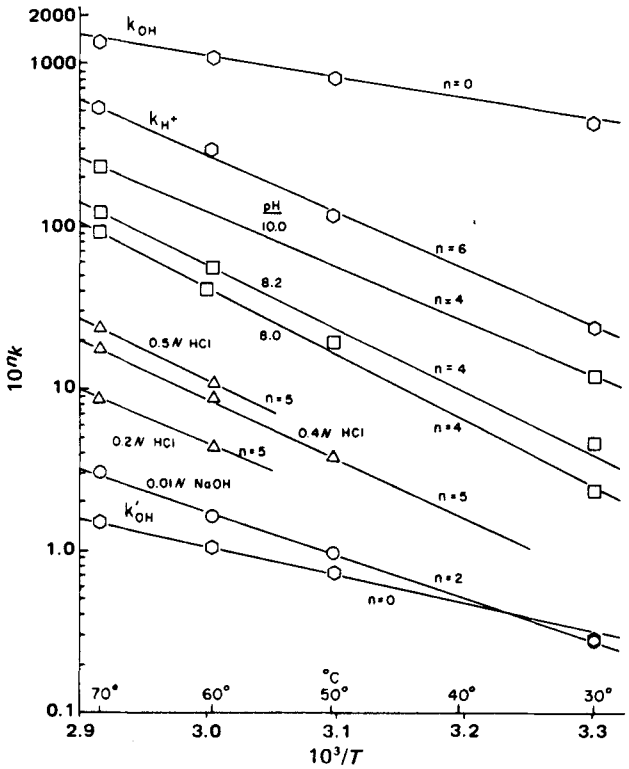


Figure 8—*Arrhenius plots of apparent first-order rate constants (k expressed in minutes^{-1}) under different pH conditions and at constant hydrochloric acid and sodium hydroxide conditions where T = 273+ °C. In addition, the Arrhenius plots are given for the microscopic catalytic constants, k_{OH}, k'_{OH}, and k_{H^+}, that were obtained from the fitted log k–pH profiles at the various temperatures given in Fig. 7.*

for the alkaline branch characterizing the hydroxide-ion attack on uncharged I at pH values above the plateau, pH ~11. The determined constants and the kinetic pKa′ values that define the plateaus are listed in Table I.

Other estimates of biomolecular rate constants, referenced to concentrations rather than activities, can be obtained from plots of apparent first-order rate constants against hydrochloric acid concentrations in accordance with:

$$k = k_{HCl}[HCl] \qquad \text{(Eq. 10)}$$

and the k_{HCl} values were 4.44×10^{-4} liter/mole/min at 70° and 2.16×10^{-4} liter/mole/min at 60°. The lack of pH-independent solvolysis was indicated by the fact that these plots passed through the origin.

Significance of Arrhenius Parameters—The Arrhenius parameters

Table II—Parameters for Temperature Dependence [a] of the Apparent Rate Constants for the Solvolysis of I

Rate Constant	Slope (S)	ΔH_a, kcal/mole	ln P
k in 0.01 M NaOH, min^{-1}	6098	12.1	14.25
k in 0.2 M HCl, min^{-1}	8440	16.8	15.18
k in 0.4 M HCl, min^{-1}	8531	17.0	16.24
k in 0.5 M HCl, min^{-1}	7701	15.3	14.12
k in pH 8.0 phosphate buffer, min^{-1}	9447	18.9	23.00
k in pH 8.2 phosphate buffer, min^{-1}	9009	17.9	21.87
k in pH 9.0 borate buffer, min^{-1}	8400	16.7	20.39
k in pH 10.0 borate buffer, min^{-1}	7712	15.3	18.69
k_{OH} [b], liters/mole/min	3886	7.72	11.74
k_{OH} [c], liters/mole/min	2972	5.91	15.93
k_{H^+} [d], liters/mole/min	8062	16.0	15.97

[a] $\ln k = \ln P - S/T = \ln P - (\Delta H_a/R)(1/T)$, where R = 1.987 cal/degree and T = 273+ °C. [b] Bimolecular rate constant for the attack of hydroxide-ion activity on the nonprotonated drug species as determined from the best fit of log k–pH profiles at the several temperatures. [c] Bimolecular rate constant for the attack of hydroxide-ion activity on the protonated drug species as determined from the best fit of log k–pH profiles at the several temperatures. [d] Bimolecular rate constant for the attack of hydrogen-ion activity on the protonated drug species as determined from the best fit of log k–pH profiles at the several temperatures.

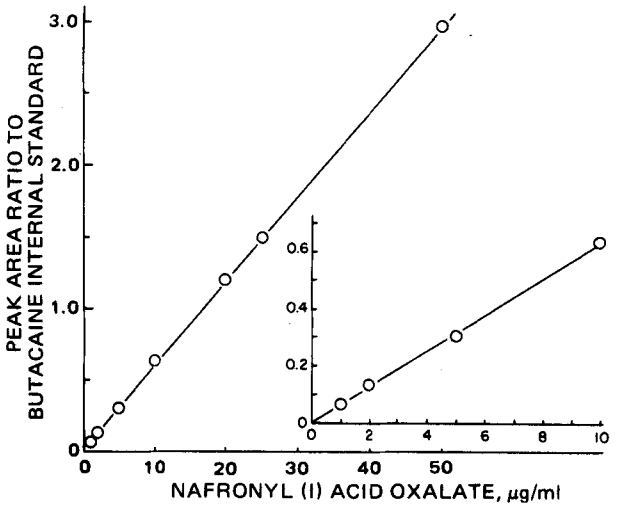


Figure 9—*HPLC calibration curves of the relative peak area ratio with respect to butacaine (the internal standard) against concentration (micrograms per milliliter) of nafronyl (I) acid oxalate in human plasma in pH 3.5 acetate buffer–acetonitrile (50:50); the flow rate was 2.5 ml/min.*

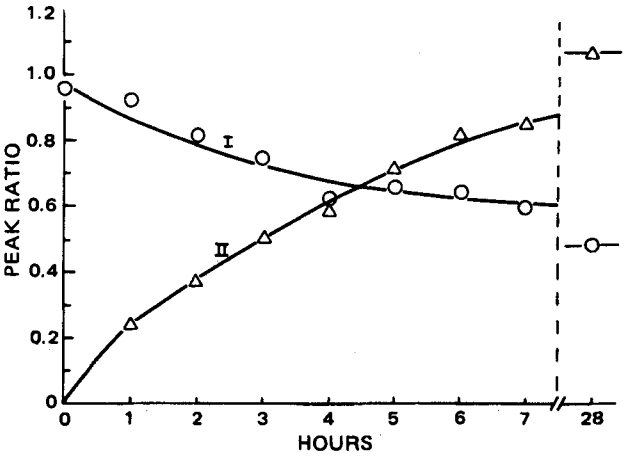


Figure 10—*Plots of peak area ratio of I (○) and peak height ratios of II (△) with respect to butacaine (the internal standard) against time for the degradation of I in fresh dog plasma at 24.5° with an initial concentration of 1.25 μg/ml of plasma.*

for the determined microscopic rate constants (Table II) show a higher heat of activation for the hydroxide-ion attack on the neutral species (7.72 kcal/mole) than on the coulombic charge-expedited attack on the protonated species (5.91 kcal/mole). The greater resistance of protonated I to hydrogen-ion attack is manifested by the higher heat of activation of 15.2 kcal/mole. The reasonable fits of the log k–pH profiles at the several temperatures were confirmed by the linearity of the Arrhenius plots for the derived constants (Fig. 8).

Effects of Substrate Concentrations and Buffers on I Solvolysis—Studies were conducted at several concentrations of I for given pH values, and there was no significant effect on the apparent hydrolysis rate constant. Phosphate buffer concentrations from 0.043 to 0.13 M at pH 6.4 and 70° showed no significant buffer effects on the apparent first-order rate constant. There was a significant, but small, phosphate buffer effect at pH 8.1 and 70° with μ = 0.1, where the k ($\times 10^3$ min^{-1}), $[KH_2PO_4]$, and $[Na_2HPO_4]$ values were, respectively: 9.08, 9.14×10^{-4}, 1.58×10^{-2}; 10.62, 1.37×10^{-3}, 2.36×10^{-2}; and 11.64, 1.83×10^{-3}, 3.15×10^{-2}; and where:

$$K^{70°}_{pH\,8.1} = 8.52 \times 10^{-3} + 0.096\,[H_3PO_4^{2-}] \qquad \text{(Eq. 11)}$$

The half-life of I in 10% ammonia at 30° was 336 min.

Pharmaceutical Significances of I Stability Studies—The high solvolytic instability of I, even in mildly alkaline solutions, requires great care in the formulation of liquid and solid dosage formulations so that the microscopic environment is as acidic as possible. Alkaline lubricants and excipients should not be used, and the granulation should be kept as free of water as possible. Even at 30° and pH 7, the half-life of I in aqueous solution is predicted to be only 7 days (Fig. 7). A pH of 5.4 is the maximum that can be tolerated at 30°, with a loss of 10% of I to hydrolysis over 3 years. Thus, I must be assayed immediately and must not be stored in samples of biological tissues and fluids.

The k value was calculated from:

$$k(\text{min}^{-1}) = \ln 0.1/(3 \times 365 \times 24 \times 60) = 1.46 \times 10^{-6}\ \text{min}^{-1} \qquad \text{(Eq. 12)}$$

and this rate constant is predicted to be at the pH value of 5.4 at 30° (Fig. 7). In Europe, I is distributed as the oxalate, and aqueous solutions should be pharmaceutically stable. The microscopic environment of dosage forms can be maintained at the pH of maximum stability with such an acid salt. A 0.1-mg/ml solution has a pH of 4.0 and a predicted half-life of 20 years at 30°.

Extractions of I into organic solvents may be conducted from alkalinized solutions if such extractions are performed immediately. At 30° and pH 10, the half-life of I is 12 hr but only 21 and 7 min in 0.5 and 2.5 N NaOH, respectively. An extraction procedure using 10% ammonia (10) is satisfactory if it is carried out immediately, giving a half-life of 5 hr.

Partition as a Function of pH; Application to Design of Analytical Methods—The oil–water partition coefficient, K, was calculated from the observed peak areas from the HPLC analyses, in which the areas were shown to be directly proportional to concentration, by:

$$K = \frac{(A_1 - A_2)V_{aq}/V_{org}}{A_2} \qquad \text{(Eq. 13)}$$

where A_1 and A_2 are the peak areas of the aqueous phase before and after extraction, respectively, and V_{aq} and V_{org} are the respective volumes of the admixed buffer and chloroform solutions.

A similar procedure was used for the determination of the oil–water partition coefficient of the derived acid (II), except that the volume of chloroform used (V_{org}) also was 1.0 ml.

The partitioning of I into chloroform from various buffer solutions of acetate, phosphate, and borate was studied at pH 3.5, 4.4, 5.4, 6.4, 7.0, 8.0, 9.0, and 10. In all cases above pH 3.5, the partition coefficients (Eq. 1) were >100, indicating almost complete extraction. The partition coefficient was 74 at pH 3.5 and 7.7 in 0.01 M HCl.

Studies of the partitioning of the acid (II) showed complete extraction

Table III—Mobile Phases and Retention Times in HPLC Separations

Flow Rate, ml/min	Mobile Phase[a] pH	Mobile Phase[a] Buffer–Acetonitrile	Retention Time, min: Plasma Components	II	Butacaine	I	Adequate Separation of II and Plasma Components?
2.0	4.5	50:50	3.42	3.24	4.73–4.90	7.04–7.15	No
2.5	3.5	50:50	3.45	4.00	>15	>20	Somewhat
1.0	5.0	50:50[b]	6.95	6.00	9.14	12.74	Yes
1.5	4.0	55:45[c]	3.28	3.6	8.54	12.83	No
1.4	4.0	55:45	3.33	4.07–4.36	9.40–10.00	14.06–14.85	Yes
1.5	3.75	55:45	3.66	4.03	8.37	12.36	Somewhat
1.0	5.0	60:40	6.33–6.34	7.05	—	12.52	Yes
2.5	4.5	60:40	4.75	4.65	6.15	9.90	No
2.2	4.5	60:40	4.74	4.57	6.00	9.71	No
2.5	3.5	60:40	11.4	13.0	—	>25	Yes

[a] All buffer was comprised of 0.1 M acetate buffer except that of last row, which was 0.01 M acetate buffer. [b] Contains 1% tetrahydrofuran. [c] In this system, heparin gave a peak at 2.58 min and thiopental gave a peak at 2.80 min.

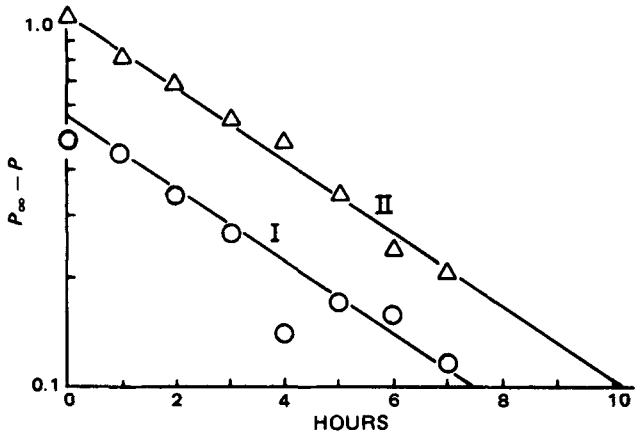


Figure 11—*Semilogarithmic plot of log* ($P_\infty - P$) *versus time for the loss of I and the production of II in fresh dog plasma at 24.5°. The* P *value represents peak area ratios for I and peak height ratios for II with respect to the internal standard, butacaine. The* P_∞ *values were those obtained at the 28-hr assays. The half-life of I for its 50% degradation in this plasma was 3.2 hr.*

of II from acetate buffers of pH 4.5 and lower and from 0.05 *M* HCl. No II was extractable from pH 9.5 borate buffer and 10% ammonia. The oil–water partition coefficients (Eq. 1) at other pH values were 80.2 at pH 5.3, 35.1 at pH 6.2, 5.17 at pH 7.15, and 0.17 at pH 8.5. All studies except the last one (in borate buffers) were conducted in phosphate buffers.

These systematic studies on the partitioning of I from aqueous media into chloroform as a function of pH showed that I can be readily extracted over the entire pH range. Thus, there is no need to alkalinize aqueous solutions, plasma, or urine, which can lead to degradation, to extract I with efficiency. Both I and II can be extracted at pH <7, and both compounds can be analyzed simultaneously with one injection into the chromatograph. Alternatively, extraction at pH 9.5 can selectively extract I, and subsequent extraction of the acidified aqueous solution can remove the acid degradation product. HPLC analytical methods were developed using these physicochemical properties.

HPLC Analytical Methods for I in Plasma Using Acetonitrile as Deproteinizing Agent—Acetonitrile can be used as both a plasma deproteinizing agent and as an extraction solvent so that an aliquot of the acetonitrile fraction can be injected directly for assay. It is ideal for injection into the chromatograph since the mobile phase is a mixture of acetonitrile and acetate buffer and the intensity of the solvent peak is minimized. Methods A and B were developed using this procedure (see *Experimental*).

There were interferences from blank plasma in Method A for II, with a major plasma peak at 2.56 min under the given HPLC conditions, but not with the I peak. The calibration curves were similar to Fig. 2. The baseline of the chromatogram was linear, and the standard errors of the estimates for the 0–2.5-μg/ml range were 140 ng/ml of plasma for the peak area and 41 ng/ml for the peak heights when 100 μl of plasma was analyzed. These data indicated sensitivities of 300 and 100 ng/ml for the peak areas and peak heights, respectively, using 100 μl of plasma. The linear regression equations for the peak area (A) and peak height (P) for 0–50 μg/ml are: C (±0.617) μg/ml = −0.11 (±0.27) + 4.82 (±0.06)A (n = 10, r^2 = 0.990) and C (±0.23) μg/ml = −0.19 (±0.10) + 9.71 (±0.05)P (n = 9, r^2 = 0.998). For 0–2.5 μg/ml and n = 4: C (±0.14) μg/ml + 0.06 (±0.14) + 4.17 (±0.37)A (r^2 = 0.9849) and C (±0.041) μg/ml = −0.02 (±0.04) + 8.88 (±0.23)P (r^2 = 0.9987). The relative area, A, is 10^{-6} times the actual area units measured. The relative peak height, P, is 10^{-2} times the actual centimeters measured. The values in parentheses are the standard errors.

The peak area ratios of I to butacaine are plotted as a function of the concentration of I in 1 ml of plasma in Fig. 9 for Method B. The standard error of the estimate was 239 ng/ml of plasma for the 0–50-μg/ml range, and it was 151 ng/ml of plasma for the 0–10-μg/ml range.

The linear regression equation for concentration (C in micrograms per milliliter of plasma) against the peak area ratio (PAR) for 0–50 μg/ml is C (±0.24) μg/ml = −0.28 (±0.13) + 16.99 (±0.09) PAR (n = 7, r^2 = 0.9998). For 0–10 μg/ml, it is: C (±0.15) = 0.02 (±0.12) + 15.95 (±0.34) PAR. The values in parentheses are the standard errors.

HPLC Analytical Methods for I in Plasma Using Haloalkane Extractions—Remarkable reproducibility was obtained for calibration curves of the peak area against the plasma concentration performed on different days by Method C. The standard error estimated for the 0–10-μg/ml range was 185 ng/ml of plasma.

The linear regression equation for concentration, expressed as micrograms per milliliter of plasma, against the relative peak area (A) for 0–10 μg/ml of plasma is C (±0.185) μg/ml = 0.211 (±0.089) + 0.879 (±0.015)A (n = 9, r^2 = 0.9980).

The standard error estimated by Method D was 86 ng/ml of plasma for the 0–5-μg/ml range of I and 192 ng/ml of plasma for the 0–5-μg/ml range of II from the linear regression of concentration on the peak area ratio to the internal standard, butacaine. The presence of a finite intercept in the regression of II indicated a small interference from plasma constituents at the retention time of II under these analytical conditions.

The linear regression equation for concentration, expressed as micrograms per milliliter of plasma, against the peak area ratio (PAR) for 0–5 μg/ml of I is C (±0.0864) μg/ml = −0.002 (±0.056) + 1.404 (±0.031) PAR (n = 5, r^2 = 0.9985). For 0–5 μg/ml of II, it is C (±0.192) μg/ml = −0.94 (±0.20) + 1.189 (±0.065) PAR (n = 4, r^2 = 0.9946). The values in parentheses are the standard errors.

HPLC Separation of I and Its Derived Acid (II) from Plasma Impurities and Applications to I Stability in Plasma—The HPLC separation of I, butacaine sulfate (internal standard), and II was readily effected with a mobile phase of buffer–acetonitrile (50:50 or 60:40) where 0.01–0.10 N acetate buffers were used and the pH was adjusted to 3.5–5.0 (Figs. 1A and 1B and Table III).

Unfortunately, plasma has some constituents that appear close to, or concomitantly with, the retention time of II and could interfere with its assay at 224 nm. The plasma constituent extracted with chloroform or methylene chloride (Fig. 1C) produced a peak that frequently interfered with the peak of II (Figs. 1B and 1D). However, variations in the mobile phase composition did permit satisfactory separations (Figs. 1E–1H). A valid HPLC system (Fig. 1H and Method B) was chosen to analyze for the stability of 1.25 μg of nafronyl (I) oxalate/ml of freshly withdrawn heparinized dog plasma. The peak area ratio (with respect to the internal standard) calibration curves were valid for the I assay, but the peak height ratios were best for the assay of II since a slight interference with a plasma component gave a significant positive intercept in its peak area ratio calibration curve.

The peak area and height ratios of I and II are given in Fig. 10 for fresh dog plasma spiked with I at 24.5°. Appropriate semilogarithmic plots of these data are given in Fig. 11 and are consistent in their estimates of a 3.2-hr half-life for the stability of I in dog plasma. Pharmacokinetic studies where plasma samples were not assayed immediately would be suspect.

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