their stomachs should have been full of solid material as a result of coprophagy.

The data presented here show that the stomach-emptying-controlled rabbit can be a satisfactory model for evaluation of GI absorption characteristics of these drugs with limited or regional solubility.

CONCLUSION

Chiou *et al.* (5) concluded that the rabbit was not a useful animal for drug absorption studies because it was almost impossible to obtain an empty stomach in the rabbit by using the conventional fasting method and because the fasted state markedly prolonged the stomach emptying time. This conclusion has been very instructive to many investigators. However, in this study it was found possible to simulate the stomach emptying rate of rabbits to that of humans by taking adequate steps to empty the stomach and to feed the animals with a special soft diet. When the stomach emptying rate is controlled, the rabbit can be used as an animal model for predicting bioavailability of oral dosage for human subjects.

Results of bioavailability studies on oral dosage forms will be reported later.

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Kinetics of Hydrolysis of Fenclorac

C. M. WON [x], J. J. ZALIPSKY, D. M. PATEL, and N. H. REAVEY-CANTWELL

Abstract □ The kinetics of hydrolysis of fenclorac were studied to determine its stability in aqueous solution at different pH's and temperatures. For this study, a stability-specific liquid chromatographic assay method was developed to separate fenclorac from its hydrolysis product, α-hydroxy-3-chloro-4-cyclohexylbenzeneacetic acid. The *k*–pH profile in the 0–12 pH range in various buffer solutions shows that fenclorac is stable in its undissociated form in strongly acidic media and is unstable in neutral and alkaline media. The instability of fenclorac in aqueous solution is proportional to the degree of ionization of the carboxyl group in the 1–4 pH range and is independent of pH above 4. The rate-determining step in the mechanism of hydrolysis of fenclorac involves ionization of the carbon–chlorine bond. The ionization is catalyzed by an intramolecular nucleophilic attack on the α-carbon by the dissociated carboxyl group, resulting in the formation of an unstable intermediate, a three-membered ring lactone. This unstable intermediate rapidly hydrolyzes to the final hydrolysis product. This mechanism is supported by experimental evidence such as the medium effect, positive salt effect, common ion effect, and substituent effect. Arrhenius parameters for the hydrolysis of fenclorac and its 3-nitro substituted analog were obtained.

Keyphrases □ Fenclorac—kinetics of hydrolysis, stability in aqueous solution, various pH's and temperatures, liquid chromatographic analysis □ Hydrolysis—fenclorac in aqueous solutions, kinetics, various pH's and temperatures, liquid chromatographic analysis □ Stability—fenclorac in aqueous solutions, various pH's and temperatures, liquid chromatographic analysis □ Liquid chromatography—analysis, fenclorac in aqueous solutions □ Anti-inflammatory agents—fenclorac, kinetics of hydrolysis, stability in aqueous solution, various pH's and temperatures, liquid chromatographic analysis

In the search for a new, nonsteroidal, anti-inflammatory compound for clinical utility, a series of substituted benzeneacetic acids was synthesized (1). Fenclorac (I) (α,3-dichloro-4-cyclohexylbenzeneacetic acid) diethylammonium salt exhibited promising anti-inflammatory activities. The anti-inflammatory activity of fenclorac was demonstrated in rats using the carrageenan paw edema assay[1].

A simple, sensitive analytical method was needed to follow the hydrolysis of relatively unstable fenclorac in

[1] G. Nuss *et al.*, to be published.

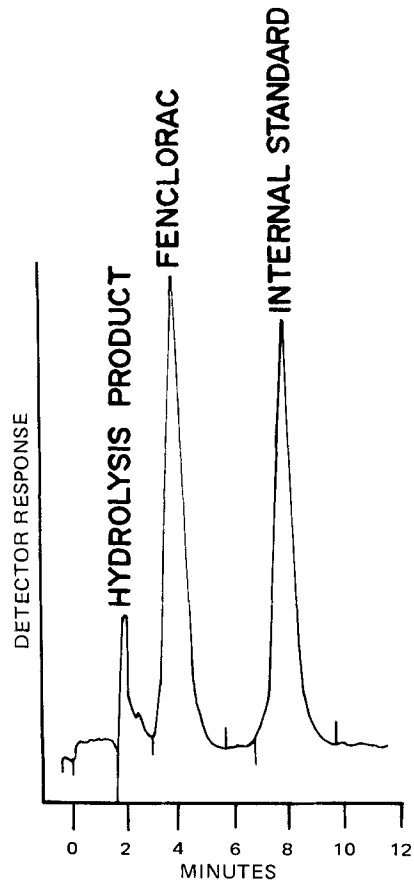


Figure 1—*Typical chromatogram of fenclorac, its hydrolysis product, α-hydroxy-3-chloro-4-cyclohexylbenzeneacetic acid, and the internal standard, triphenylethylene.*

aqueous solution. Previously reported methods for analysis of substituted aryl- and alkylphenoxyalkyl carboxylic acids, based on paper chromatographic separation and ion-pair formation with bromcresol green (2) and GLC assay (3), were not suitable. Therefore, a rapid stability-specific liquid chromatographic assay was developed for kinetic assay.

This report is concerned with the study of the kinetics and the mechanism of the hydrolysis of fenclorac in aqueous solution to utilize the basic information for pharmaceutical formulation design.

EXPERIMENTAL

Analytical Apparatus—A liquid chromatograph[2] equipped with a 254-nm UV detector and an electronic integrator[3] was used. The column was 50-cm × 7.9-mm (i.d.) stainless steel packed with chemically bonded octadecyl hydrocarbon on 30-μm porous silica[4].

Chromatographic Conditions—The solvent system was composed of methanol–water–acetic acid (50:36:14). The flow rate was 4.4 ml/min, and the pressure required to maintain this flow rate was 500 psi. The slightly different solvent systems used for α-chloro-4-cyclohexylbenzeneacetic acid and α-chloro-3-nitro-4-cyclohexylbenzeneacetic acid were methanol–water–acetic acid (54:40:6) and methanol–water–acetic acid (40:46:14), respectively.

Calibration Curve—Separate solutions containing 0.192, 0.384, 0.576, and 0.768 mg/ml of fenclorac standard and 36 μg/ml of internal standard, triphenylethylene[5], in methanol were injected into the column through a six-port injection valve with a loop capacity of 35 μl.

[2] Model 830, DuPont Instruments, Wilmington, Del.
[3] Model 3380A, Hewlett-Packard, Avondale, Pa.
[4] Permaphase ODS, DuPont Instruments, Wilmington, Del.
[5] Eastman Kodak, Rochester, N.Y.

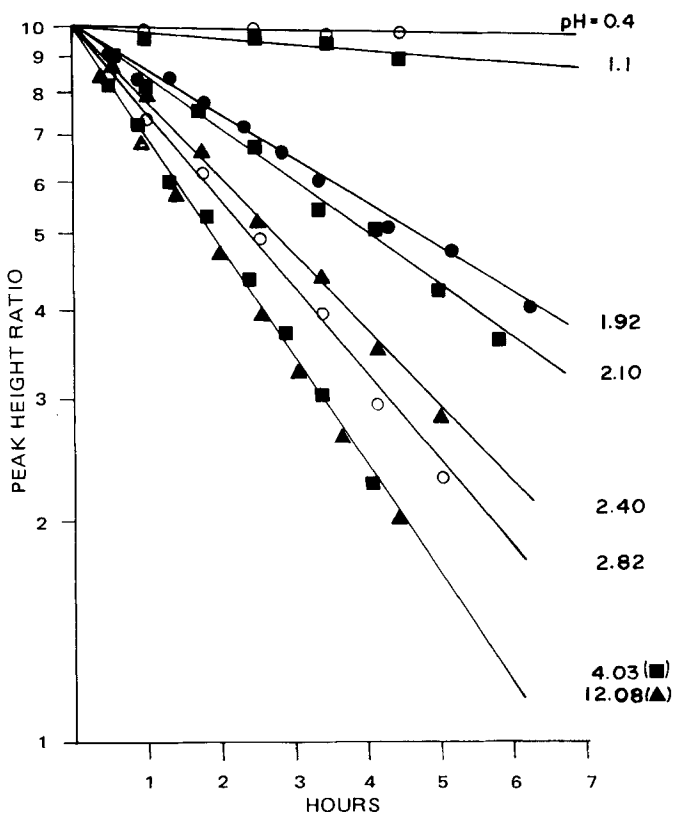


Figure 2—*Typical semilogarithmic plots of the ratio of the peak height of fenclorac to that of the internal standard, triphenylethylene, against time for the hydrolysis of fenclorac at 37.0° and the given pH.*

Kinetic Procedure—All kinetic experiments were performed at an ionic strength of 0.1 with sodium nitrate. Buffers employed were glycine (pH 2.1–2.8), formate (pH 3.3–4.0), acetate (pH 4.4), and carbonate (pH 10.1). An aliquot (0.8 ml) of a methanolic stock solution of fenclorac (50 mg/ml) was transferred into 100 ml of buffer solution equilibrated to the temperature under study. The low solubility of fenclorac in water at pH ≤ 4 necessitated the use of a stock solution of a lower concentration (1 mg/ml). The samples were withdrawn at various time intervals and analyzed as described.

Assay Procedure—Five milliliters of sample solution was cooled in an ice–water bath. An aliquot (4.0 ml) of the cooled sample was introduced into a vial with 4.0 ml of an internal standard solution. The internal standard solution contained 6 mg of triphenylethylene in 6 ml of acetic acid and 94 ml of methanol. The mixed sample was injected into the column through a six-port injection valve with a 35-μl capacity loop. A larger injection loop (1.39 ml) and a lower concentration of internal standard (1.2 μg/ml) were used for the sample solutions studied at pH ≤ 4.0.

RESULTS AND DISCUSSION

Liquid Chromatography—A typical liquid chromatogram of fenclorac (t_R = 4.6 min), its hydrolysis product (t_R = 2.4 min), and the internal standard (t_R = 9.7 min) is shown in Fig. 1. The plot of the ratio of peak height or area of fenclorac to that of internal standard *versus* the concentration of fenclorac showed a linear relationship between 0.192 and 0.768 mg/ml. The calibration curve had a mean slope of 1.945 mg^{-1},

Table I—Apparent First-Order Rate Constant in Hydrolysis of Fenclorac at 37.0° and pH 7.04 as a Function of Phosphate Buffer Anion, $[HPO_4^{2-}]$

$[HPO_4^{2-}]$, $M \times 10^{-2}$	10^4 k, sec^{-1}
1.4	1.15
2.8	1.13
7.0	1.11
11.2	1.11

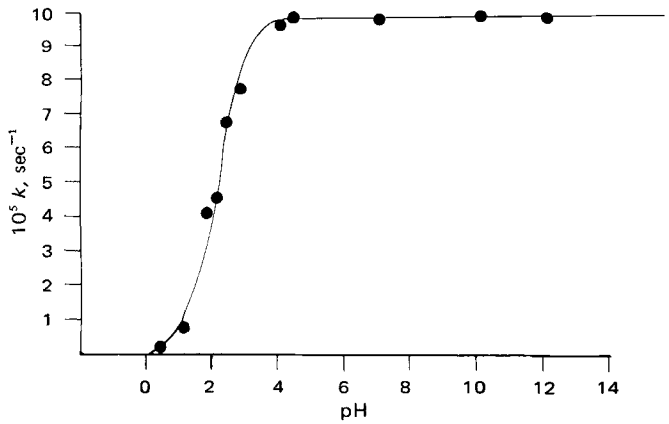


Figure 3—*The k–pH profile for the intramolecular nucleophilic catalysis in the hydrolysis of fenclorac in water (μ = 0.1) at 37.0°*

a mean Y-intercept of 0.014, and a mean correlation coefficient of 0.9998. These data indicate that the liquid chromatographic method can be used with a single-point standard.

Rate Constant—The apparent first-order rate constants were estimated from the slopes of appropriately plotted experimental data using:

$$\log R = \log R_0 - kt/2.303 \qquad \text{(Eq. 1)}$$

where R_0 was the initial ratio of peak height of fenclorac to that of the internal standard, and R was the ratio at any given time t. The reaction was irreversible, and no fenclorac peak was observed when the reaction went to completion. At any given pH, the semilogarithmic plots of the peak height ratio *versus* time showed linearity. Typical first-order plots at various pH values are given in Fig. 2. The hydrolysis of fenclorac was first order with respect to substrate over a 50-fold variation in initial concentration.

General Acid–Base Catalysis—The rate constants were independent of buffer concentration at constant pH and ionic strength when the phosphate buffer concentration was changed eightfold (Table I). This independence indicates that the hydrolysis is not due to the possible catalytic properties of the phosphate ions.

***k*–pH Profile**—The k–pH profile (Fig. 3) was constructed from the first-order rate constants and pH values at 37.0°. The first-order rate constant is independent of pH above 4 and is a sigmoid function of pH below 4. The hydrolysis rate is proportional to the degree of ionization of the carboxyl group, and the first-order rate constant for this system can be formulated as:

$$k = k_{AH}f_{AH} + k_Af_A \qquad \text{(Eq. 2a)}$$

$$k = \frac{k_{AH}[H^+] + k_AK_a}{[H^+] + K_a} \qquad \text{(Eq. 2b)}$$

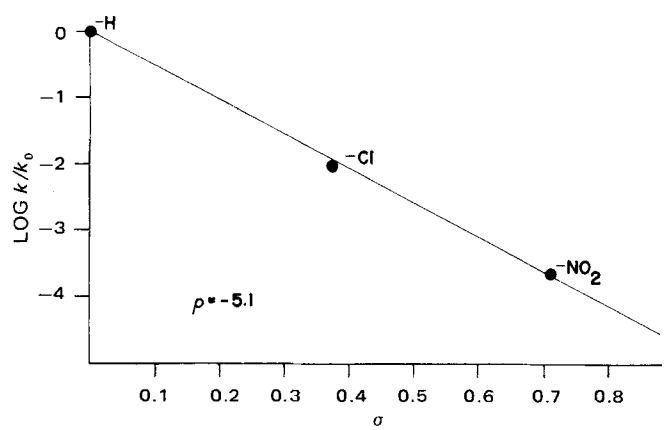


Figure 4—*Linear free energy relationship between the rates of hydrolysis of α-chloro-4-cyclohexylbenzeneacetic acid containing electron-withdrawing substituents and the Hammett substituent constants.*

Table II—Apparent First-Order Rate Constants in Hydrolysis of Fenclorac at 37.0° and pH 7.04 as a Function of Ionic Strength with Sodium Nitrate (k') and with Potassium Chloride (k), and Relative Rates of the Intermediate with Cl^- and Solvent (k_{Cl}/k_s)

μ	$10^5 k'$, sec^{-1}	$[Cl^-]$, M	$10^5 k$, sec^{-1}	k_{Cl}/k_s
0.096	9.86	0	9.86	—
0.446	11.7	0.35	10.2	0.42
0.846	13.6	0.75	10.5	0.39
1.596	14.4	1.50	11.1	0.20

where k_{AH} and k_A are the rate constants for the hydrolysis of undissociated and dissociated fenclorac, respectively; and f_{AH} and f_A are the fractions of undissociated and dissociated species, respectively. Since k_{AH} is negligible compared to k_A, Eq. 2*b* is reduced to:

$$k = \frac{k_AK_a}{[H^+] + K_a} \qquad \text{(Eq. 3)}$$

When $[H^+] \ll K_a$, Eq. 3 is reduced to $k = k_A$.

Thus, the k value becomes k_A when the rate constant is independent of pH above 4. The pKa value (pKa = 2.13) determined from the kinetic data using Eq. 3 agrees with that determined by the solubility method[6].

Salt and Common Ion Effects—The hydrolysis of fenclorac in phosphate buffer at pH 7.04 was tested for a salt effect using sodium nitrate up to an ionic strength of 1.596. An approximately 50% increase in the rate constant resulted by a change in ionic strength from 0.1 to 1.6 (Table II). A positive salt effect indicates generation of charge in the transition state, making the transition state more polar than the reactant. The first-order rate constant was not significantly increased when potassium chloride was used in place of sodium nitrate (Table II). The common ion effect, characteristic of an S_N1 reaction, can be taken into account by a reversible ionization mechanism (4) (Scheme I).

The apparent first-order rate constant, k, when the common ion effect is considered, is:

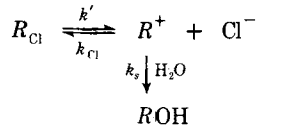
$$k = \frac{k'}{1 + (k_{Cl})/(k_s)[Cl^-]} \qquad \text{(Eq. 4)}$$

The ratio k_{Cl}/k_s (Table II) was estimated from the rate constants k' and k at a given ionic strength using sodium nitrate and potassium chloride, respectively.

Medium Effect—By changing the solvent from water to 50% aqueous methanol, the first-order rate constant for the hydrolysis of fenclorac at 50.0° and pH 5–12 decreased 6.5-fold ($k = 9.9 \times 10^{-5}$ sec^{-1}). The direction of the medium effect corresponds to that of a salt effect. This finding indicates that the solvent with greater polarity (water) stabilizes the transition state to the greater extent by solvation.

Substituent Effect—It was of interest to determine the electronic effect of different substituents (hydrogen and nitro) at the 3-position of the benzene ring on the hydrolysis rate of these analogs and to compare the effect with that of the 3-chloro substituent in fenclorac. The first-order rate constants were determined for fenclorac and the unsubstituted analog at pH 7.04 and 25.0°. The rate constant for the nitro substituent was extrapolated from its Arrhenius plot. The hydrolysis rate constants of the unsubstituted analog ($k = 1.67 \times 10^{-3}$ sec^{-1}) increased 100-fold from that of fenclorac, and that of the nitro-substituted analog ($k = 3.56 \times 10^{-7}$ sec^{-1}) decreased 50-fold. When the logarithm of k/k_0, where k_0 is the rate constant for the unsubstituted analog, was plotted against Hammett's σ-constants of the substituents, a linear relationship with $\rho = -5.1$ was obtained (Fig. 4). The reaction constant obtained was comparable to that of typical S_N1 reactions, *e.g.*, the solvolysis of 2-chloro-2-phenylpropane in 90% aqueous acetone with $\rho = -4.54$ (5).

From these data, it is seen that the different 3-substituents on the aromatic ring significantly affect the rate of hydrolysis. The large substit-



Scheme I

[6] B. Crugman *et al.*, Research Division, William H. Rorer Inc., personal communication.

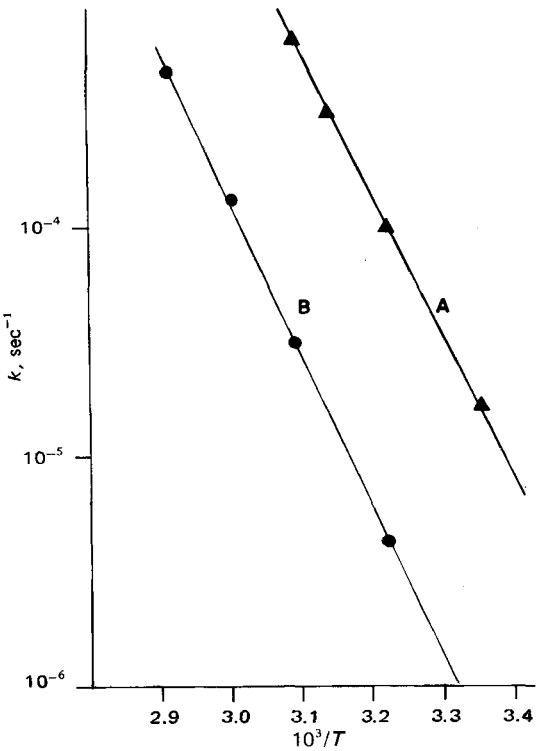


Figure 5—*Arrhenius plots for hydrolysis of fenclorac (A) and the 3-nitro-substituted analog (B).*

uent effect on the hydrolysis rate apparently is due to a change of the activation energy rather than the entropy of activation (Table III). This statement, however, is inconclusive, since the temperature dependence of the substituent effect was studied only for two compounds.

Rate Dependency on Temperature—The first-order rate constants were determined in phosphate buffer (pH 7.04) at four temperatures for fenclorac and the 3-nitro analog (Table III). The Arrhenius equation gives the quantitative relation of the rate constant and temperature. The logarithmic form of the equation is:

$$\log k = \frac{E_a}{2.303RT} + \log A \qquad \text{(Eq. 5)}$$

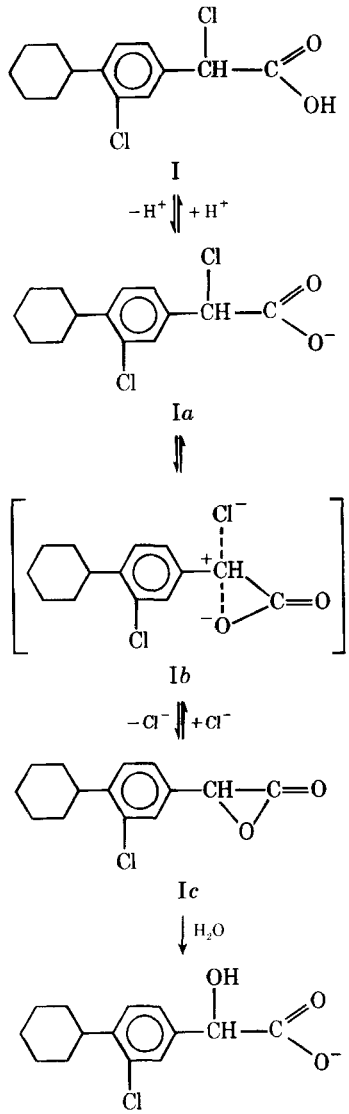
where E_a is the activation energy, and A is the frequency factor. The Arrhenius plots of the logarithms of the first-order rate constants *versus* $1/T$ are shown in Fig. 5. The Arrhenius parameters were calculated from the plots in accordance with Eq. 5. The values of E_a, log A, and $S^{\ddagger}$ (entropy of activation) are included in Table III.

Mechanism for Hydrolysis of Fenclorac—It has been well demonstrated (6–9) that α-bromopropionate hydrolyzes by the intramolecular displacement of bromine by the carboxyl anion and that the lactone formation follows the ionization of the carbon–bromine bond.

Table III—Apparent First-Order Rate Constants at Different Temperatures, Arrhenius Parameters, and Entropies of Activation for Hydrolysis of Fenclorac and the 3-Nitro-Substituted Analog at pH 7.04

	Fenclorac, 10^5 k, sec^{-1}	3-Nitro Analog, 10^5 k, sec^{-1}
Temperature		
25.0°	1.62	0.0356[a]
37.0°	9.95	0.273
45.0°	31.0	—
50.0°	65.7	3.07
60.0°	—	13.0
70.0°	—	47.2
E_a, kcal	28.3	33.0
log A	15.93	17.75
$S^{\ddagger}$, eu	6.2	10.4

[a] Extrapolated value.



Scheme II

Scheme II is consistent with the proposed mechanism for the hydrolysis of fenclorac. The sequence explains the intramolecular catalysis of carboxyl anion in the ionization of the carbon–chlorine bond and the formation of an unstable intermediate, lactone.

Only the dissociated carboxyl anion participates in the intramolecular catalysis by nucleophilic attack on the α-carbon. The general base mechanism would be ruled out in the intramolecular catalysis, because no general acid–base catalysis was observed (Table I).

Since the increase in the rate constant was observed with an increasing salt concentration and polarity of the medium, it can be deduced that the transition state (Ib) has high ionic character (9). As the carbon–chlorine bond ionizes, a positive charge develops on the α-carbon, and the electron-withdrawing substituents destabilize the transition state by pulling electrons from already electron-deficient α-carbon and thus decrease the hydrolysis rate as shown by the substituent effect. The intermediate Ic, a three-membered ring lactone, would be trapped either by solvent water in the fast step to produce hydrolysis product Id or by added Cl^- to reverse the reaction as observed through the common ion effect.

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Quantitative Determination of Amitriptyline and Its Principal Metabolite, Nortriptyline, by GLC–Chemical Ionization Mass Spectrometry

W. A. GARLAND

Abstract □ A quantitative GLC–mass spectrometry assay was developed for the determination of the tricyclic antidepressant amitriptyline and its desmethyl metabolite (nortriptyline) in human plasma. The assay utilizes selective ion detection to monitor in a GLC effluent the MH^+ molecular ions of amitriptyline and nortriptyline generated by isobutane chemical ionization. The procedure, which utilizes deuterated analogs of amitriptyline and nortriptyline as internal standards, requires 1 ml of plasma and can measure 1 ng/ml of amitriptyline and 0.5 ng/ml of nortriptyline. The curves relating the amounts of amitriptyline and nortriptyline added *versus* the amounts found over a 100-fold range of amitriptyline and nortriptyline concentrations are straight lines with intercepts of approximately zero and slopes of unity. Analyses of plasma samples from three subjects receiving 50 mg of amitriptyline orally, three times a day, gave an average plasma concentration of 115 ± 42 ng/ml for amitriptyline and 109 ± 20 ng/ml for nortriptyline. Similar analyses of the plasma of three subjects who had received a single 50-mg oral dose of amitriptyline showed an average maximum plasma concentration of 25 ± 10 ng/ml for amitriptyline and 10 ± 4 ng/ml for nortriptyline. Seventy-two hours after administration, the average plasma amitriptyline and nortriptyline levels were 3 ± 1 and 3 ± 2 ng/ml, respectively.

Keyphrases □ Amitriptyline—GLC–mass spectrometric analysis, human plasma □ Nortriptyline—GLC–mass spectrometric analysis, human plasma □ GLC–mass spectrometry—analyses, amitriptyline and nortriptyline in human plasma □ Antidepressants—amitriptyline and nortriptyline, GLC–mass spectrometric analyses, human plasma

Amitriptyline and nortriptyline are used extensively for treating psychic depression (1). Nortriptyline is also generated metabolically in humans from amitriptyline (2, 3). Indeed, it has been suggested that the action of amitriptyline is mediated through its nortriptyline metabolite (4). Therapeutically, these compounds are reported to be effective over a relatively narrow range of plasma concentrations (5), although large interindividual variations in plasma concentrations are observed with similar dosing schedules (6–8). In addition, there are conflicting reports concerning the relative amelioration of depression as a function of plasma amitriptyline and/or nortriptyline levels (5, 8, 9).

Research on problems associated with amitriptyline therapy has been hindered somewhat by the lack of a sufficiently sensitive and specific assay for determining low levels of amitriptyline in plasma, *e.g.*, no single-dose amitriptyline pharmacokinetic data are available. Two flame-ionization GC assays suitable only for determining "steady-state" plasma concentrations of amitriptyline and nortriptyline were described (10, 11). Both assays require 3–5 ml of plasma and have a sensitivity limit of 25 ng/ml.

Recently, a GC procedure using a nitrogen detector was reported (12). It has a quoted sensitivity of 5 ng/ml for amitriptyline and 10–15 ng/ml for nortriptyline (2 ml of plasma extracted), but it cannot determine nortriptyline in humans following a therapeutic single dose of amitriptyline. Furthermore, it is only marginally suitable for amitriptyline, since the typical maximum concentration of amitriptyline after a 50-mg dose is only 15–25 ng/ml.

An electron-capture GC method for the determination of the heptafluorobutyrate derivatives of nortriptyline and some of its metabolites was reported (13). This procedure requires 4 ml of plasma and has a reported sensitivity of 10 ng/ml.

This report describes a GLC–mass spectrometric assay requiring 1 ml of plasma that is capable of determining 1 ng/ml of amitriptyline and 0.5 ng/ml of nortriptyline. The method, which utilizes chemical ionization GLC–mass spectrometry (14) with isobutane functioning both as a reagent gas and GLC carrier gas, is suitable for measuring amitriptyline and nortriptyline in humans following single-dose amitriptyline administration. To obtain sufficient sensitivity, selective ion detection is used to monitor the MH^+ ion of both amitriptyline and either nortriptyline or the trifluoroacetyl derivative of nortriptyline. Known quantities of deuterated analogs of both amitriptyline and nortriptyline are added to the plasma as internal standards.

EXPERIMENTAL

Apparatus—A quadrupole mass filter system[1] and data system[2] were used in conjunction with the gas chromatograph[3]. The GLC column, 1.21 m (4 ft) × 2 mm i.d., was silanized and packed with 3% OV-17 on 100–

[1] Finnigan model 1015D.
[2] Finnigan model 6000.
[3] Finnigan model 9500.