Prediction of Stability in Pharmaceutical Preparations XIX: Stability Evaluation and Bioanalysis of Clofibric Acid Esters by High-Pressure Liquid Chromatography

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Abstract □ Specific, sensitive, reversed-phase high-pressure liquid chromatographic assays of clofibric acid esters, clofibrate and etofibrate, and their hydrolysis products, clofibric acid and its monoglycolate and nicotinic acid and its monoglycolate, have been developed in aqueous solution and in biological fluids. Sensitivities of 100 ng/ml of injected mobile phase, a 10-fold increase over existing methods, are reported. Plasma concentrations as low as 200 ng/ml can be analyzed easily in the miscible phase after acetonitrile denaturation. The compounds and their products can be extracted with haloalkane solvents. The extracts were evaporated, reconstituted, and assayed in minimal amounts of mobile phase, resulting in sensitivities of 10 ng/ml of plasma. Conditions are presented that minimize interferences with plasma components. The assay was used to determine the stability of the clofibric acid esters in aqueous solutions, to establish log k–pH profiles at various temperatures, and to evaluate Arrhenius parameters. Hydrolysis was by specific acid–base catalysis. The initial product of etofibrate solvolysis at pH > 6 is the monoglycol ester of clofibric acid; at pH < 3, it is the monoglycol ester of nicotinic acid. Clofibric acid esters are highly unstable to mild alkali (1–3 hr at pH 10 and 30°); even in the estimated pH range of maximum stability, they have half-lives of 100–200 days at 30°. They have half-lives of 4–7 min at 37.5° in fresh dog plasma, and data presented indicate that clofibric acid monoglycolate is an inital product of etofibrate solvolysis.

Keyphrases □ Clofibric acid—esters, stability evaluation and bioanalysis by high-pressure liquid chromatography □ High-pressure liquid chromatography—stability evaluation and bioanalysis of clofibric acid esters □ Stability—prediction and bioanalysis using high-pressure liquid chromatography, clofibric acid esters

The pharmacokinetics of clofibrate, the ethyl ester of clofibric acid, a drug that decreases cholesterol and triglyceride levels (1–4), has not been reported. Only the derived clofibric acid (Scheme I), presumably the only observable material in plasma from clofibrate administration, has been monitored in plasma and urine.

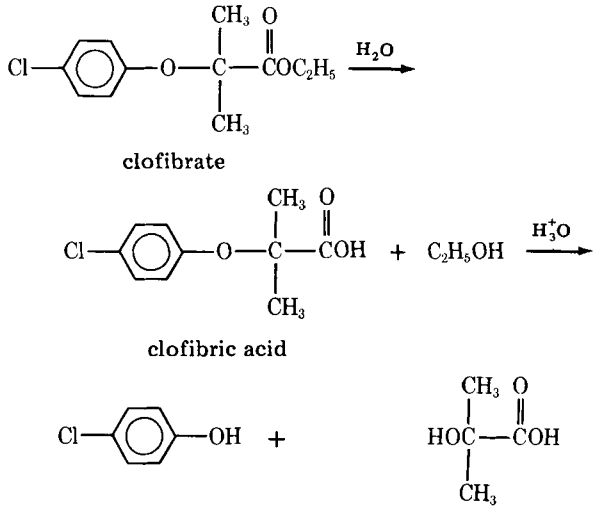
BACKGROUND

Thorp (1) stated that clofibrate was rapidly hydrolyzed by tissue and serum to clofibric acid. His assay was of low sensitivity and was based on spectrophotometric determination in dilute alkali of p-chlorophenol. Subsequent authors (5–7) stated that clofibrate was rapidly hydrolyzed during or after absorption.

GLC assays of clofibric acid have shown sensitivities of 1 μg/ml of plasma (8–10). Recently, a GLC method was proposed (11) for the simultaneous assay of clofibric acid and clofibrate with a sensitivity limit of 10 μg/ml. It was proposed that sensitivity could be increased to 1 μg/ml by evaporation and reconstitution in less solvent.

An evaluation of potential toxicity of the clofibric acid product of clofibrate led to temporary withdrawal of clofibrate from the market in 1979 (12). This fact renewed interest in other clofibric acid esters such as etofibrate (Scheme II), a glycol diester of clofibric acid and nicotinic acid, another hypolipidemic agent. It was postulated that efficacy could be effected through the intact molecule or the monoglycolate esters rather than through the biohydrolytic release of clofibric acid, the presumed source of the adverse reaction (12, 13).

Differences in hypolipidemic effects between clofibrate and etofibrate and possible differences in their rates of clofibric acid release *in vivo* had been discerned (12). The monoester products of microsomal transformations (Scheme II) and their etofibrate precursor were characterized by TLC, GLC, and mass spectroscopy (12), and studies were conducted on their stabilities to GI and blood esterases (13).



Scheme I—Hydrolysis of clofibrate.

This paper presents specific and simultaneous assays of clofibrate, clofibric acid, and etofibrate and its monoesters (Schemes I and II). The high-pressure liquid chromatographic (HPLC) and extracting techniques developed resulted in sensitivities of 10 ng/ml of plasma, a 100-fold increase over existing methods. The determination of the partitioning of these compounds into organic solvents as a function of pH permitted optimal extraction conditions. The methods were applied to the determination of the log k–pH stability profiles of the clofibric acid esters and their stabilities in plasma. Optimum conditions were established for their assays in biological fluids where transformations during storage and analysis did not occur.

EXPERIMENTAL

Materials—The following analytical grade materials were used: di-[1] and monobasic sodium[2] and potassium[3] phosphates, anhydrous sodium acetate[2], acetic acid[2], volume concentrates of sodium hydroxide and hydrochloric acid[4], boric acid[1], ACS methylene chloride[5], and GLC, TLC, and UV grade chloroform and acetonitrilie[6]. Pure (GLC and TLC) clofibrate, etofibrate, clofibric acid, nicotinic acid, and clofibric acid monoglycolate reference standards[7] were used as received. The nicotinic acid monoglycolate[7] contained 5% glycol dinicotinate by TLC and GLC since further purification by distillation was not possible. The monoester

[1] Baker analyzed reagent, J. T. Baker Chemical Co., Phillipsburg, N.J.
[2] Mallinckrodt Chemical Works, St. Louis, MO 63160.
[3] Fisher Scientific Co., Chemical Manufacturing Division, Fair Lawn, N.J.
[4] Harleco, Philadelphia, PA 19143.
[5] Mallinckrodt, Paris, KY 40361.
[6] Burdick & Jackson Laboratories, Muskegon, MI 49442.
[7] Clofibrate, batch 12976, analysis 1349; clofibrate, batch 12479, analysis 3579; clofibric acid, batch 1517, analysis F 1341; nicotinic acid, batch 22179, analysis F5951; clofibric acid monoglycolate, batch VR 27679; and nicotinic acid monoglycolate, batch 27679 Merz and Co., Chemische Fabrik, Eckenheimer Landstrasse 100, 6000 Frankfurt (Main), West Germany.

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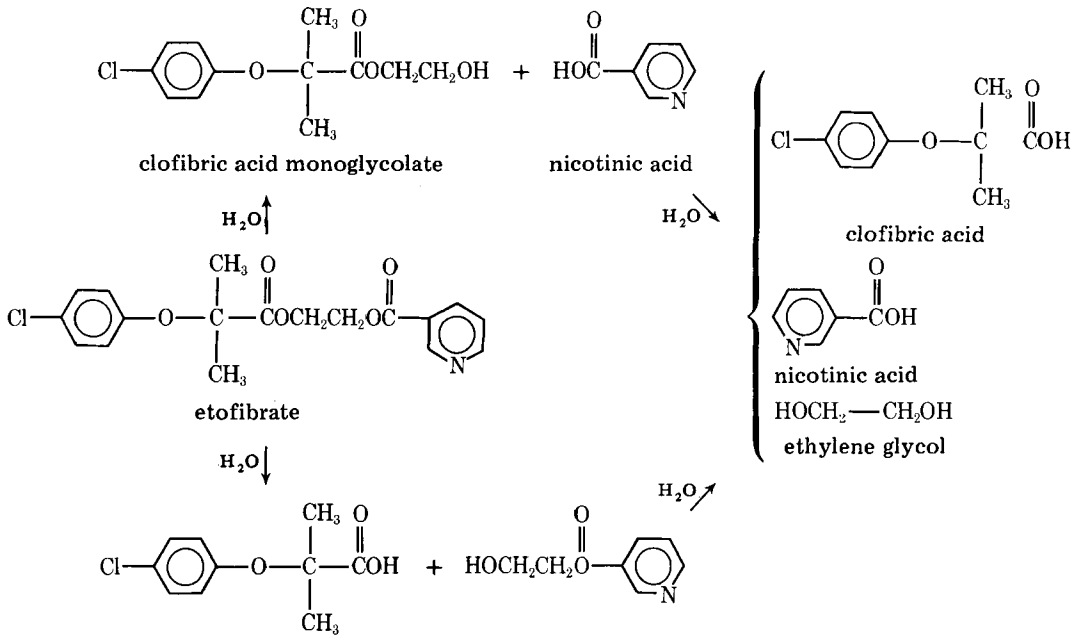


Scheme II—Pathways of etofibrate hydrolysis.

is thermolabile, and considerable transesterification takes place at distillation temperatures.

Apparatus—A high-pressure liquid chromatograph[8] equipped with a variable wavelength UV detector[8] was used with a reversed-phase alkylphenyl column[9] and a radial compression C_{18} reversed-phase column[10]. A guard column[11] was used. Areas under the chromatogram peaks were measured by an integrator[12].

HPLC Procedures—Prepared buffer solutions of sodium acetate of known molarity were adjusted to a measured pH with acetic acid and filtered through a 0.45-μm filter. Solutions were generally 0.10 or 0.05 *M* and were adjusted to pH 3.74 or 3.5, respectively. The mobile phases were mixtures of this buffer and acetonitrile or methanol. Their composition and the HPLC conditions and retention times of various substances are given in Table I. The mixed mobile phase was degassed under vacuum with simultaneous ultrasonication for 1 min.

The variable-wavelength detector was adjusted to 225 nm for the simultaneous analysis of clofibrate and clofibric acid or of etofibrate, clofibric acid monoglycolate, and clofibric acid. The maximum absorbance of clofibrate and clofibric acid was 223.5 nm with a weak absorbance maximum at 277 nm and a negligible absorbance at 254 nm in water. The detector was set in the 263–268-nm range for the simultaneous analysis of nicotinic acid and nicotinic acid monoglycolate where the solvent peaks had less interference than at 225 nm. Both nicotinic acid and its monoglycolate had maximum absorbances at 263 and 225 (or less) nm in mobile phase III with a minimal absorbance at 238 nm.

Solubilities of Clofibric Acid Esters—Plots of the spectrophotometric absorbance of clofibrate in water at its λ_{max} of 223.5 nm against molecular concentration at room temperature showed an initial straight line through the origin of slope 1138, the molar absorptivity. Absorbance at higher concentrations was characterized by a line of slope 67.4 and an intercept of 0.42 absorbance. These two straight line segments intersected at a clofibrate concentration of 4.0×10^{-4} *M*, which was the solubility of clofibrate in water at room temperature. A previously reported (13) solubility was 0.004% (1.65×10^{-4} *M*) at 22° in pH 7.4 phosphate buffer.

Etofibrate (1 mg) was added to 10 ml of various solutions in the pH 1–11 range (theoretically 2.75×10^{-4} *M*). The solutions were vortexed and filtered in a 0.45-μm syringe system. An aliquot (1.00 ml) of the filtrate was diluted to 3.00 ml with acetonitrile that was 1.69×10^{-4} *M* in the internal standard, thymol. A 25-μl aliquot was injected onto HPLC System III (Table I), and the etofibrate concentration was determined from a prepared calibration curve, concentration *versus* peak height ratio, of etofibrate to thymol.

At room temperature, the solubility of etofibrate, pKa 6.41 (12), at pH values ≥ 4 was 2.0×10^{-5} *M* with solubilities of 3.8×10^{-5} *M* at pH 3, 1.99×10^{-4} at pH 2, and $> 2.5 \times 10^{-4}$ *M* at pH 1. A previously reported (13) solubility was 0.002% (5.5×10^{-5} *M*) at 22° in pH 7.4 phosphate buffer. Thus, the best procedure in alkaline stability studies at 20–30° was to add pH 2 solutions of etofibrate to the reaction solutions at the higher pH values so that the final concentration was $\sim 10^{-5}$ *M* in etofibrate.

The solubility of clofibric acid monoglycolate was reported previously (13) as 0.4% (0.0155 *M*) in pH 7.4 phosphate buffer.

Hydrolysis Kinetics—Weighted amounts of clofibrate, sufficient to give $1.4–3.8 \times 10^{-4}$ *M* clofibrate solvents in 10 ml of solvent or $2.9–3.3 \times 10^{-4}$ *M* etofibrate in 20 ml of hydrochloric acid, were dissolved with vortexing in buffer thermally preequilibrated at the reaction temperature. Aliquots (50 and 200 μl for clofibrate and etofibrate acid solvolysis, respectively) were taken at timed intervals and mixed with reaction-quenching solution (50 and 400 μl for clofibrate and etofibrate acid solvolysis studies, respectively), which contained the internal standard, morphine sulfate for clofibrate studies and thymol for etofibrate. The quenching solution had sufficient hydrochloric acid or sodium hydroxide and acetate buffer solution to adjust the final pH of the quenched solution to the 4–5 range of high stability.

Either a 25% methanol concentration in, or mobile phase composition of, the quenched solution of the clofibrate solvolysis studies gave a finer separation of the solvent front from clofibric acid in the chromatogram of System I (Table I). The quenched solution for all etofibrate studies in acid solution always had the same composition as the mobile phase and was injected onto System III. In these clofibrate studies, the total concentrations of clofibric acid and clofibrate in the assayed quenched solutions were $0.7–2.0 \times 10^{-4}$ and $2–5 \times 10^{-5}$ *M* in morphine, respectively. The total concentration of etofibrate and its acid solvolysis products was 1×10^{-4} *M* in the assayed quenched solution and 1.75×10^{-4} *M* in thymol.

The samples were tightly capped and kept refrigerated until inserted in the automatic injector to inject 25 μl. Some fast reaction studies in the alkaline range were effected by vigorous mixing, for a few seconds, 5 ml of thermally preequilibrated stock solutions of clofibrate with 5 ml of thermally equilibrated sodium hydroxide of twice the concentration desired in the final reacting mixture to be maintained in the constant-temperature bath.

System II (Table I) was used for the clofibrate solvolysis studies in borate buffer (pH 9–10 at 60°) and in 0.01, 0.03, and 0.06 *N* NaOH at 30° and for all other solvolysis studies for etofibrate that were not done in acid. The quenching solutions were prepared from concentrated buffer solutions and acetonitrile so that the solvent compositions of the quenched solutions to be injected approached those of the mobile phases.

[8] Model 6000A solvent delivery system and U6K injector equipped with a variable-wavelength UV detector, model 450, and a WISP automatic injector system, Waters Associates, Milford, MA 01757.

[9] μBondapak alkylphenyl, Waters Associates, Milford, MA 01757.

[10] Radial compression RCM 100 with C_{18}, Waters Associates, Milford, MA 01757.

[11] Bondapak Phenyl/Corasil, Waters Associates, Milford, MA 01757.

[12] Hewlett-Packard model 3380A integrator, Avondale, Pa.

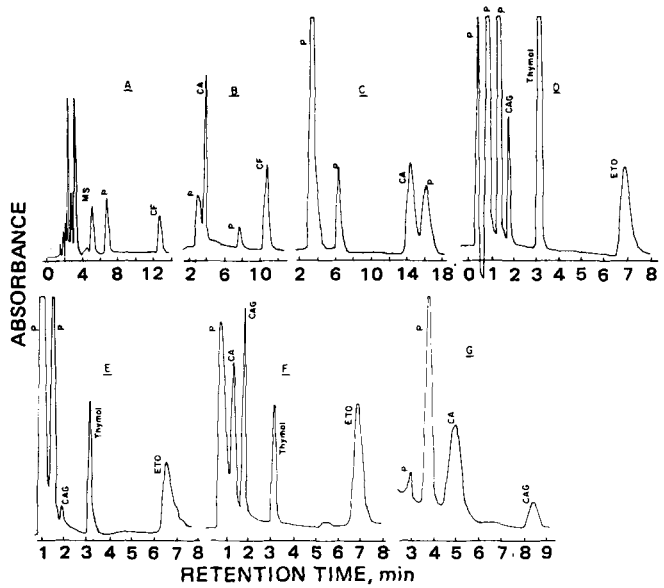


Figure 1—*Reversed-phase chromatograms at 225-nm UV detection for plasma assays of clofibrate (CF), etofibrate (ETO), clofibric acid (CA), clofibric acid monoglycolate (CAG), and the internal standard, thymol or morphine sulfate (MS). The HPLC systems and their mobile phases are given in Table I. Key: A, CF, and CA (6.25 × 10^{-6} M in 1 ml of plasma at pH 2.5, extracted with 4 ml of chloroform of which 2 ml was evaporated and reconstituted in 100 μl of mobile phase that was 3.13 × 10^{-5} M in MS) on System I; B, CF and CA (5 × 10^{-5} M in plasma mixed 1:1 with acetonitrile) on System III; C, CA (2 × 10^{-5} M in 1 ml of plasma at pH 2.5, extracted with 4 ml of chloroform of which 3 ml was evaporated and reconstituted in mobile phase) on System IV; D, assay of ETO degrading for 7.0 min in fresh dog plasma at 37.5° (initially 5.89 × 10^{-5} M in plasma, 0.5 ml adjusted to pH 2.5, extracted with 3 ml of methylene chloride, of which 1 ml was evaporated and reconstituted in 0.75 ml of acetonitrile that was 1.625 × 10^{-5} M in thymol) on System VI; E, assay of ETO degrading for 5.3 min in fresh dog plasma at 37.5° (initially 3.5 × 10^{-5} M in plasma, mixed 1:3 with acetonitrile) on System VI; F, ETO, CA, and CAG (5 × 10^{-5} M in outdated human plasma) and mixed 1:2 with acetonitrile that was 1.625 × 10^{-5} M in thymol) on System VI; and G, assay of ETO degrading for 12 min in fresh dog plasma (initially 5 × 10^{-5} M in plasma, mixed 1:3 with acetonitrile) on System VII.*

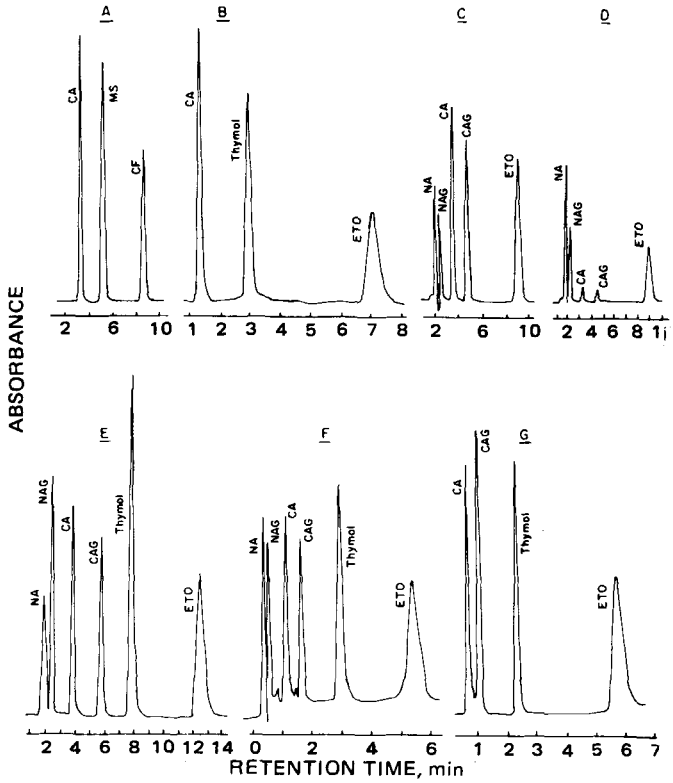


Figure 2—*Reversed-phase chromatograms at 225-nm UV detection (unless stated otherwise) of clofibrate (CF), etofibrate (ETO), clofibric acid (CA), clofibric acid monoglycolate (CAG), nicotinic acid monoglycolate (NAG), nicotinic acid (NA), and the internal standards, morphine sulfate (MS) and thymol, injected into the mobile phase. The HPLC systems and their mobile phases are given in Table I. Key: A, CF and CA (2.5 × 10^{-5} M) and MS (2.94 × 10^{-5} M) on System I; B, CF and CA (7.5 × 10^{-6} M) and thymol (1.63 × 10^{-5} M) on System II; C and D, NA, NAG, CA, CAG, and ETO (2.5 × 10^{-4} M) on System I (225 and 263 nm, respectively); E, NA, NAG, CA, CAG, and ETO (5 × 10^{-5} M) and thymol (1.75 × 10^{-4} M) on System III; F, NA, NAG, CA, CAG, and ETO (5 × 10^{-6} M) and thymol (1.625 × 10^{-5} M) on System II; and G, ETO, CA, and CAG (7.5 × 10^{-6} M) and thymol (1.625 × 10^{-5} M) in System VI.*

Table I—Composition, Conditions, and Retention Times of Clofibrate (CF), Clofibric Acid [a] (CA), Morphine Sulfate (MS), Thymol, Etofibrate (ETO), Clofibric Acid Monoglycolate (CAG), Nicotinic Acid Monoglycolate [b] (NAG), and Nicotinic Acid [b] (NA) in Various HPLC Systems [c]

HPLC [d] System	Mobile Phase	Flow Rate, ml/min	Pressure psi	Column	Typical Retention Times, min: CF	ETO	CA	CAG	NAG	NA	MS	Thymol	Plasma Interferences
I	50:50 acetonitrile–0.1 *N* acetate, pH 3.75	1.5	700	Akylphenyl	10.7	—	3.37	—	—	—	4.68	—	<3.75
II	50:50 acetonitrile–0.05 *N* acetate, pH 3.5	6.0	900	RCM 100 C_{18} (8-mm i.d.)	9.2	5.4	1.20	1.65	0.51	0.35	—	3.10	<3.0
III	45:55 acetonitrile–0.1 *N* acetate, pH 3.75	1.5	750	Alkylphenyl	10.6	12.05	3.92	5.83	2.43	1.93	4.71	7.78	<2.65, 7.55
IV	35:65 methanol–0.1 *N* acetate, pH 3.75	1.5	1600	Alkylphenyl	>40	—	14.4	—	—	—	6.51	—	<4.2 6.30, 16.23
V	50:50 methanol–0.05 *N* acetate, pH 3.50	3.0	1500	RCM 100 C_{18} (5-mm i.d.)	—	>40	5.4	—	<0.3	<0.3	—	—	0–2, 7–8
VI	50:50 acetonitrile–0.05 *N* acetate, pH 3.50	3.0	1000	RCM 100 C_{18} (5-mm i.d.)	—	5.8	0.6	1.35	—	—	—	2.5	0–1
VII	30:70 acetonitrile–0.5 *N* acetate, pH 3.50	3.0	600	RCM 100 C_{18} (5-mm i.d.)	—	—	5.1	8.6	—	—	—	—	0–1.5, 3.8

[a] Additional studies were performed for clofibric acid using the alkylphenyl column. The systems all had plasma interferences with clofibric acid and were, with (parenthetical) retention times (minutes) of clofibric acid: 50:50 acetonitrile–0.1 *N* acetate, pH 7 (1.8); 40:60 acetonitrile–0.1 *N* acetate, pH 3.75 (4.21); 50:50 methanol–0.1 *N* acetate, pH 3.75 (5.62); and 50:50 methanol–0.1 *N* acetate, pH 7.0 (3.36), with plasma interferences at 2.49, 3.47, 4.03, and 7.08 min. Some separation was effected with 40:60 methanol–0.1 *N* acetate, pH 3.75 (8.97) with plasma peaks at 4.81, 5.83, and 7.56 min. System IV gave the best separation of clofibric acid and plasma peaks that interfered with the peak for morphine. [b] Nicotinic acid and nicotinic acid monoglycolate were better separated on the RCM 100 C_{18} column with 35:65 acetonitrile–0.05 *N* acetate, pH 3.5, with retention times of 0.60 (NA), 1.00 (NAG), 2.50 (CA), and 3.25 min (CAG) where ETO and thymol hung up on the column. [c] All peaks were detected spectrophotometrically at 225 nm, except that NAG and NA were detected at 266 nm. [d] Fifty microliters were injected into Systems II and V–VII; 25 μl was used in the other systems.

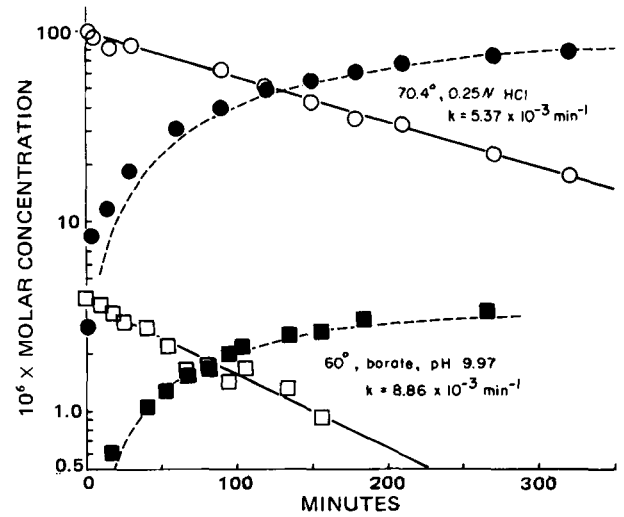


Figure 3—*Semilogarithmic plots of clofibrate (solid line) and clofibric acid (dashed line) concentrations* versus *time for solvolyses in the acidic (System I) and buffered regions (System II). The samples were injected in pH 4–5 mobile phase. The clofibric acid concentrations were fitted to [clofibric acid] = C_∞ (1 − e^{-kt}), where C_∞ is the clofibric acid concentration formed at infinite time and* k *(Table III) is the apparent first-order rate constant for the first-order degradation of clofibrate.*

This procedure minimized negative injection peaks.

In these clofibrate studies, the total concentrations of clofibrate and clofibric acid in the degrading and quenched solutions were 6.91 and 3.46 × 10^{-4} *M*, respectively. The maximum concentrations of etofibrate and its solvolysis products in the degrading solutions at higher temperature and in the quenched solution were 4 and 1 × 10^{-5} *M*, respectively. Etofibrate was maintained in solution under these conditions.

Calibration curves, usually at 1.0–40 × 10^{-5} *M* clofibrate (Systems I and III) or 0.1–1.25 × 10^{-5} *M* clofibrate (System II), were constructed with appropriate amounts of concentrated stock solutions of clofibrate, clofibric acid, etofibrate, clofibric acid monoglycolate, nicotinic acid monoglycolate in acetonitrile, nicotinic acid in pH 5 acetate buffer, and either thymol (acetonitrile) or morphine sulfate (aqueous as internal standards in 2 ml of mobile phase. Either 25 or 50 μl was injected onto the HPLC systems (Table II).

Buffer Solutions for Solvolyses Studies—Phosphate buffers were prepared by mixing 0.10 *N* (for clofibrate solvolyses) and 0.05 *N* (for etofibrate solvolyses) mono- and dibasic potassium phosphate to obtain the desired pH. Borate buffers were prepared by mixing 0.10 or 0.05 *N* boric acid and sodium borate, or by mixing 0.025 *N* boric acid with concentrated sodium hydroxide to an appropriate pH. Appropriate amounts of sodium chloride were added to maintain 0.1 ionic strengths.

The pH meter was standardized at the reaction temperature with two standard buffers with known pH values at that temperature that straddled the pH of the desired reaction solution. The pH values of the prepared buffered solutions were read at the reaction temperature.

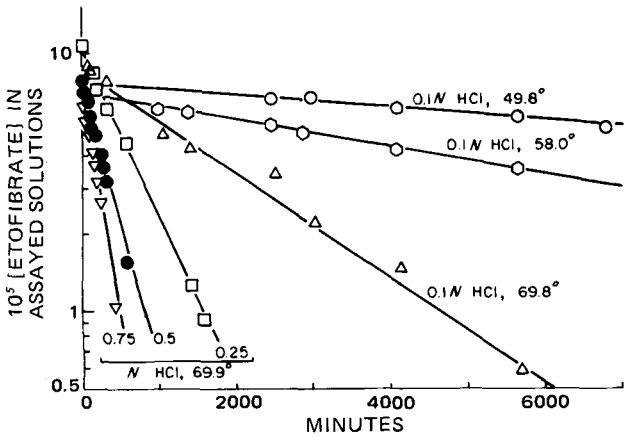


Figure 4—*First-order plots of etofibrate concentrations in hydrochloric acid* versus *time. The reacting solutions before dilution for injection on System III and pH 4–5 mobile phase were 2.9–3.3* × 10^{-4} M.

Partition Studies as Functions of pH—Solutions of 1.25 × 10^{-5} *M* clofibric acid and clofibrate, separate and in combination, were prepared in 0.1 and 0.01 *N* HCl, in 0.1 *N* acetate buffer adjusted to pH 3.0, 4.0, and 5.0, and in 0.1 *N* phosphate buffer adjusted to pH 6.0 and 7.0. Aliquots (2 ml) were extracted with 2 ml of methylene chloride with 5 min of mild shaking. After centrifugation for 5 min, the aqueous layer was removed by aspiration. Then 1 ml of the organic layer was evaporated under nitrogen in a reaction vial and reconstituted in 100 μl of acetonitrile–water containing morphine sulfate (3.13 × 10^{-5} *M*) as an internal standard; 25 μl was injected onto the HPLC system with mobile phase I (Table I).

The quantity extracted was determined from a calibration curve (Table II). Clofibric acid was extracted 102 ± 5% at pH 1–4, 60% at pH 5, 16% at pH 6, and 0 and 0.3% at pH 7.0 when alone and admixed with clofibrate. Clofibrate was extracted 85 ± 3% over this pH range with no significant effects when alone; but when combined with clofibric acid, it showed only 20% extraction.

When 1.00 ml of pH 2.5 aqueous solution containing 1.25 × 10^{-5} *M* clofibric acid and clofibrate was extracted with 4 ml of methylene chloride, analysis revealed that 104 ± 4% of the clofibric acid and 57 ± 2% of the clofibrate were extracted. When 1.00 ml of plasma adjusted to pH 2.5 was used, there was an interference of the plasma with the clofibric acid peak, but the recovery of clofibrate was similar.

When the same procedure was used on spiked pH 2.5 aqueous solution with chloroform as the extracting solvent, 78 ± 3% of the clofibrate and 95 ± 2% of the clofibric acid were extracted. From pH 2.5 plasma, 89% of the clofibrate was extracted, whereas the plasma impurity interfered with the clofibric acid assay.

Similar studies were made on 2 ml of buffer solutions that were 5.56 × 10^{-6} *M* each in etofibrate and clofibric acid and its monoglycolate at unit pH intervals of 1–10. Each buffer solution was extracted with 3 ml of methylene chloride by shaking for 10 min, aspirating off the aqueous phase, and evaporating under nitrogen 2 ml of the methylene chloride layer. The residue was reconstituted in 500 μl of mobile phase that was 1.50 × 10^{-5} *M* in the compounds and 1.63 × 10^{-5} *M* in thymol. A 50-μl aliquot was injected using System II. All of the etofibrate and clofibric acid monoglycolate was extracted at pH 2–10, with 77% of the etofibrate extracted at pH 1.0. The clofibric acid extraction was 97–100% at pH 1–4, 69.5% at pH 5, 20.3% at pH 6, and negligible at pH ≥7.

Assays of Clofibrate and Clofibric Acid in Plasma—*Procedure A: Acetonitrile Deproteinization of Plasma*—Acetonitrile (0.5 or 1.0 ml) was added to an equal volume of human or dog plasma. The mixture was vortexed and centrifuged for 10 min at 3000 rpm; the internal standard morphine sulfate was added to the supernate, and 25 μl was injected in System I (Fig. 1A) or III (Table I). The latter system adequately, but not completely, separated the peak of a plasma impurity and clofibric acid in this procedure (Fig. 1B) but gave a good calibration curve for both (Study F, Table II) at plasma concentrations of 5.0–40 × 10^{-5} *M*. When such acetonitrile-denatured plasma was stored overnight and reassayed,

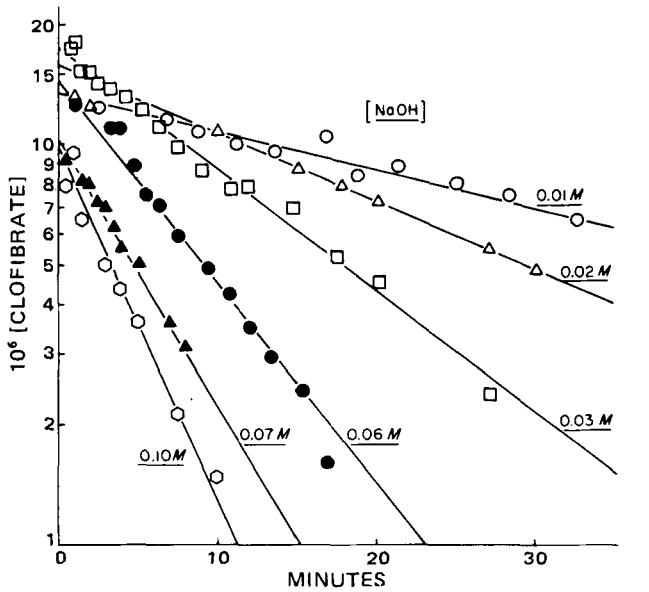


Figure 5—*First-order plots of clofibrate concentrations* versus *time at 30° in various concentrations of sodium hydroxide (System I at 0.02, 0.07, and 0.10* M *NaOH and System II at 0.01, 0.03, and 0.06* M *NaOH).*

Table II—Typical Statistics[a] of HPLC Assays of Clofibrate (CF), Clofibric Acid (CA), Clofibric Acid Monoglycolate (CAG), Etofibrate (ETO), Nicotinic Acid Monoglycolate (NAG), and Nicotinic Acid (NA) in Accordance with $10^5\ C \pm s_{10^5 c.x} = (m \pm s_m)X + b \pm s_b$

Study	HPLC System	$10^5\ M$ Range	Compound	Clofibrate Study X^b	$s_{10^5 c.x}$	m	s_m	b	s_b	n	xxx in $r^2 = 0.99xxx$
A	I	2.5–25	CF	PHR	0.64	7.00	0.26	−1.16	0.54	4	735
		2.5–40	CA		0.51	4.62	0.08	−1.41	0.37	5	992
		2.5–40	CF	10^{-3}PA	0.63	0.01096	0.00022	−0.76	0.44	5	882
			CA		0.37	0.00987	0.00012	−2.09	0.27	4	970
		2.5–40	CF	PAR	0.10	6.44	0.21	−0.44	0.07	5	997
			CA		0.28	5.71	0.05	−1.78	0.21	4	982
B	I	1.0–20	CF	PHR	0.09	6.17	0.03	−0.17	0.06	6	988
			CA		0.16	3.41	0.03	−0.13	0.11	6	965
C	II	0.1–1.0	CF	PH	0.007	0.1929	0.0019	−0.010	0.006	5	972
		0.1–1.25	CA		0.017	0.0627	0.0012	−0.0003	0.017	5	897
		0.1–1.0	CF	PHR	0.007	1.696	0.017	−0.019	0.006	5	971
		0.1–0.75	CA		0.0096	0.585	0.011	−0.028	0.010	4	926
D	II	0.1–1.25	ETO	PH	0.021	0.116	0.003	0.02	0.02	6	813
				PHR	0.039	0.982	0.039	0.04	0.03	6	387
		0.1–1.25	CAG	PH	0.008	0.0725	0.0006	−0.003	0.006	6	977
				PHR	0.011	0.628	0.007	0.0009	0.009	6	947
		0.1–1.25	CA	PH	0.016	0.0643	0.0011	−0.006	0.013	6	892
				PHR	0.017	0.557	0.009	0.005	0.013	6	887
		0.1–1.25	NAG	PH	0.020	0.123	0.003	−0.010	0.016	6	833
				PHR	0.027	0.372	0.010	−0.03	0.01	6	709
		0.1–1.25	NA	PH	0.048	0.109	0.005	−0.04	0.04	6	058
				PHR	0.030	0.335	0.010	−0.07	0.03	6	628
E	III	1–10	ETO	PH	0.35	0.91	0.03	−0.13	0.27	5	503
		1–12.5	ETO	PHR	0.34	12.96	0.44	−0.08	0.26	6	535
				10^{-3}PA	1.25	0.0308	0.0040	1.11	0.86	6	600[c]
				PAR	0.27	7.81	0.22	−0.04	0.21	6	697
		1–12.5	CAG	PH	0.29	0.72	0.02	−0.23	0.23	6	666
				PHR	0.90	11.13	0.93	−1.16	0.70	6	289[d]
				10^{-3}PA	0.29	0.0566	0.0017	−0.23	0.23	6	649
				PAR	0.33	12.82	0.43	−0.17	0.25	6	560
		1–12.5	CA	PH	0.26	0.61	0.02	0.05	0.20	6	725
				PHR	0.24	8.70	0.21	0.09	0.18	6	759
				10^{-3}PA	0.24	0.0506	0.0012	0.03	0.18	6	765
				PAR	0.20	11.48	0.23	0.09	0.15	6	838
		1–12.5	NAG	PH	0.40	1.34	0.05	−0.25	0.32	6	346
				PHR	0.33	4.76	0.16	−0.09	0.25	6	562
				10^{-3}PA	0.25	0.208	0.005	−1.09	0.21	6	752
				PAR	0.28	10.84	0.31	−0.20	0.22	6	671
		1–12.5	NA	PH	0.26	1.28	0.03	−0.14	0.21	6	713
				PHR	0.19	4.53	0.09	0.03	0.15	6	847
				10^{-3}PA	0.42	0.163	0.009	0.05	0.44	5	151
				PAR	0.08	9.74	0.08	−0.44	0.07	6	972
F	III	5.0–40[e]	CF	PH	1.09	2.72	0.10	−1.84	1.00	5	568
			CA		0.59	1.55	0.03	−2.93	0.56	5	876
			CF	PHR	1.44	11.5	0.58	−1.55	1.3	5	247
			CA		1.00	6.55	0.23	−2.68	0.94	5	637
G	IV	0.25–2.0[f,g]	CA	PH	0.063	0.45	0.02	−0.13	0.06	5	413
				10^{-3}PA	0.068	0.0016	0.0001	−0.05	0.06	5	315
H	VI	0.4–5.0[h]	ETO	PH	0.21	0.365	0.020	−0.001	0.165	6	831[i]
				PHR	0.26	4.78	0.31	0.11	0.19	6	302[i]
			CAG	PH	0.081	0.2289	0.0047	−0.26	0.07	6	832
				PHR	0.106	3.00	0.08	−0.14	0.08	6	711
			CA	PH	0.160	0.341	0.025	−0.02	0.27	5	973[i]
				PHR	0.101	4.30	0.19	0.26	0.15	5	593
I	VI	0.5–5.0[j]	ETO	PH	0.042	0.465	0.005	−0.07	0.03	6	954
			CAG	PH	0.046	0.305	0.004	−0.05	0.04	6	944
J	VII	0.5–5.0[j]	CAG	PH	0.032	0.357	0.003	−0.26	0.03	6	973
			CA	PH	0.163	0.524	0.022	0.22	0.12	6	303

[a] The concentrations, C, of clofibrate and clofibric acid are the molarities injected into the mobile phase except for Studies F and G. Regressions were performed on $10^5 \times C$. The constants m and b are the slope and intercept, respectively, where $s_{10^5 c.x}$, s_m, and s_b are the standard errors of estimate (14) of $10^5 \times$ concentration on the measured parameter of the slope and intercept, respectively. The dependent variables, X, are the parameters of measurement, *i.e.*, peak height (PH), $10^{-3} \times$ peak area (PA), peak height ratio to that of the internal standard (PHR), and peak area ratio to that of the internal standard (PAR). The values of n and r^2 are the numbers of data pairs and the square of the correlation coefficient, respectively. [b] The internal standard was $5.9 \times 10^{-5}\ M$ morphine sulfate in the injected solutions of Studies A, B, and F; $1.625 \times 10^{-5}\ M$ thymol in Studies C, D, and H; and $1.75 \times 10^{-4}\ M$ thymol in Study E. [c] For $0.93xxx$. [d] For $0.97xxx$. [e] Concentration in original plasma diluted 1:1 with acetonitrile and supernate injected. [f] Concentration in original plasma that was extracted and evaporated, with the residue reconstituted in 100 μl of mobile phase before injection. [g] Plasma components interfered with the peak of morphine. [h] Concentration in original plasma (outdated human) that was diluted 1:2 with acetonitrile and the supernate injected. [i] For $0.98xxx$. [j] Concentration in original dog plasma diluted 1:3 with acetonitrile and the supernate injected. Both Studies I and J were effected on same spiked plasma studies.

the peak height ratios to the internal standard did not change significantly, although the clofibric acid–plasma peak interference was more pronounced.

Clofibric acid concentrations were best determined by injecting another aliquot into System IV (Table I and Fig. 1C), although plasma components did interfere with the morphine sulfate peak.

Procedure B: Chloroform Extraction of Plasma—Plasma (0.5 or 1.0 ml) was extracted with 4 ml of chloroform with mild shaking for 10 min and was centrifuged for 10 min at 3000 rpm. The aqueous layer was aspirated, and 2 or 3 ml of chloroform was evaporated to dryness and reconstituted in 100 μl of mobile phase containing morphine sulfate as the internal standard. This procedure is more sensitive than Procedure A. Aliquots can be injected into Systems I–III for clofibrate assay (Fig. 1A) and into System IV, where plasma interferences are minimized, for clofibric acid assay (Fig. 1C and Study G, Table II).

Assays of Etofibrate and Its Solvolytic Products in Plasma—*Procedure C: Acetonitrile Deproteinization of Plasma*—Acetonitrile, with or without internal standard thymol, was added to 0.5, 1, or 2 ml of

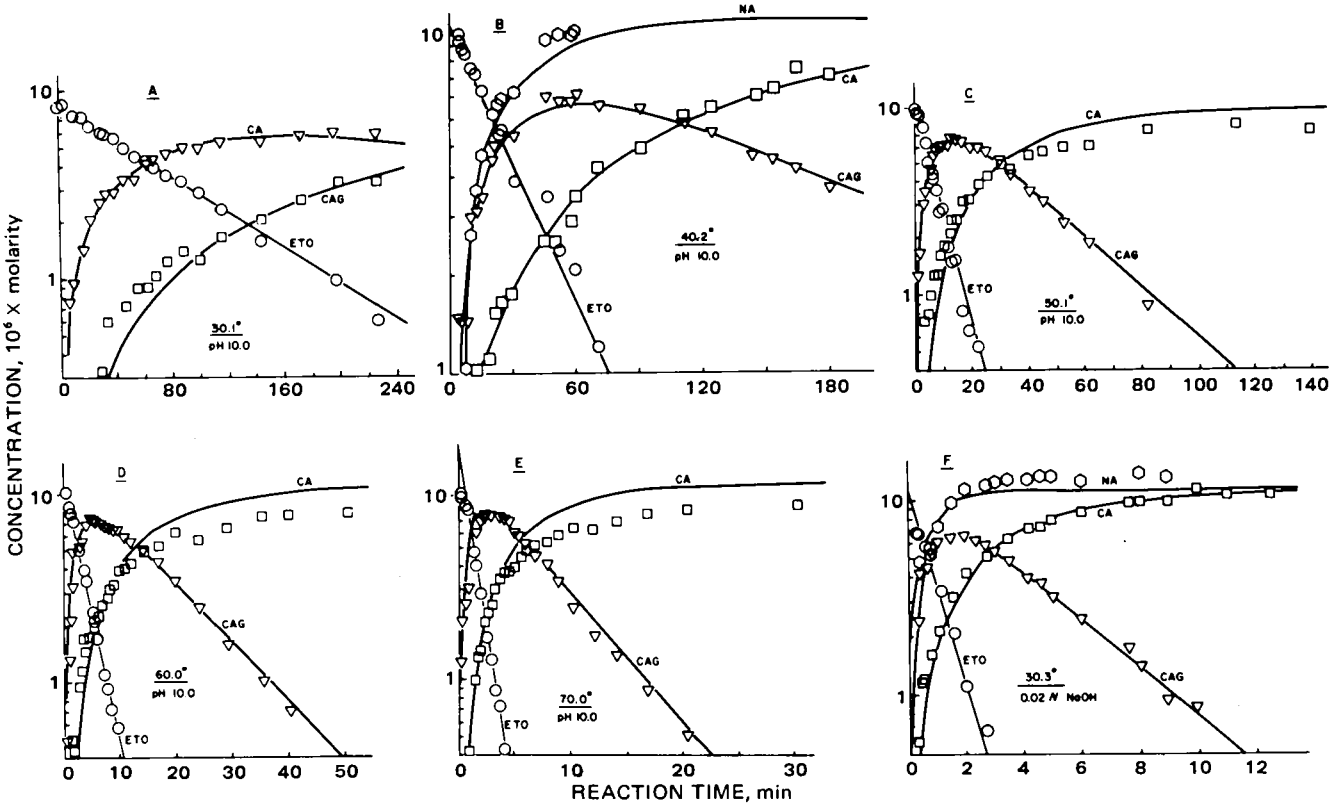


Figure 6—*Semilogarithmic plots of assayed concentrations of etofibrate (ETO), clofibric acid monoglycolate (CAG), clofibric acid (CA), and nicotinic acid (NA)* versus *time for etofibrate degradation in borate buffers and sodium hydroxide. The lines through the CAG, CA, and NA values were calculated from the kinetic parameters given in Table IV.*

human or dog plasma in 1:1, 2:1, 3:2, or 3:1 ratios. The mixture was vortexted and centrifuged for 10 min at 3000 rpm, and an aliquot was injected into Systems II, V, or VI (Table I). At 1:1 and 1:2 mixture ratios, Systems II and VI gave minimal or negligible plasma peak interferences with etofibrate, clofibric acid monoglycolate, and thymol (Fig. 1E) but significant interferences with clofibric acid in fresh dog plasma (Fig. 1E and Study I, Table II). Good calibration curves were established for clofibric acid in outdated human plasma (Fig. 1F and Study H, Table II) at plasma concentrations of $0.4–5.0 \times 10^{-5}$ *M*. Plasma constituents had retention times similar to those of nicotinic acid and nicotinic acid monoglycolate in these systems and thus interfered with their assay.

Clofibric acid in plasma was best assayed separately in System IV (Fig. 1C), V, or VII (Fig. 1G and Study J, Table II) where it was well separated from plasma interferences, although etofibrate was held on the column. The last system could also assay for clofibric acid monoglycolate. Etofibrate-spiked plasma ($1.28 \times 10^{-}$ *M*) diluted 1:2 with acetonitrile showed no change in the etofibrate peak for more than 24 hr.

Procedure D: Methylene Chloride Extraction of Plasma—Plasma (1.0 ml) was adjusted to pH 2.5 with 125 μl of 1 *N* HCl with vortexing, extracted with 3 ml of methylene chloride, shaken at low speed for 10 min, and centrifuged for 10 min at 3000 rpm. The aqueous protein phase was aspirated and discarded. An aliquot of the methylene chloride layer (1.00 ml) was evaporated under nitrogen at room temperature and reconstituted in mobile phase that was 1.625×10^{-5} *M* in thymol, and an aliquot was injected onto System VI. This system assayed for etofibrate and clofibric acid monoglycolate (Fig. 1D) but had plasma peak interferences with the other solvolytic products of etofibrate. Clofibric acid could be assayed by injection of an aliquot into System IV (Fig. 1C), V, and possibly VII (Fig. 1G) where plasma interferences with clofibric acid peaks were minimal.

Clofibric acid could be assayed to 5×10^{-6} *M* concentrations in plasmas by reextracting 1 ml of methylene chloride into 1.00 ml of pH aqueous buffer with System VI.

RESULTS AND DISCUSSION

Reversed-Phase HPLC Assays of Clofibric Acid Esters and Their Products for Degradation Studies—HPLC Systems I–III were used for clofibric acid esters and their degradation products. Typical chromatograms (Studies A–C and F of Table II) are given in Figs. 2A–2G. The aliquots of degrading solutions taken with time were neutralized to the relatively stable region of pH 4–5. The quenched solution had the same composition as the mobile phase used. Morphine sulfate or thymol, added in the quenching solution, were internal standards.

Calibration curves of concentration against peak height, peak areas, and their ratios to the respective parameters of the internal standard showed excellent linear regressions (Table II). In general, it could not be concluded that one measured parameter was significantly better than another.

The standard errors of estimation (14) of clofibrate and clofibric acid concentrations were $\sim 0.6 \times 10^{-5}$ *M* (1.46 μg/ml) and 0.3×10^{-5} *M* (0.64 μg/ml), respectively, for calibration curves prepared with System I (Study A of Table III) in the $2.5–40 \times 10^{-5}$ *M* range (5–100 μg/ml) and indicated analytical sensitivities of 1–3 μg/ml. The standard errors of estimation of concentration of etofibrate and its degradation products were $\sim 0.30 \times 10^{-5}$ *M* (1.0 μg/ml) for calibration curves prepared with System III (Study E of Table II) in the $1–12.5 \times 10^{-5}$ *M* range (3.6–45 μg/ml of etofibrate) and indicated analytical sensitivities of 2.0 μg/ml.

The radial compression systems gave sharper peaks, lessened retention time, and heightened sensitivities for clofibrate, etofibrate, and their degradation products in Systems II and V–VII (Table I) when injected in the mobile phase. The standard errors of estimation of concentrations were $1–2 \times 10^{-7}$ *M* for clofibrate (24–48 ng/ml), etofibrate (36–72 ng/ml), and clofibric acid (21–52 ng/ml) and its monoglycolate (26–52 ng/ml) (Studies C and D of Table II with System II and 225 nm detection) for calibration curves in the $1–12.5 \times 10^{-6}$ *M* range (200–2500 ng/ml). Thus, analytical sensitivities of 100 ng/ml were indicated. System VI gave similar results (Fig. 2G). Solvent front interferences with nicotinic acid and nicotinic acid monoglycolate on these systems at 225-nm detection were minimized at 263-nm detection, but sensitivities were 200–400 ng/ml (Study D of Table II and Figs. 1B and 1F).

Decreased acetonitrile and methanol content in the mobile phase with acetate buffer increased the retention times of all components and permitted separation of clofibric acid from plasma components (Table I). The use of methanol in the mobile phase gave higher retention times than acetonitrile and well-separated plasma components and clofibric acid peaks (Fig. 1C).

Reversed-Phase HPLC Assays of Clofibric Acid Esters and Their Solvolytic Products in Plasma—Partition studies showed that clofibrate, etofibrate, and clofibric acid monoglycolate could be well extracted

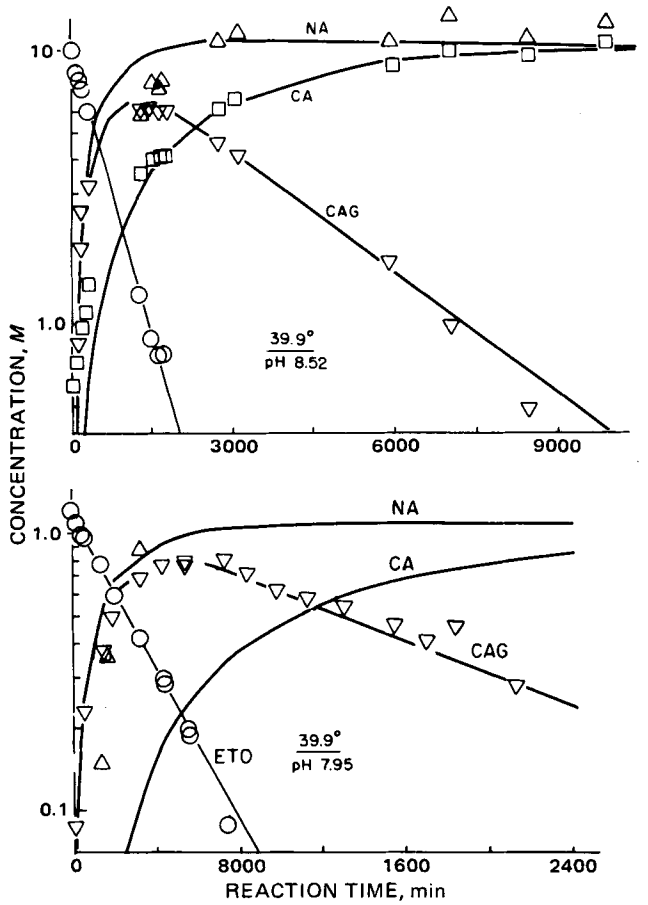


Figure 7—*Semilogarithmic plots of assayed concentrations of etofibrate (ETO), clofibric acid monoglycolate (CAG), clofibric acid (CA), and nicotinic acid (NA)* versus *time for etofibrate degradation in phosphate buffers. The lines through the CAG, CA, and NA values were calculated from the kinetic parameters given in Table IV.*

at a pH > 2 with chloroform or methylene chloride (Procedures B and D). Clofibric acid was completely extracted at pH < 4 and negligible at pH > 7. The haloform extracts could be evaporated to dryness and reconstituted in 100 μl of mobile phase for injection. Assays of clofibrate,

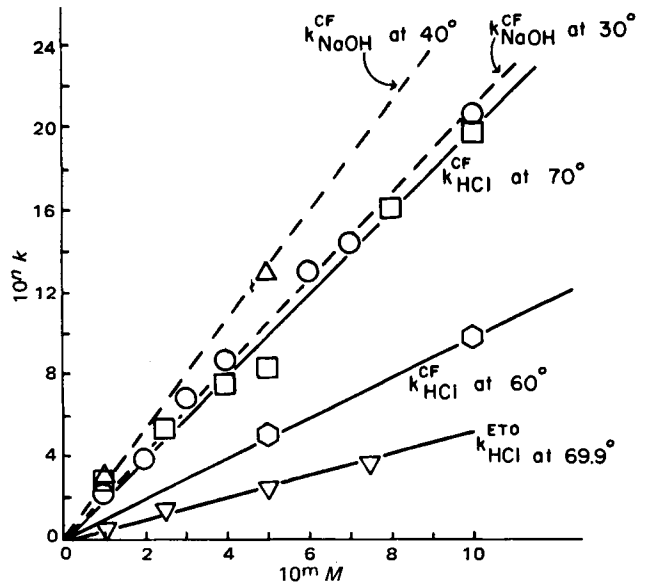


Figure 8—*Plots of first-order rate constants of clofibrate (k^{CF}) and etofibrate (k^{ETO}) hydrolysis* versus *molarities of sodium hydroxide (dashed line, n = 2, m = 2) and hydrochloric acid (solid line, n = 3, m = 1) at several temperatures.*

Table III—First-Order Rate Constants (k, min^{-1}) for the Hydrolysis of Clofibrate

Temperature	Sodium Hydroxide Concentration	10^3k	pH[a]
30.2°	0.010	22.2	11.76
30.5°	0.020	38.9	12.05
30.1°	0.030	69.0	12.23
30.3°	0.040	86.3	12.35
30.1°	0.060	130.5	12.52
29.9°	0.070	144.5	12.58
29.5°	0.100	206	12.73
40.0°	0.010	30.8	11.46
40.0°	0.050	129	12.14
	Hydrochloric Acid Concentration		
61°	0.500	5.0	0.39
60.1°	1.000	9.7	0.12
70.8°	0.100	3.03	1.05
70.4°	0.250	5.37	0.74
70.5°	0.400	7.55	0.54
70.4°	0.500	8.23	0.45
70.4°	0.800	16.1	0.24
70.4°	1.00	19.6	0.13
	Buffer		
60.0°	Phosphate	0.043	6.00
60.0°	Phosphate	0.043	7.00
61.0°	Phosphate	0.349	8.00
60.8°	Borate	1.55	9.01
60.0°	Borate	8.66	9.97
70.0°	Phosphate	0.072	6.00
70.0°	Phosphate	0.110, 0.087	7.00

[a] The pH values for sodium hydroxide and hydrochloric acid solutions were calculated from $pH = pK_w + \log f_{NaOH}[NaOH]$ and $pH = -\log f_{HCl}[HCl]$, respectively, from the pK_w values and activity coefficients, f_{NaOH} and f_{HCl}, available in the literature (15) for the designated temperatures. The pK_w values at respective temperatures were: 13.83, 30°; 13.52, 40°; 13.26, 50°; 13.02, 60°; and 12.78, 70°

etofibrate, and clofibric acid monoglycolate could be effected in Systems I (Fig. 1A), II, III, and VI (Fig. 1D and Table I). While the clofibric acid peaks had retention times similar to plasma components in this system, no such interferences occurred with Systems IV (Fig. 1C), V, and VII (Table I). These extraction procedures with reconstitution with System II and V–VII provided assays sensitive to 10 ng/ml of plasma from 1 ml of plasma.

A more facile, but less sensitive assay used acetonitrile deproteinization of plasma (Procedures A and C). The supernate, after addition of acetonitrile to plasma in 1:1, 2:1, 3:2, or 3:1 ratios, was injected directly into the system. The standard error of estimation of clofibrate and clofibric acid concentrations was 1–1.5 × 10^{-5} M (2–3 μg/ml) for calibration curves prepared with System III (Fig. 1B and Study F of Table II) for plasma concentrations of 5–40 × 10^{-5} M (10–100 μg/ml). Analytical sensitivities of 5–6 μg/ml were indicated.

The use of the radial compression systems increased plasma assay sensitivities *via* acetonitrile denaturation (Figs. 1E–1G). The standard errors of estimation of plasma concentrations of etofibrate and clofibric acid and its monoglycolate were 0.5–2 × 10^{-6} M (100–400 ng/ml) for calibration curves prepared with System VI or VII (Studies H–J of Table II) for plasma concentrations in the 4–50 × 10^{-6} M range (0.8–10 μg/ml). Thus, analytical sensitivities of 0.2–0.8 μg/ml were indicated.

Kinetics of Solvolyses—At constant pH, clofibrate and etofibrate degrade by apparent first-order processes. Representative semilogarithmic plots of concentrations *versus* time are given for hydrochloric acid solutions (Figs. 3 and 4), sodium hydroxide solutions (Figs. 5 and 6), borate buffers (Figs. 3 and 6 and Tables III and IV), and phosphate buffers (Fig. 7), where the apparent first-order rate constant, k, was obtained from the slopes in accordance with:

$$\log C = -\frac{kt}{2.303}t + \log C_0 \qquad \text{(Eq. 1)}$$

where C and C_0 are the concentrations at time t and time zero, respectively.

The pH values of the degrading buffer solutions were experimentally determined at the study temperature. The pH values for hydrochloric acid were calculated from

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

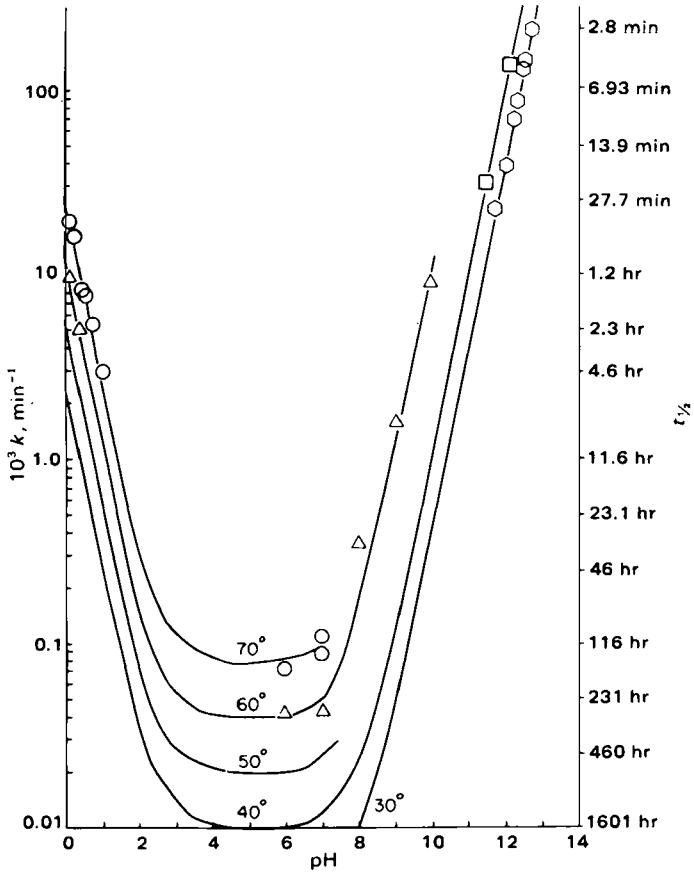


Figure 9—*Fitted log* k–*pH profiles for the hydrolyses of clofibrate at various temperatures. Corresponding half-lives are given on the right.*

where γ is the mean activity coefficient for the hydrochloric acid solution. For sodium hydroxide:

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

where γ is the mean activity coefficient for the sodium hydroxide (15). Values of $pK_w = -\log K_w$ are given in Footnote *f* of Table I; K_w is the hydrolysis constant for water.

The aparent first-order rate constants for clofibrate, etofibrate, or clofibric acid monoglycolate hydrolysis can be characterized in terms of actual concentrations of strong acid or alkali as:

$$k = k_{HCl}[HCl] \qquad \text{(Eq. 4)}$$

and:

$$k = k_{NaOH}[NaOH] \qquad \text{(Eq. 5)}$$

The bimolecular rate constants, k_{HCl} and k_{NaOH}, at various temperatures were obtained from the slopes of plots of k *versus* these concentrations (Table V).

Log *k*–pH Profiles (16) and Solvolysis Routes–When activities of hydrogen, a_H, and hydroxide, a_{OH}, ions are considered as catalytic species:

$$a_H = 10^{-pH} \qquad \text{(Eq. 6)}$$

$$a_{OH} = 10^{-pOH} = 10^{-(pK_w - pH)} \qquad \text{(Eq. 7)}$$

the acid and alkaline branches of the log k–pH profiles (Figs. 9–11) that characterize the attack of hydrogen- and hydroxyl-ion activities, respectively, conform to:

$$\log k = \log k_H - pH \qquad \text{(Eq. 8)}$$

and:

$$\log k = \log k_{OH} + pH - pK_w \qquad \text{(Eq. 9)}$$

The profiles at the temperatures where no studies were made were constructed so as to be consistent with the Arrhenius parameters, ΔH_a and ln P, calculated or determined from the slopes and intercepts of the Arrhenius plots (Fig. 12).

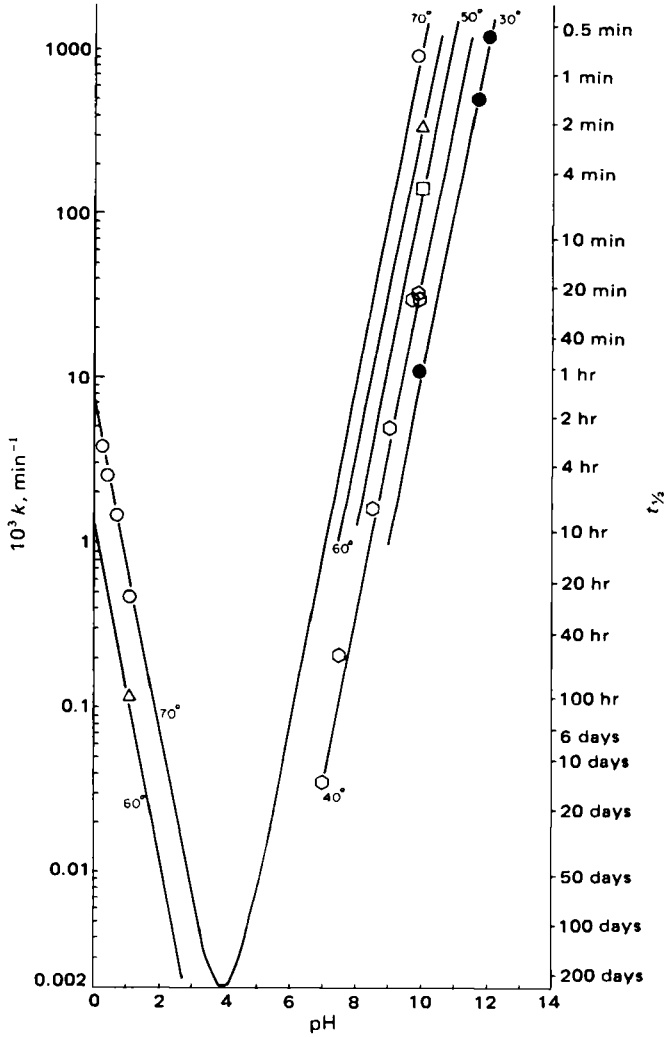


Figure 10—*Fitted log* k–*pH profiles for the hydrolyses of etofibrate at various temperatures. Corresponding half-lives are given on the right.*

The constants are given in Table V and can be used to calculate the apparent first-order rate constant for hydrolysis at any pH and temperature using:

$$k = k_H a_H + k_{OH} a_H + k_0 \qquad \text{(Eq. 10)}$$

when Eqs. 6 and 7 and the Arrhenius equation:

$$\ln k_H \text{ or } \ln k_{OH} = \ln P - \Delta H_a/1.987(1/T) \qquad \text{(Eq. 11)}$$

where T is degrees plus 273° and ΔH_a is in calories per mole, are considered.

A pH-independent component, k_0^{CF}, had to be considered in the case of clofibrate solvolysis (Fig. 9 and Table V) to account for first-order rate constants between pH 6 and 7 that were higher than those predicted from the sum of the extrapolation of the acid and alkaline branches of the clofibrate log k–pH profile.

The predicted minimum in the log k–pH profile for etofibrate (Fig. 10) is probably not exact but should be displaced a few 10ths of a pH unit to the right since etofibrate is protonated in the region of acid solvolysis. In addition, hydrogen-ion attack on a nonprotonated compound would be faster.

Pharmaceutical Significances of Stability Studies—The high solvolytic instability of clofibric acid esters, even in mildly alkaline solutions, requires that the microscopic environment be kept as acidic as possible (pH 4) in the formulation of liquid and solid dosage forms. Alkaline lubricants and excipients should not be used, and granulations should be kept as free of water as possible. Even at 30° and pH 4–6, the half-lives of clofibrate and etofibrate in aqueous solutions are predicted (Figs. 9 and 10) to be 120 and 240–2000 days, respectively; at pH 7, they

Table IV—First-Order Rate Constants (k_1, min^{-1}) for the Hydrolysis of Etofibrate and the Glycol Esters of Clofibric Acid (k',min^{-1}) and Nicotinic Acid (k'', min^{-1})

Temperature	Hydrochloric Acid Concentration	(k'',min^{-1}) 10^3k	$10^3k''$[a]	pH[b]	f[c]
49.8°	0.100	0.055	—	1.11	0.00
58.0°	0.100	0.188	—	1.08	0.00
69.8°	0.100	0.474	—	1.11	0.00
69.9°	0.250	1.46	—	0.74	0.16
69.9°	0.500	2.58	0.75	0.45	0.00
69.9°	0.750	3.76	1.0	0.27	0.05
	Sodium hydroxide concentration		$10^3k'$		
29.5°	0.010	497	175	11.77	1.00
30.3°	0.020	1187	285	12.97	0.90
	Buffer				
30.1°	Borate	10.9	3.0	9.98	0.94
40.2°		32.6	6.37	9.96	0.83
40.2°		30.3	5.70	9.95	0.85
40.2°		29.7	5.00	9.85	0.75
39.9°		4.94	0.962	9.02	1.00
39.9°		1.58	0.290	8.52	0.82
40.0°	Phosphate	0.314	0.0724	7.95	0.95
39.9°		0.206	0.032	7.53	1.00
39.9°		0.035	—	6.94	1.00
50.1°	Borate	138	31	9.98	1.00
59.95°		339	72	10.03	1.00
70.0°		898	160	9.91	1.00

[a] Estimates are crude for the hydrolysis of the glycol ester of nicotinic acid since studies were not carried out long enough. [b] The pH values for sodium hydroxide and hydrochloric acid solutions were calculated from $pH = pK_w + \log f_{NaOH}[NaOH]$ and $pH = -\log f_{HCl}[HCl]$, respectively, from the pK_w values and activity coefficients, f_{NaOH} and f_{HCl}, available in the literature (15) for the designated temperatures. [c] Estimated fraction of clofibrate hydrolyzed to the monoglycol ester of clofibric acid where 1 − f is the estimated fraction hydrolyzed to clofibric acid and the monoglycol ester of nicotinic acid.

would be 160 and 20 days, respectively. Thus, these compounds must be assayed immediately and not stored in samples of biological tissues and fluids. Extraction from plasma into haloalkane solvents or mixing plasma with equivalent volumes of acetonitrile can maintain the integrity of the esters for several days prior to assay.

Routes of Solvolyses—The primary product of clofibrate solvolysis is clofibric acid at all pH values (Fig. 3), although *p*-chlorophenol can be generated in strong acidic solutions (Scheme I).

The routes of etofibrate solvolysis differ for the acidic and alkaline regions. The initial products of acid hydrolysis were nicotinic acid monoglycolate and clofibric acid (Scheme II), and no clofibric acid monoglycolate was observed (Fig. 13). The initial products of solvolyses at pH > 7 were nicotinic acid and clofibric acid monoglycolate, and the latter subsequently hydrolyzed to clofibric acid and ethylene glycol (Figs. 6 and 7). No nicotinic acid monoglycolate was observed within the level of analytical sensitivities.

If the products of etofibrate (ETO) solvolyses in the alkaline region are only clofibric acid monoglycolate (CAG) and nicotinic acid (NA) and if no glycol ester of nicotinic acid (NAG) (Scheme II) is generated, then the concentration of clofibric acid monoglycolate may be calculated from the expression (17):

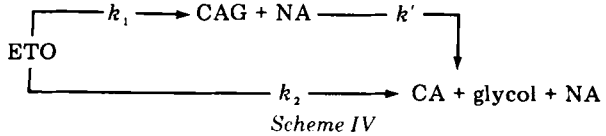
$$[CAG]' = [ETO]_0 \frac{k}{k' - k}(e^{-kt} - e^{-k't}) \qquad \text{(Eq. 12)}$$

where $[ETO]_0$ is the initial etofibrate concentration and $[CAG]'$ is clofibric acid monoglycolate concentration on the presumption that the sole route of hydrolysis is as shown in Scheme III, where k and k' are the apparent first-order rate constants for the sequential hydrolyses.

$$ETO \xrightarrow{k} CAG + NA \xrightarrow{k'} CA + glycol + NA$$

Scheme III

If nicotinic acid monoglycolate is generated but is so low in concentration that it cannot be observed, Scheme IV could be postulated.



Scheme IV

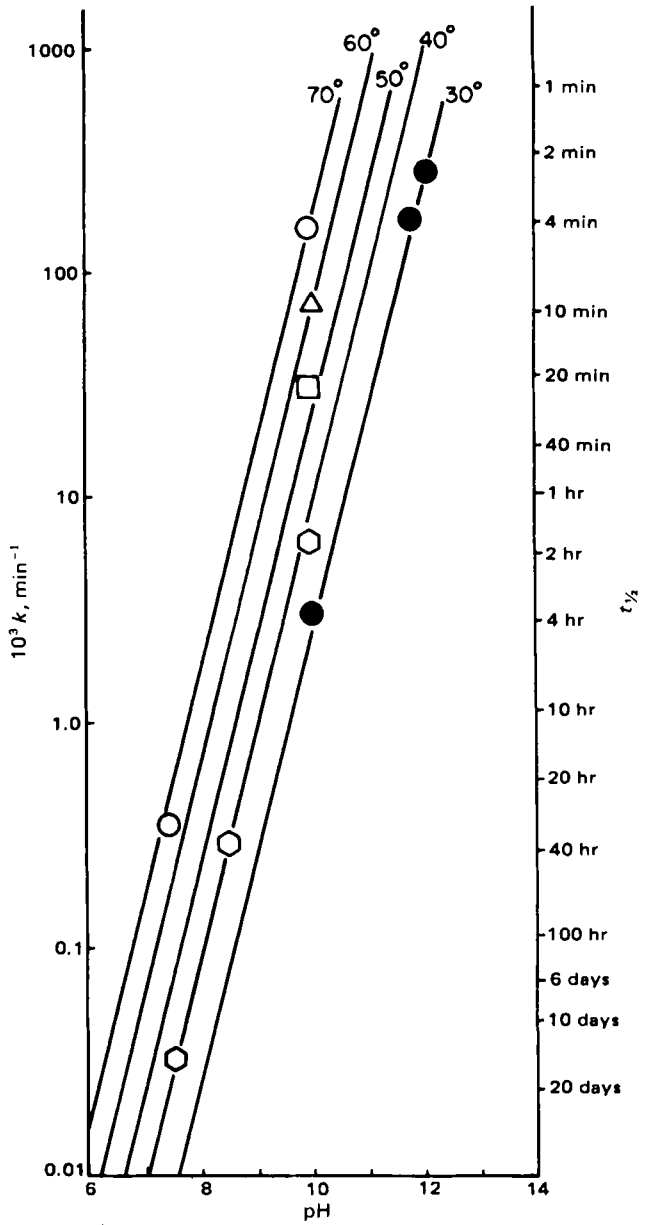


Figure 11—*Fitted log k-pH profiles for the hydrolyses of clofibric acid monoglycolate at various temperatures. Corresponding half-lives are given on the right.*

There is no storage in this form due to its extremely rapid hydrolysis, and $k = k_1 + k_2$ and (18):

$$[CAG] = [ETO]_0 \frac{k_1}{k' - k}(e^{-kt} - e^{-k't})$$

$$= f[ETO]_0 \frac{k}{k' - k}(e^{-kt} - e^{-k't}) \qquad \text{(Eq. 13)}$$

where:

$$f = [CAG]/[CAG'] = k_1/k \qquad \text{(Eq. 14)}$$

This signifies that the ratio, f, of the experimental concentration of clofibric acid monoglycolate to the theoretical concentration, calculated on the premise of no nicotinic acid monoglycolate intermediate, is constant at all specified times.

The apparent first-order rate constant, k', for the solvolysis of clofibric acid monoglycolate could be obtained from the terminal slope of the plots of its generation with time (Figs. 6 and 7) from etofibrate. It was also obtained by the apropriate adjustment of the f and k' values of Eq. 13 that gave the best fit to the time course of generated clofibric acid monoglycolate in Figs. 6 and 7. These values are listed in Table IV and demonstrate that negligible etofibrate is solvolyzed through a nicotinic acid monoglycolate intermediate above a pH of 7. Separate studies of

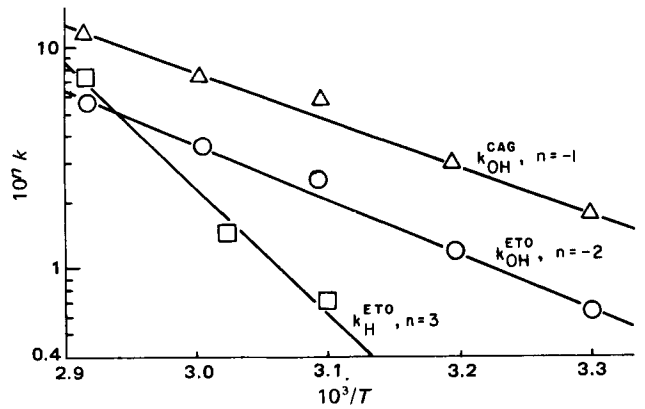


Figure 12—*Arrhenius plots of bimolecular rate constants for the alkaline solvolyses of etofibrate (ETO) and clofibric acid monoglycolate (CAG) and the acid solvolysis of etofibrate (ETO).*

clofibric acid monoglycolate solvolyses at these pH values confirmed the k' values estimated by this procedure. The lines through the concentrations of nicotinic and clofibric acids in Figs. 6 and 7 were drawn in accordance with:

$$[NA] = [ETO]_0 (1 - e^{-k_1 t}) \quad \text{(Eq. 15)}$$

$$[CA] = [ETO]_0 - [ETO] - [CAG] \quad \text{(Eq. 16)}$$

where [CAG] was calculated from Eq. 13.

A similar approach was used to estimate k'', the apparent first-order rate constant at a given pH for the acidic hydrolysis of nicotinic acid monoglycolate (Fig. 13). However, the k'' values given in Table IV are crude estimates since these kinetic studies were not conducted for adequate lengths of time.

Stabilities of Clofibric Acid Esters in Plasma—Clofibrate stability was monitored by HPLC assay in fresh dog plasma. The apparent half-lives of $1–2 \times 10^{-5}$ *M* clofibrate in plasma were 9.75 min at 24° and 4.57 min at 37.6° using Procedure B (Fig. 14). When Procedure A was used for assay of degrading 1.83×10^{-4} *M* clofibrate in fresh plasma at 24.6°, degradation was apparent zero order (7.0×10^{-7} mole/liter/min) and an apparent time for half-degradation was 130 min (curve A of Fig. 15). A similar study by Procedure A, conducted at room temperature, showed a half-degradation time of 125 min for 5.8×10^{-3} *M* clofibrate in fresh plasma. The fact that apparent first-order degradation of clofibrate with an apparent half-life of 10 min at 24° occurs at low concentrations (curve B of Figs. 14 and 15) indicates that the plasma enzymes responsible for clofibrate solvolysis are readily saturated and give slower degradation rates at higher plasma concentrations.

The stability of etofibrate in fresh dog plasma assayed by Procedure C gave apparent half-lives of 4.4 min for 3.6×10^{-5} *M* etofibrate in two separate studies (Fig. 16).

The apparent half-life for an initial plasma concentration of etofibrate ($[ETO]_0 = 5.9 \times 10^{-5}$ *M*), assayed by Procedure D, was 6.9 min ($k = 0.100$ min^{-1}) with an apparent terminal half-life of 11.2 min for clofibric acid monoglycolate solvolyses ($k' = 0.0619$ min^{-1}) (Fig. 16). The maximum peak height ratio of 0.37 for this compound at 10 min corresponded to an approximate concentration of 2.85×10^{-5} *M* clofibric acid monoglycolate. On the assumption that the degradation pathway is solely through clofibric acid monoglycolate (Scheme III), the maximum concentration, $[CAG]_{max}$, can be estimated (17) from:

$$[CAG]_{max} = [ETO]_0 (1/k')(k/k')^{k'/(k'-k)} = 2.7 \times 10^{-5} M \quad \text{(Eq. 17)}$$

The coincidence of this calculated maximum and that experimentally determined provides strong evidence that clofibric acid monogycolate is the primary product of etofibrate solvolysis in plasma.

With Procedure C at 37.5°, 1.19×10^{-4} *M* etofibrate in plasma showed a half-life of 5.7 min ($k = 0.121$ min^{-1}) by the solvolysis route through clofibric acid monoglycolate. The latter had a half-life of 5.8 min (0.119 min^{-1}) when the data were fit as described by Eqs. 13, 14, and 16; $f = 1$ (Fig. 17). Solubility in plasma is expected to be much greater than in buffer due to high protein binding (13). Using Procedure C, clofibric acid monoglycolate, 4.25×10^{-5} *M* in plasma, showed a half-life of 11.5 min (Fig. 17).

Table V—Estimated Kinetic Parameters (16) for the Hydrolyses of Clofibrate (k^{CF}), Etofibrate (k^{ETO}), and the Glycol Esters of Clofibric Acid (k^{CAG}) and Nicotinic Acid (k^{NAG})

Microscopic Rate Constant	30°	40°	50°	60°	70°	ΔH_a	ln P^a
k_{OH}^{CF}[b], liters/mole/min	2.7	3.3	—	—	—	3.8[c]	7.3
k_{NaOH}^{CF}[d]	2.11	2.71	—	—	—	4.8[c]	8.7
$10^3 k_H^{CF}$[b]	—	—	—	12	25	16.2[c]	20
$10^3 k_{HCl}^{CF}$[d]	—	—	—	9.8	20	16.6[c]	20.4
$10^3 k_0^{CF}$[d], min^{-1}	—	—	—	0.04	0.08	16[c]	13.6
k_{OH}^{ETO}[b], liters/mole/min	63.5	119	251	351[f]	554	11.4[a]	23.1
$10 k_H^{ETO}$[b]	—	—	0.70	1.4	7.1	26.4[a]	33.8
k_{OH}^{CAG}[b]	17.9	29.5	59.2	75.3	111.5	9.8[a]	19.1
$10^3 k_H^{NAG}$[b]	—	—	—	—	2.2	—	—
$10^3 k_{HCl}^{NAG}$[d]	—	—	—	—	5.2	—	—

[a] These heats of activation (kilocalories per mole) and ln *P* values were obtained from Fig. 12 in accordance with the Arrhenius function $\ln k = \ln P - (\Delta H_a/R)(1/T)$, where $R = 1.987$ cal/degree and ΔH_a is in calories per mole. The values of k_{OH}^{ETO} obtained from the best linear fit of this function were used in the log *k*–pH profile at 50 and 70° and were 210 and 590, respectively. Similarly, the values of k_{OH}^{CAG} at 50° was 47.5. [b] Bimolecular rate constants in liters/mole/min for hydrogen- and hydroxide-ion catalysis obtained from intercepts of the alkaline and acid branches, respectively, of the log k *versus* pH profile (Fig. 9 for clofibrate, Fig. 10 for etofibrate, and Fig. 11 for the monoglycol ester of clofibric acid) where the logarithm of the apparent first-order rate constant for solvolysis is $\log k = \log k_{OH} + pH - pK_w$, where pH is calculated from $pH = pK_w + \log f_{NaOH}[NaOH]$ in the former case, and $\log k = \log k_{aH} - pH$, where $pH = -\log f_{HCl}[HCl]$ in the latter. The pK_w values and activity coefficients, f_{NaOH} and f_{HCl}, were obtained from the literature (15). [c] Estimates of heats of activation (kilocalories per mole) of these rate processes were calculated from $\Delta H_a = 1.987 \times (\ln k_{T_1} - \ln k_{T_2})/[(1/T_2) - (1/T_1)]$, where T_1 and T_2 are the respective temperatures in degrees Kelvin (°C + 273) for the two studies. [d] Bimolecular rate constants for sodium hydroxide- and hydrochloric acid-catalyzed solvolysis obtained from slopes of plots of the apparent first-order rate constants against sodium hydroxide and hydrochloric acid, respectively (Fig. 8). [e] Apparent first-order rate constants for pH-independent solvolysis of clofibrate. [f] Actually 58.0°.

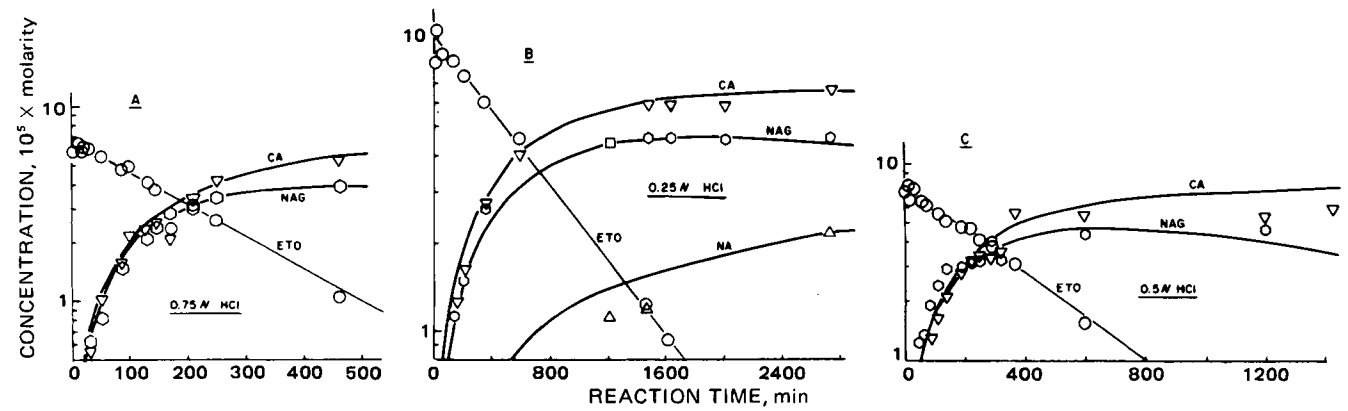


Figure 13—*Semilogarithmic plots of assayed concentrations of etofibrate (ETO) and solvolysis products, nicotinic acid monoglycolate (NAG), clofibric acid (CA), and nicotinic acid (NA), degrading at 69.9° in hydrochloric acid with an original concentration of 3×10^{-4} M etofibrate.*

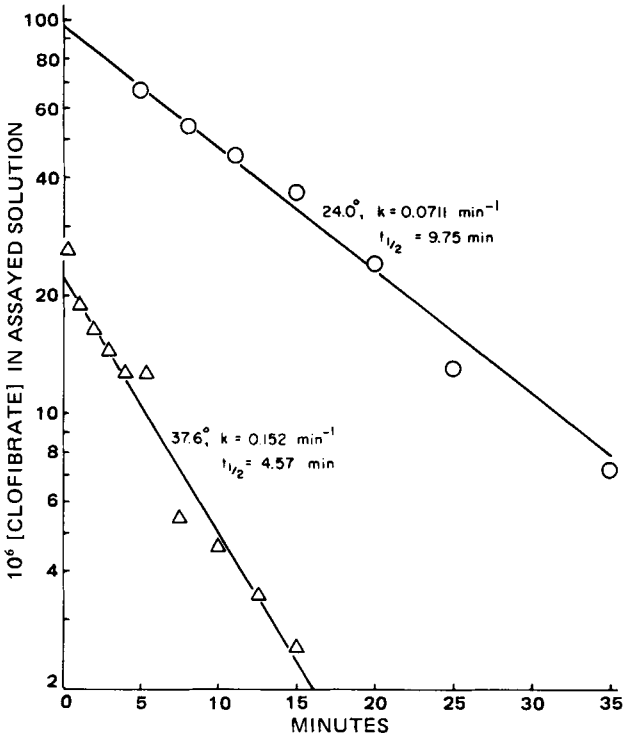


Figure 14—*Semilogarithmic plots of clofibrate concentrations with time in fresh dog plasma where blood was withdrawn 2–4 hr before by Procedure B. Aliquots (3.00 and 2.00 ml) of the 4-ml chloroform extracts of 1.00 ml of plasma were evaporated and reconstituted in 100 μl of mobile phase for injection in the 24 and 37.6° studies, respectively. The respective initial clofibrate concentrations were 1.00 and 1.77 × 10^{-5} M.*

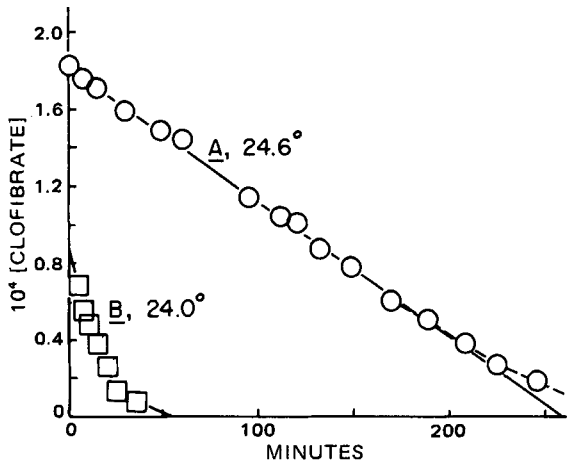


Figure 15—*Linear plots of clofibrate concentration with time at 24.5° in fresh dog plasma, 1.5 hr after withdrawal, as assayed by Procedure A for curve A with an initial plasma concentration of 1.83 × 10^{-4} M and as assayed by Procedure B for curve B with an initial plasma concentration of 1 × 10^{-5} M. The ordinate is for assayed concentrations (curve B) and plasma concentrations (curve A).*

In summary, etofibrate was readily hydrolyzed at 37.5° in fresh dog plasma with apparent half-lives of 4.4–6.9 min. The major product of this solvolysis was the glycol monoester of clofibric acid with a half-life of 5.8–11.5 min. Although the 4–5-min half-life of etofibrate determined previously (13) in diluted human blood (2.5% blood in isotonic pH 7.4 phosphate buffer) agreed with the present values, the half-lives of clofibrate (24 min) and clofibric acid monoglycolate (48 min) in diluted blood (15% blood in isotonic pH 7.4 phosphate buffer) were much greater than the present 5.8–11.5 min in whole dog plasma.

It was claimed (13) that the major route of etofibrate solvolysis in diluted blood is at the clofibric acid ester linkage, in contrast to the present findings in undiluted dog plasma that the major route is by the splitting

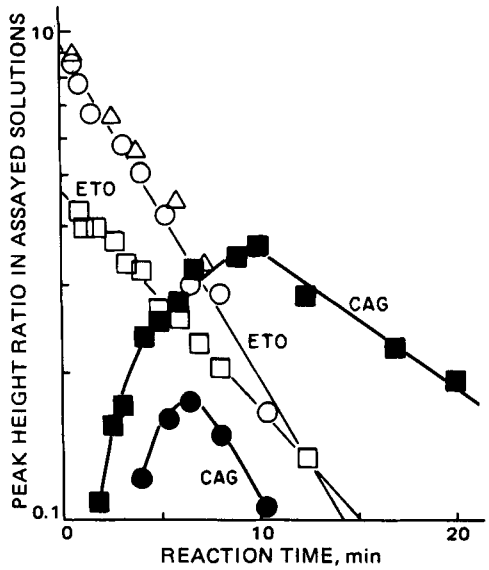


Figure 16—*Semilogarithmic plots of assayed etofibrate (open symbols) and clofibric acid monoglycolate (solid symbols) concentrations with time at 37.5° in fresh dog plasma. Procedure C was used for assay of degrading 3.6 × 10^{-5} M etofibrate in original plasma (○, ●, ▲). Procedure D was used for the assay of degrading 5.9 × 10^{-5} M etofibrate in original plasma (□, ■).*

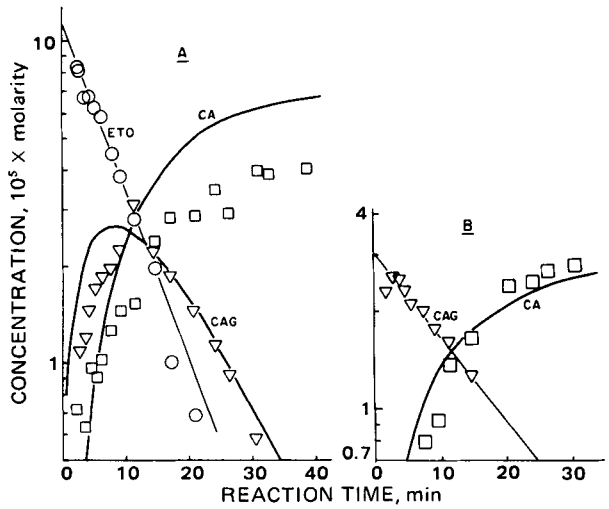


Figure 17—*Semilogarithmic plots of assayed (Procedure C) concentrations of etofibrate (ETO), clofibric acid monoglycolate (CAG), and etofibric acid (CA) for degradation of 1.19 × 10^{-4} M etofibrate (A) and 4.23 × 10^{-5} M clofibric acid monoglycolate (B) by Procedure C in fresh dog plasma. The lines through the CAG and CA values in A were calculated in accordance with Eqs. 13 and 16 for f = 1 (Eq. 14). The line through the CA values in B was calculated in accordance with [CA] = $[CAG]_0(1 - e^{-k_2 t})$.*

of the nicotinic acid ester linkage. The differences in results could be due to the fact that the present study used fresh dog plasma and the earlier study used diluted human blood with etofibrate concentrations in great excess of its solubility. The solvolyses of etofibrate and the half esters in highly diluted blood were not first order in the previous study as they were in the present study. The previous first-order semilogarithmic plots of diluted blood concentrations *versus* time showed a diminishing slope indicating decreasing esterase activity with time, which is contrary to what would be expected for saturable enzyme kinetics.

These discrepancies need clarification by further studies.

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Effect of 4-Dimethylaminomethyl-1-(3-hydroxyphenyl)-1-nonen-3-one Hydrochloride and Related Compounds on Respiration in Rat Liver Mitochondria

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Abstract □ 4-Dimethylaminomethyl-1-(3-hydroxyphenyl)-1-nonen 3-one hydrochloride (II) was shown to stimulate respiration in rat liver mitochondria at levels of 2.5 μmoles or less; but at levels higher than 5.0 μmoles, respiration was inhibited when succinate and 3-hydroxybutyrate were the substrates. Compound II caused inhibition of respiration in the presence of glutamate over the dose range studied. The stimulating effect of II was attributed to its functioning as an uncoupling agent. Its inhibiting properties were considered to be due to its behaving like antimycin A in blocking transport of electrons between cytochromes b and c_1. The effect of II on respiration in mitochondria varied with the pH of the medium. A conjugated styryl ketone, which contained a nuclear hydroxy function and was structurally related to II, also stimulated respiration at low doses while inhibiting respiration at higher concentrations. Etherification of the hydroxy group led to compounds in which only stimulation of respiration was noted.

Keyphrases □ Respiration, mitochondria—effect of 4-dimethylaminomethyl-1-(3-hydroxyphenyl)-1-nonen-3-one hydrochloride and styryl ketone derivatives □ Mannich bases—prepared from styryl ketones, effect on respiration in mitochondria □ Styryl ketones—preparation of Mannich bases

A number of Mannich bases (I) derived from styryl ketones containing chlorine or hydrogen atoms in the aromatic ring have been shown to inhibit the electron transport chain in mitochondria of both rat liver cells and yeast (1, 2). These compounds were considered to exert their effect by competition with coenzyme Q_{10} (1). Recently, 4-dimethylaminomethyl-1-(3-hydroxyphenyl)-1-nonen-3-one hydrochloride (II), which is a Mannich base with a nuclear hydroxy group, was synthesized (3). Although it had little action against murine P-388 lymphocytic leukemia in contrast to some dichlorinated derivatives of I (4), it displayed anti-inflammatory analgesic properties as well as antifungal activity (3).

Therefore, the questions were asked whether II would affect mitochondrial function and, if so, whether its site of action would differ from that in series I. In addition, if II affected the electron transport chain, which is found in the inner membrane of the mitochondria, it could be a carrier group to which other molecular entities may be attached *via* the hydroxyl group. Furthermore, two related α,β-unsaturated ketones, III*a* and III*d*, differed in their activity towards P-388 lymphocytic leukemia (3), which could be due to a variation in effect on mitochondria. Thus, it was of interest to examine the effects of II, III*a*, III*d*, and the related compounds III*b* and III*c* on the electron transport chain in rat liver mitochondria.

DISCUSSION

Table I indicates the effect of II on respiration in rat liver mitochondria using three different substrates. In the case of succinate and 3-hydroxybutyrate, respiration was stimulated at low concentrations; at higher doses of II, inhibition of respiration was noted. When glutamate was the substrate, no stimulation of respiration was noted and only inhibition of respiration was found over the dose range studied.

The inhibiting effect of II at 5 μmoles with succinate as the substrate was considered. The related compounds I*a* and I*b* caused inhibition of respiration by competition with coenzyme Q_{10}, which could be reversed by addition of coenzyme Q_{10} (1). Therefore, a possible site for the respiration-inhibiting action of II might be similar. However, addition of coenzyme Q_{10} to mitochondria whose respiration had been inhibited by II did not alter the percentage of respiration inhibition, so the mode of action of II appears to differ from that of I.

A number of compounds inhibit respiration by blocking the transport

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