intravenous administration of a 10-mg dose of folinic acid to a healthy 57-kg volunteer. These data are similar to those reported by Rothenberg *et al.* (3) following the administration of folinic acid to two healthy subjects. The disposition of folinic acid was characterized by a two-compartment open model. The peak plasma concentration was 3.5×10^{-6} *M*. The distribution and elimination half-lives were 0.27 and 3.7 hr, respectively. These half-lives are different from those obtained by Mehta *et al.* (2) using a microbiological assay, probably because the HPLC assay reported here and the assay of Rothenberg *et al.* (3) do not differentiate the stereoisomers, whereas the microbiological assay measures only the *l* isomer.

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Plasmolysis, Red Blood Cell Partitioning, and Plasma Protein Binding of Etofibrate, Clofibrate, and Their Degradation Products

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Abstract □ Etofibrate (I), the ethylene glycol diester of clofibric and nicotinic acids, degrades almost equally through both half-esters with half-lives of ~10 and 1 min in fresh dog and human plasma, respectively. The nicotinate V degrades with half-lives of ~12 hr and 50 min in fresh dog and human plasma, respectively. Ester III and clofibrate VI degrade by saturable Michaelis–Menten kinetics in fresh human plasma, with similar maximum initial rates and respective terminal first-order half-lives of 12 and 26 min. Tetraethyl pyrophosphate at 100 μg/ml inhibited human plasma and red blood cell esterases permitting plasma protein binding and red blood cell partitioning studies. The red blood cell–plasma water partition coefficient was 5.4 for 0.2–80 μg/ml of I. Clofibrate (VI) showed a saturable erythrocyte partitioning that decreased from 7.8 (10 μg/ml) to 1 (50 μg/ml). The strong binding of I and VI to ultrafiltration membranes necessitated the determination of their plasma protein binding by the method of variable plasma concentrations of erythrocyte suspensions to give 96.6% (0.2–80 μg/ml) and 98.2% (13.6–108.4 μg/ml) binding, respectively. Methods for the determination of the parameters of saturable and nonsaturable plasma protein binding for unstable and membrane-binding drugs by the method of variable plasma concentrations in partitioning erythrocyte suspensions are presented.

Keyphrases □ Etofibrate—degradation products, plasmolysis, red blood cell partitioning, plasma protein binding □ Clofibrate—degradation products, plasmolysis, red blood cell partitioning, plasma protein binding □ Plasmolysis—etofibrate, clofibrate, degradation products □ Red blood cell partitioning—etofibrate, clofibrate, degradation products □ Plasma protein binding—etofibrate, clofibrate, degradation products

Clofibric acid derivatives and nicotinic acid are well established therapeutic agents for the treatment of hyperlipidemia, a major risk factor in coronary artery disease (1, 2). Etofibrate [2-nicotinoyloxyethyl 2-(4-chlorophenoxy)-2-methylpropionate, (I)], the ethylene glycol diester of clofibric and nicotinic acids, is effective in lowering triglycerides and cholesterol in controlled clinical trials (3, 4). The greater lipid-lowering effect of etofibrate in rats than simultaneously administered equimolar amounts of clofibric and nicotinic acids permitted the postulation that both etofibrate and/or its derived half-esters may have pharmacological activity (5). It was reported (6) that both half-esters were detectable after incubation of rat microsomes with etofibrate and that they appeared to be more stable in biological fluids than their etofibrate precursor.

The solution stabilities of etofibrate (I), clofibrate (VI), and derived monoesters were reported previously (7). The possible solvolytic routes are given in Schemes I and II. Conditions were established for optimal extractions and log k–pH profiles for solution solvolyses were constructed for various temperatures using specific high-performance liquid chromatographic (HPLC) assays. Preliminary studies in fresh dog plasma (7) indicated that a considerable fraction of etofibrate rapidly hydrolyzed in dog plasma to produce the ethylene glycol ester of clofibric acid (III), which also hydrolyzed rapidly. This is in contrast to other reported studies in diluted human blood (8).

This paper reports on the human red blood cell–plasma partitioning and human plasma protein binding of etofibrate (I), clofibrate (VI), and their degradation products using the esterase inhibitor, tetraethyl pyrophosphate. Improved HPLC assays of higher sensitivity were applied to complete studies of the human and dog plasmolytic routes and degradation rates. In addition, complete methods for the determination of the parameters of saturable and nonsaturable plasma protein binding for unstable and membrane-bound drugs by the method of variable plasma concentrations in partitioning erythrocyte suspensions are presented.

EXPERIMENTAL

Methods—Acetic acid[1], sodium acetate[1], dibasic sodium phosphate[1], monobasic potassium phosphate[1], *tert*-butyl alcohol[2], sodium chloride[2],

[1] Mallinckrodt Inc., Parris, KY 40361.
[2] Fisher Scientific Co., Fair Lawn, NJ 07410.

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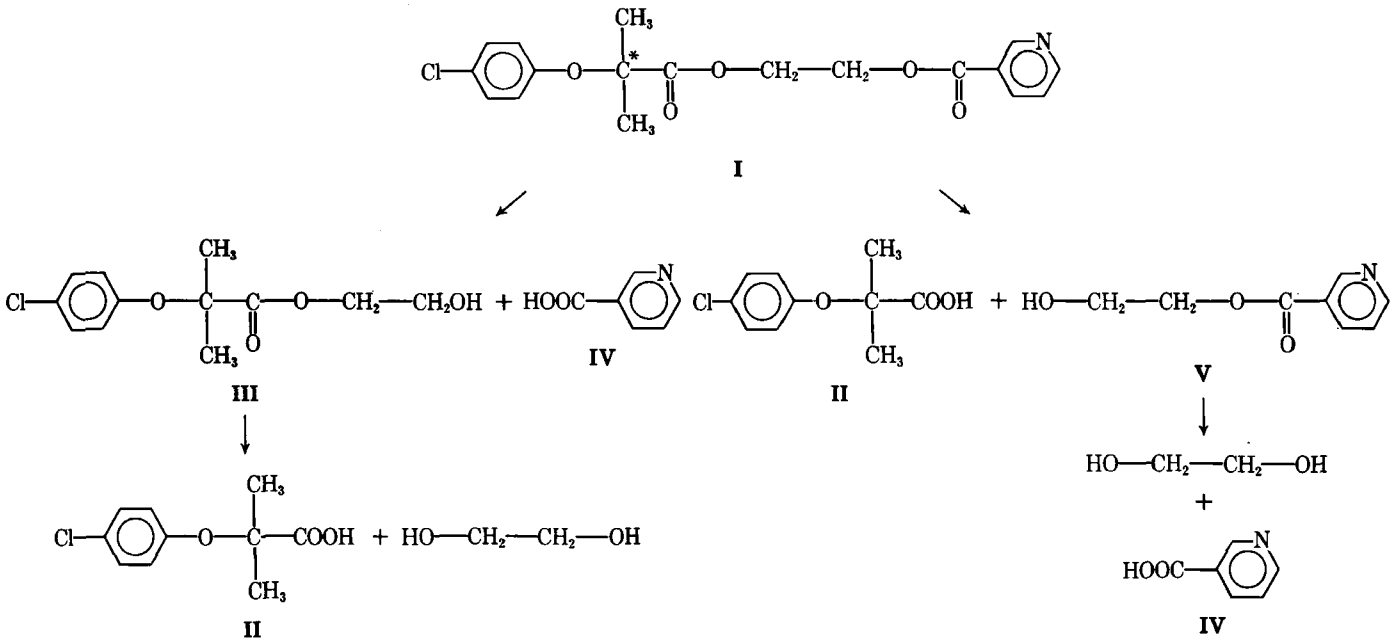
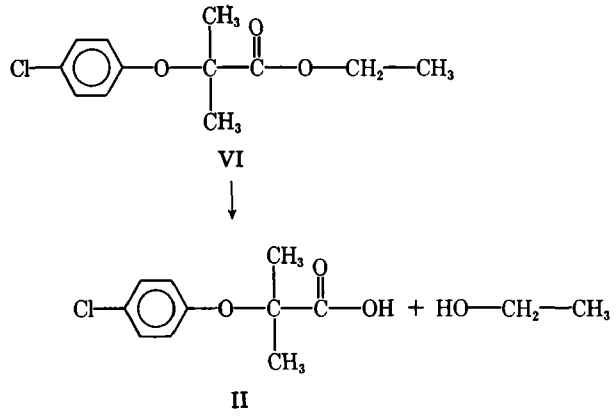


Scheme I—Pathways of etofibrate hydrolysis. (*) ^{14}C atom of radiolabeled etofibrate.



Scheme II—Pathway of clofibrate hydrolysis.

tetrabutylammonium phosphate[3], and volumetric concentrations of hydrochloric acid[4] were analytical grade. Chloroform[2], ethyl acetate[1], methanol[2], and acetonitrile[2] were HPLC or UV grade. The following also were used: sodium chloride injection USP[5], sodium heparin injection USP[6], and disposable syringes[7]. Tetraethyl pyrophosphate[8] was used as enzyme inhibitor. Thymol[2] and phenylurea[4] were internal standards for the HPLC systems. Etofibrate[9] (I), clofibric acid[9] (II), 2-hydroxyethyl 2-(4-chlorophenoxy)-2-methylpropionate[9] (III), nicotinic acid[9] (IV), 2-hydroxyethyl nicotinate[9] (V), and clofibrate[9] (VI) were used as received. For protein-binding studies, ultrafiltration cones[10], cone supports[11] and tubes[10], molecular filters[11], and a sample filtration assembly[11] were used.

Specifically ^{14}C-labeled etofibrate[9] (Scheme I) was used for plasma protein binding and red blood cell partition studies. A toluene-based scintillation[12] cocktail was used in the radioactive assay.

Apparatus—The HPLC system consisted of single pump[13], a radial compression module[14] equipped with a C_8 or C_{18} column[13] (8 mm i.d.), and an automatic injector[15]. The variable-wavelength UV detector[16] was set at 225 or 254 nm, with a strip chart recorder[17]. Radioactivity was assayed with a liquid scintillation counter[18]. A laboratory centrifuge was used[19].

HPLC Procedures—Three HPLC systems were used: System A for I, II, and III; System B for IV and V; and System C for VI.

System A—Prepared solutions of 0.05 *M* sodium acetate were adjusted to a measured pH 3.75 with 0.05 *M* acetic acid. The mobile phase was a mixture of this buffer and acetonitrile (50:50). The flow rate of 2 ml/min gave almost no back pressure (0–500 psi) on the C_8 column. The wavelength of the UV detector was 225 nm.

System B—The mobile phase was a mixture of an aqueous solution of 0.008 *M* tetrabutylammonium phosphate and methanol (25:75). The flow rate of 2 ml/min produced a back pressure of 400–700 psi with the C_{18} column. The wavelength of the UV detection was 254 nm.

System C—The mobile phase was a mixture of 0.05 *M* acetate buffer (pH 3.75) and acetonitrile (37:63). The flow rate of 2.2 ml/min gave a back-pressure of 1100 psi on the C_{18} column. The wavelength of the UV detector was 225 nm.

HPLC Analytical Methods in Plasma for I, II, and III—*Extraction Procedure (Method a)*—Aliquots (0.2 or 0.5 ml) of fresh, heparinized dog or human plasma were adjusted to pH 2.5 with 25 or 62.5 μl of 1 *N* HCl and extracted with 2 or 5 ml of chloroform for 10 min with slow shaking. Aliquots (1.6 or 4 ml) of the organic phase were evaporated to dryness under a nitrogen stream. The residue was reconstituted with mobile phase containing the internal standard (thymol), and 50 μl was injected into HPLC system A.

Acetonitrile Deproteinization (Method b)—An equal volume of acetonitrile containing the internal standard (thymol) was added to a plasma sample, vortexed at high speed for 30 sec, and centrifuged at 3000 rpm for 10 min. A 50-μl aliquot of the supernatant was injected into HPLC system A.

HPLC Analytical Methods in Plasma for IV and V—*Extraction Procedure (Method c)*—Fresh plasma (0.5 ml) was adjusted to pH 2 with 65 μl of 1 *N* HCl and extracted with 2 ml of an ethyl acetate–*tert*-butyl alcohol mixture (50:50) for 10 min with slow shaking. After centrifugation for 10 min at 3000 rpm, 1.8 ml of the organic phase was evaporated under a nitrogen stream. The residue was reconstituted with mobile phase containing phenylurea as an internal standard, and 50 μl was injected into HPLC system B.

Acetonitrile Deproteinization (Method d)—Acetonitrile (2 or 2.5 ml) was added to plasma (0.2 or 0.5 ml). The mixture was vortexed for 30 sec and centrifuged for 10 min at 3000 rpm. Aliquots of the clear supernatant

[3] Eastman Kodak Co., Rochester NY 14650.
[4] Ricca Chemical Co., Arlington, TX 76012.
[5] McGaw Laboratories, Irvine, CA 92714.
[6] The Upjohn Co., Kalamazoo, MI 49001.
[7] Monoject, Division of Sherwood Medical, A Brunswick Co., St. Louis, MO 63103.
[8] Chemical Procurement Laboratories, College Point, N.Y.
[9] Merz & Co., Frankfurt am Main, West Germany.
[10] Type 2100 CF-50; Amicon Corp., Lexington, MA 02173.
[11] Types PTGC and PSAC; Millipore Corp., Bedford, MA 01730.
[12] ScintiVerse; Fisher Scientific Co., Fair Lawn, NJ 07410.
[13] Model M 6000A; Waters Associates, Milford, MA 01751.
[14] Waters Associates, Milford, MA 01751.
[15] Model Wisp 710A; Waters Associates, Milford, MA 01751.
[16] LC 75 Spectrophotometer; Perkin-Elmer, Norwalk CT 06856.
[17] Fisher recordall series 5000; Fisher Scientific Co., Pittsburgh, PA 15219.
[18] Tricarb 460 CD; Packard Instrument Co. Inc., Downers Grove IL 60515.
[19] International Equipment Co., Needham Heights, MA 02194.

(1 or 2.5 ml) were evaporated to dryness under a nitrogen stream. The residue was reconstituted with mobile phase containing phenylurea as internal standard, and 50 μl was injected into HPLC system B.

HPLC Analytical Methods in Plasma for VI (Method e)—Appropriate volumes of acetonitrile containing the internal standard (etofibrate) were added to 0.2 or 0.5 ml of plasma. The mixture was vortexed and centrifuged at 3000 rpm for 10 min. Aliquots of the clear supernatant were injected into HPLC system C.

Purification of Radiolabeled Etofibrate—^{14}C-Labeled etofibrate (0.03 mg), of presumed specific activity 1.47×10^8 dpm/mg, was dissolved in 1 ml of acetonitrile. A 100-μl aliquot, mixed with 10 ml of scintillation fluid[12] was dark-adapted for 12 hr before liquid scintillation counting. The ^{14}C-labeled etofibrate in acetonitrile solution (100 μl) was injected into HPLC system A, monitored by the UV detector, and the eluant was collected in 30-sec intervals for 20 min. Scintillation fluid (10 ml) was added to each collection before counting. Approximately 20% of the counts of the injected sample lay outside the etofibrate fraction (retention time range: 8–13 min) identified by its UV peak. The ^{14}C-labeled etofibrate was purified by combining the collected etofibrate fractions obtained by repetitive injections of 100-μl aliquots into the chromatograph and extracting twice with chloroform. An aliquot (500 μl) of the aqueous phase was counted and showed no significant radioactivity (39 cpm), indicating complete extraction. The chloroform extract was evaporated under a nitrogen stream and the residue reconstituted in acetonitrile. A 50-μl aliquot of this purified ^{14}C-labeled etofibrate solution was injected into the chromatograph, and the 30-sec fractions of eluant (up to 20 min) were counted; 94.4% of the total injected radioactivity was in the etofibrate collection. The amount of etofibrate in the assayed sample was determined against a calibration curve prepared from unlabeled drug in the same system. Thus, the specific activity of the purified [^{14}C]etofibrate could be calculated (1.253×10^5 dpm/μg).

Plasmolysis Studies of I, III, V, and VI—Fresh human and dog plasma were used. The blood was centrifuged for 15 min at 3000 rpm immediately after blood withdrawal into a heparinized syringe. The plasma stability studies were performed in a water bath at 37.5° for human and 38.5° for dog plasma. The plasma was spiked with a known amount of III or V in phosphate buffer (pH 7.4) for the respective studies of their degradation. Since clofibrate (VI) had poor aqueous solubility, it was added to plasma in methanol solution (1–20 μl in 3 ml of plasma). Etofibrate in 0.05 *M* HCl was added to plasma. Aliquots (0.2 or 0.5 ml) of plasma were removed at intervals and added to tubes containing acetonitrile (methods b, d, and e) or extraction solvents (methods a and c) and immediately vortexed or shaken before assay.

The kinetics of plasmolysis were studied with and without the enzyme inhibitor tetraethyl pyrophosphate (usually 90–117 μg/ml of plasma or blood). The stability of etofibrate and clofibrate was also studied in fresh human whole blood inhibited by tetraethyl pyrophosphate. The solutions of tetraethyl pyrophosphate in phosphate buffer (pH 7.4) had to be prepared freshly before each study because of its instability. The plasma of spiked blood was assayed after separation from erythrocytes by centrifugation for 4 min at 5000 rpm.

Red Blood Cell–Buffer Partition Studies—Fresh human heparinized blood was centrifuged for 15 min at 3000 rpm and the plasma removed. Isoosmotic phosphate buffer (pH 7.4) was added to the packed red blood cells with gentle mixing. The slurry was centrifuged for 10 min at 2000 rpm, and the separated buffer was discarded; this procedure was repeated three times. A phosphate-buffered solution of tetraethyl pyrophosphate (11.7 mg/μl) was added to the packed red blood cells to give ~90 μg/ml of the inhibitor. Aliquots of this red blood cell suspension were added to previously assayed spiked phosphate buffer. The mixture was mixed carefully and allowed to equilibrate for 25 min. A hematocrit was taken, and the tube was centrifuged for 10 min at 2000 rpm. An aliquot (200 or 500 μl) of the supernatant buffer was removed for analysis. The red blood cell–phosphate buffer partition coefficients were determined as a function of drug concentration and time.

Binding to Ultrafiltration Membranes—The binding of I, VI, and their degradation products to three commonly used ultrafiltration membranes was studied[10,11]. Solutions of isoosmotic phosphate buffer (pH 7.4) were prepared with 10 μg/ml of I, VI, and their degradation products, separately and in mixture. An aliquot was taken for analysis, and 5 ml was filtered through the ultrafiltration assembly in 500-μl amounts. Aliquots (200 μl) of each sequential filtration were analyzed for drug.

Plasma Protein Binding—*Compounds II–V by the Ultrafiltration Method*—Appropriate volumes of stock solutions of II–V in acetonitrile were evaporated under a nitrogen stream to dryness. A phosphate buffer solution of tetraethyl pyrophosphate was added to 22 ml of fresh human heparinized plasma to give 95.7 μg/ml, and 8 ml of plasma was used to redissolve the compounds. Additional plasma was added to give five different drug concentrations, and the plasma samples were assayed. These plasma samples (4 ml) were filtered through pretreated ultrafiltration cones for 3 min at 3000 rpm. The first 0.8 ml of ultrafiltrate was discarded, and the next 0.5 ml was assayed. The filtration cones were pretreated by filtering 2 ml of phosphate buffer containing concentrations of drugs similar to those of the subsequently filtered plasma, *i.e.*, 5, 10, 20, 50, and 100 μg/ml with subsequent filtering of 4.0 ml of plain buffer on the premise that this latter filtration would remove the nonirreversibly bound material that would introduce error into the binding study.

Etofibrate (I) and Clofibrate (VI) by the Method of Variable Plasma Concentrations—Fresh heparinized human blood was centrifuged for 15 min at 3000 rpm. The plasma was transferred to a stoppered tube. The red blood cells were washed three times with isoosmotic phosphate buffer (pH 7.4), and the buffer phases were discarded.

The desired mixtures of acetonitrile and ^{14}C-labeled and unlabeled etofibrate in acetonitrile in a centrifuge tube were dried under a nitrogen stream. The residues were reconstituted in 40 μl of isotonic ethanol–buffer (50:50). Appropriate amounts of plasma (inhibited by 100 μg/ml of tetraethyl pyrophosphate) and isotonic phosphate buffer (pH 7.4) were mixed to give from 0 to 100% of etofibrate in each pseudoplasma. The pseudoplasma samples (1.0 ml of each) were added to the amounts of dried I needed to give concentrations of 0.2, 2.0, 8.0, 16.0, 40.0, and 80.0 μg/ml. An aliquot (200 μl) was mixed with 10 ml of scintillation fluid and counted. An aliquot (0.5 ml) of a red blood cell suspension containing 100 μg/ml of tetraethyl pyrophosphate was added to each pseudoplasma and gently mixed. Hematocrits were taken and the pseudoblood was allowed to equilibrate for 20 min. The pseudoblood samples were centrifuged for 10 min at 3000 rpm, and 200 μl of the pseudoplasma, mixed with 10 ml of a scintillation fluid, were counted by liquid scintillation.

The clofibrate protein binding was studied similarly with 20 μl of a methanolic solution containing 15, 30, 60, and 120 μg of VI added to separate 1.0-ml pseudoplasma samples before the addition of the red blood cells. The samples were analyzed by HPLC method e using mobile phase C.

RESULTS AND DISCUSSION

Reverse-Phase HPLC Assays—*Compounds I, II, and III*—HPLC system A with analytical method a using chloroform extraction, evaporation, and reconstitution provided a chromatogram (Fig. 1A) for analyses of I (retention time, 10.8 min), III (4.7 min), and II (3.4 min) in mixtures. The internal standard, thymol, had a retention time of 7.3 min. The average of the standard errors of estimate (65–185 ng/ml, $n = 8$), of the concentration of I was 99 ng/ml of plasma for the 0.4- to 20-μg/ml assay range. The average of standard errors of estimate for III (46–126 ng/ml, $n = 5$) was 102 ng/ml of plasma for the 0.4- to 10-μg/ml assay range. The average of standard errors of estimate for II (27–121 ng/ml, $n = 5$) was 72 ng/ml of plasma for the 0.4- to 10-μg/ml assay range. This HPLC system had advantages over those reported previously (1) in that mixtures of I, II, and III in plasma could be assayed simultaneously.

Compounds IV and V—The ion-pair methodology of HPLC system B with analytical method c using ethyl acetate–*tert*-butyl alcohol extraction, evaporation, and reconstitution provided chromatographic separation of V (retention time, 6.3 min) from IV (10 min) and from plasma interferences. The internal standard, phenylurea, had a retention time of 7.5 min (Fig. 1B). The average of standard errors of estimate for V (40–92 ng/ml, $n = 5$) was 67 ng/ml of plasma for the 0.4- to 10-μg/ml assay range, and for IV (49–172 ng/ml, $n = 5$) the average was 102 ng/ml of plasma for the same assay range. Specific assays for IV and V from plasma were not given previously (7), since plasma components interfered with their detection in the prior evaluated HPLC procedures.

Compound VI—HPLC system C with plasma deproteinization by analytical method e provided 225-nm UV detection of VI with a retention time of 6.1 min; the internal standard, I.S., had a retention time of 4.5 min (Fig. 1C). The standard error of estimate of VI in the 0.4- to 4-μg/ml plasma range was 51 ng/ml.

Plasmolysis of Etofibrate and Its Intermediate Solvolytic Products in Fresh Dog Plasma—Preliminary studies of the stability of etofibrate in fresh dog plasma (7) indicated apparent half-lives of 4.4–6.9 min at 37.5°: 4.4 min at an initial concentration of 3.6×10^{-5} *M*, 6.9 min at 5.9×10^{-5} *M*, and 5.7 min at 1.19×10^{-4} *M*. A repeat study in fresh dog plasma at 5.4×10^{-5} *M* (19.5 μg/ml) and 38.5° using the aforementioned HPLC assays showed a half-life of 10.6 min (Fig. 2A).

Another study (Fig. 2B) in fresh dog plasma at 3.6×10^{-5} *M* (13.3 μg/ml) showed a biphasic degradation of etofibrate at 38.5° that could

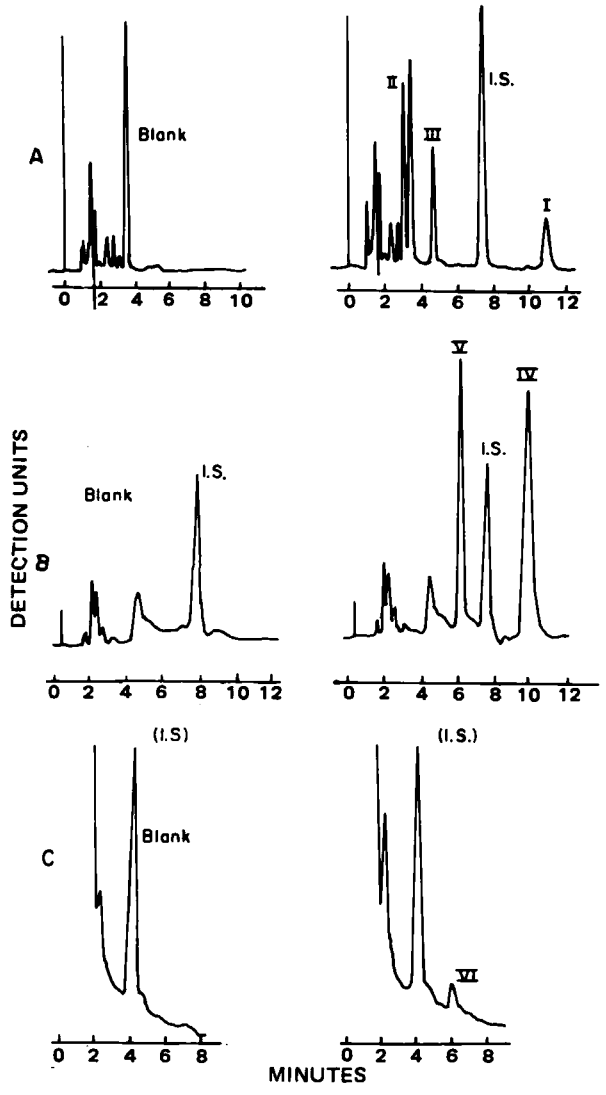


Figure 1—*Reverse-phase chromatograms of clofibric acid esters and their solvolytic products. Key: (A) Detection at 225 nm of plasma blank and plasma containing 2 μg/ml each of I, II, and III, extracted with chloroform (method a, system A). The injection volume was 50 μl; the internal standard was thymol (I.S.). (B) Detection at 254 nm of plasma blank and plasma containing 4.1 μg/ml of IV and V (method d, system B). The internal standard was phenylurea (I.S.); the injection volume was 50 μl. (C) Detection at 225 nm of plasma blank containing phenylurea as internal standard and plasma containing 0.8 μg/ml of VI (method c, system C) with 50 μl injected.*

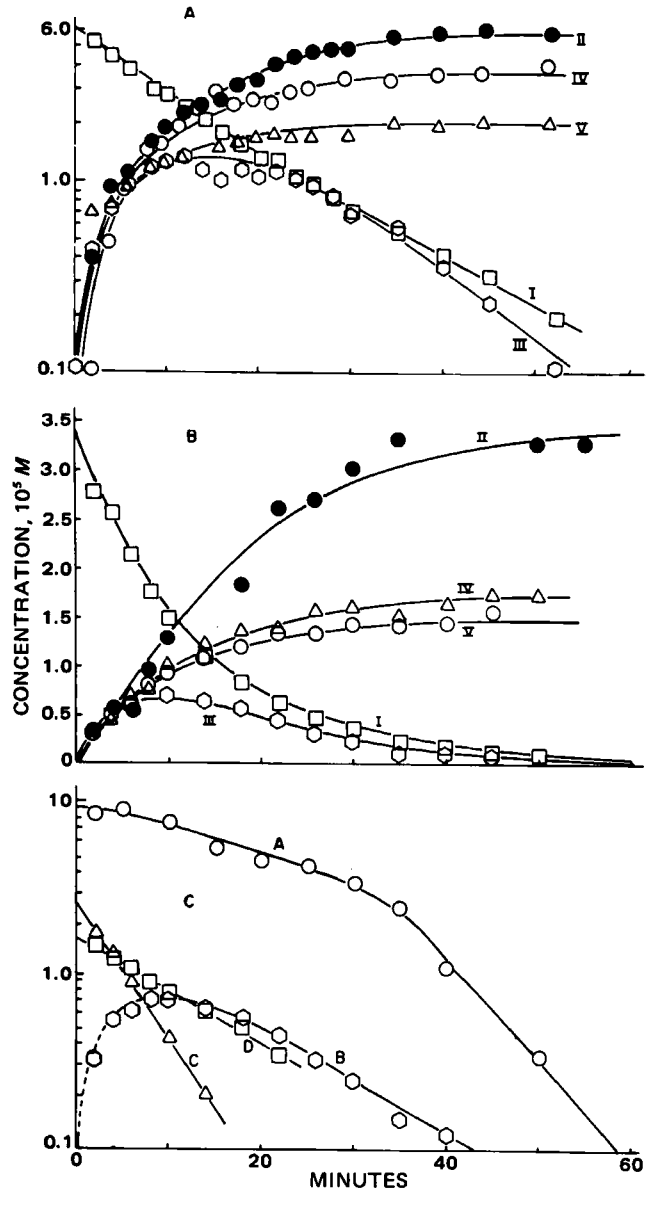


Figure 2—*(A) Semilogarithmic plots against time for 5.4 × 10^{-5} M (19.5 μg/ml) etofibrate (I) and its solvolytic products, II, III, IV, and V, in dog plasma at 38.5°. The apparent half-lives of I and III were 10.6 and 9.9 min, respectively. (B) Cartesian plots against time for 3.6 × 10^{-5} M I and its degradation products in dog plasma at 38.5°. The lines represent the predicted concentrations according to Eqs. 1 (I), 4 (V), 5 (III), 6 (IV), and 7 (II). The values used for the parameters were: f = 0.55, k_3 = 0.16616 min^{-1}, k_4 = 0.00096 min^{-1}, k = 0.07558 min^{-1}, and $[I]_0$ = 3.3 × 10^{-6} M. (C) Semilogarithmic plot against time of the III data resulting from I solvolysis in dog plasma (Fig. 2B). Curve B was calculated according to Eq. 5 with f = 0.55, k_3 = 0.166 min^{-1}, k = 0.0756 min^{-1}, $[I]_0$ = 3.3 × 10^{-6} M. Curve A shows a terminal half-life of 5.6 min for 8.08 × 10^{-5} M III in the same plasma. The appearing rate constant for III was calculated from the slope of the feathered line (curve C); the half-life of this process is 4.2 min. The appearing half-life of IV (12 min) was calculated from the slope of the sigma minus plot (curve D) of log ([IV]60 − [IV]) where $[IV]_{60}$ was the concentration of IV at 60 min.*

be fitted to $10^5 p = 14.5\, e^{-0.1438t} + 20.5\, e^{-0.058t}$ where p is in moles/liter and t is in min. The terminal half-life was 11.9 min; the apparent half-life of the first phase was 4.8 min, in the range of the half-life estimated previously (7) using assays of lower sensitivity.

The ester III previously showed apparent terminal half-lives of 11.2 min (initial etofibrate concentration of 5.9 × 10^{-5} *M*) and 5.8 min (initial etofibrate concentration of 1.19 × 10^{-4} *M*). Ester III, spiked at 4.23 × 10^{-5} *M* in fresh dog plasma had an apparent half-life of 11.5 min in the preliminary studies at 37.5° (7) and, in a present study at 8.08 × 10^{-5} *M*, of 5.6 min at 38.5° (curve A, Fig. 2C). This latter study used the greater analytical sensitivities given in this paper; the terminal phase was observed below 2.5 × 10^{-5} *M*, whereas in the preliminary study (7) the half-life was estimated from concentrations down to this value. The plasmolysis of III appears to be a saturable process (Fig. 2C). The terminal phase of the generated III in these present studies with 5.4 × 10^{-5} *M* (Fig. 2A) and 3.6 × 10^{-5} *M* (Fig. 2B and C) etofibrate in fresh dog plasma at 38.5° showed respective half-lives of 9.9, and 9.6 min. These values were well in excess of the aqueous solvolyses of these compounds, which were ~6 and 12 days at pH 7.4 and 40° for I and III, respectively.

The terminal half-lives (9.6–9.9 min) of III generated from etofibrate exceeded the terminal half-life of 5.6 min for spiked 8.08 × 10^{-5} *M* III in fresh dog plasma (Fig. 2C) and were close to the 10.6–11.9-min terminal half-lives for etofibrate. When the curve for III generated from etofibrate degradation was feathered, the resulting half-life for the appearance of III was determined as 4.2 min (curve C, Fig. 2C), close to the half-life for the loss of spiked III in plasma, indicating a probable "flip-flop."

The intermediary nicotinate (V), was more stable in fresh dog plasma than I and II (compare Figs. 2 and 3). The apparent half-life was 12.0 hr

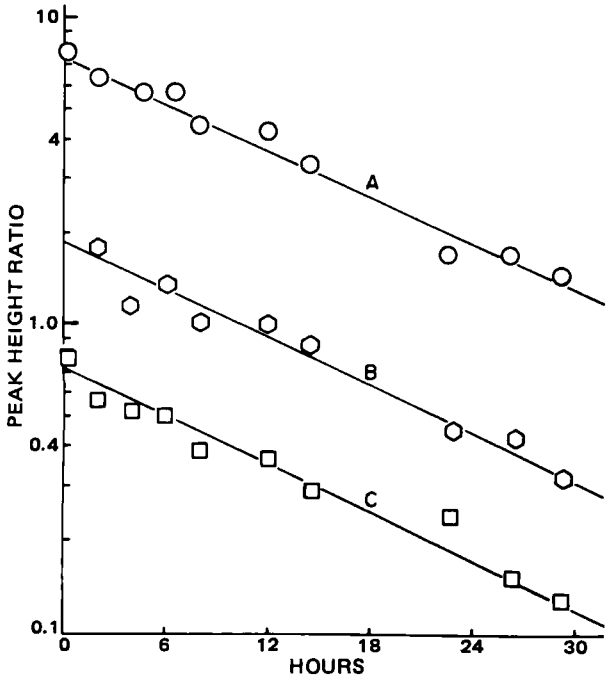


Figure 3—*Semilogarithmic plots of the peak height ratios (HPLC) of V with respect to phenylurea (internal standard) against time for the degradation in fresh dog plasma at 38.5°. Key: (A)* $C_0 = 99\ \mu g/ml$, $t_{1/2} = 12.1\ hr$; *(B)* $C_0 = 49.5\ \mu g/ml$, $t_{1/2} = 12.0\ hr$; *(C)* $C_0 = 12.4\ \mu g/ml$, $t_{1/2} = 11.9\ hr$.

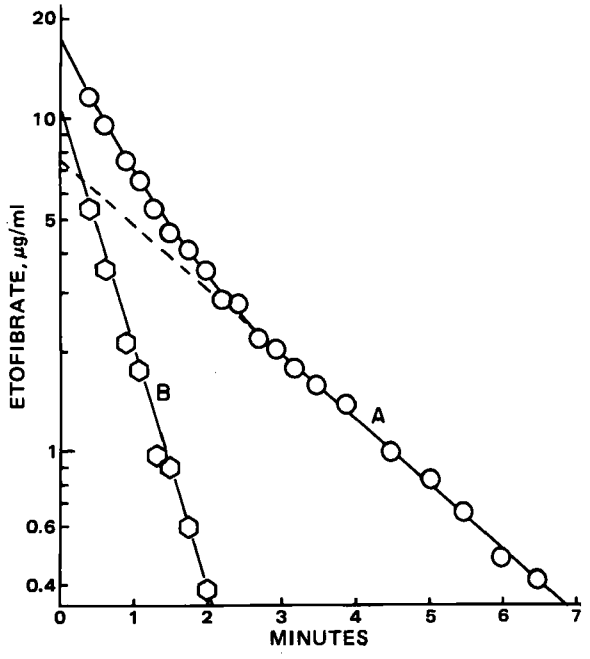


Figure 4—*Semilogarithmic plot (A) of the degradation of 18.09 μg/ml of I in heparinized human plasma at 37.5° which conforms to* C *(μg/ml)* $= 10.5\ e^{-1.59t} + 7.5\ e^{-0.444t}$ *where* t *is in minutes, and feathered curve (B) to characterize the first exponential.*

at 38.5° and was independent of the initial concentration (between 12.4 and 99 μg/ml).

The "flip-flop" phenomenon should not be, and was not, evident here, since the rate of generation of the nicotinate V is very much faster than its degradation. The slope of the semilogarithmic plot of amount of nicotinic acid not-yet-formed against time (sigma minus plot) gave an apparent half-life of ~12 min, equivalent to that for the disappearance of etofibrate and the assigned half-life for the appearance of ester III (estimated from the feathered curve of III against time).

To fit the solvolysis of etofibrate in plasma according to the model given in Scheme III, the concentration–time data from the plasmolysis of I could be reasonably approximated by a monoexponential function of the form:

$$[I] = [I]_0 \cdot e^{-kt} \qquad \text{(Eq. 1)}$$

If the products of etofibrate solvolysis were only V and II, and if no III were generated, the concentration of V could be calculated from the expression (9):

$$[V'] = [I]_0 \frac{k}{k_4 - k}\left[e^{-kt} - e^{-k_4 t}\right] \qquad \text{(Eq. 2)}$$

where $[I]_0$ is the initial concentration, [V'] is the nicotinic acid monoglycolate concentration on the presumption that I is only degrading through the V route, k is the first-order elimination rate constant for I degradation, and k_4 is the first-order rate constant for the plasmolysis of V. The ratio of experimentally observed [V] to calculated [V'] is:

$$f = [V]/[V'] \qquad \text{(Eq. 3)}$$

which was 0.55 for the studies given in Fig. 2B and C, and f is the fraction of I going through the V route. Thus the fraction of I going through the III route is $1 - f = 0.45$.

If the degradation of III can be approximated by a first-order rate constant k_3, the following set of equations describes the time course of all possible solvolysis products as a function of the initial etofibrate concentration $[I]_0$:

$$[V] = [I]_0 \cdot \frac{fk}{k_4 - k}\left[e^{-kt} - e^{-k_4 t}\right] \qquad \text{(Eq. 4)}$$

$$[III] = [I]_0 \cdot \frac{(1-f)k_3}{k - k_3}\left[e^{-k_3 t} - e^{-kt}\right] \qquad \text{(Eq. 5)}$$

$$[IV] = [I]_0 \cdot (1-f)(1 - e^{-kt}) + [I]_0 f\left[1 - \frac{1}{k - k_4}\left(ke^{-k_4 t} - k_4 e^{-kt}\right)\right] \qquad \text{(Eq. 6)}$$

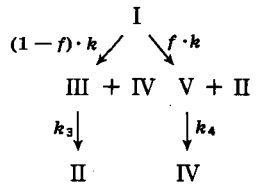
$$[II] = [I]_0 \cdot f(1 - e^{-kt}) + [I]_0 \cdot (1-f) \times \left[1 - \frac{1}{k_3 - k}\left(k_3 e^{-kt} - ke^{-k_3 t}\right)\right] \qquad \text{(Eq. 7)}$$

The lines through the experimental values in Fig. 2B and C were drawn on the basis of concentrations calculated from Eqs. 4–7 using the parameters given in the legend of the figures. The good agreement of the experimental data and calculated curves supports Scheme III and confirms the "flip-flop" phenomenon for the III curve.

Etofibrate Solvolysis in Human Plasma—A typical curve for the degradation of etofibrate in fresh heparinized human plasma is shown in Fig. 4 for 18.8 μg/ml and showed even more pronounced biphasic character than was observed in fresh dog plasma. The terminal half-life was 1.54 min at 37.5°; the half-life of the earlier phase, obtained by feathering, was 0.44 min. The nonlinearity of such semilogarithmic plots occurred with various other initial concentrations (8, 16, and 40 μg/ml) with similar terminal half-lives of 1.7 min at 38.5°. This indicated no dose dependency. An additional study at 18.7 μg of etofibrate/ml at 13° showed a biphasic curve also, with a terminal half-life of 7.7 min. The initial phase had a half-life of 0.4 min, and $10^5 p = 1.31\ e^{-1.67t} + 3.71\ e^{-0.096t}$ where p is in moles/liter and t is in min.

Since a possible explanation of this unexpected biphasic phenomenon could be the inhibition of an enzymatic process by formed solvolytic products, the solvolyses of 21.5 μg/ml of etofibrate were repeated in the same fresh human plasma in the absence and presence of 23.8 μg/ml of II, III, IV, or V added 20 sec before the addition of etofibrate. There were no significant differences in the etofibrate degradations, with terminal half-lives of 1.7 min in the same plasma with or without any of these added compounds. Thus, product inhibition or slowing of an enzymic process could not be concluded.

An alternative explanation could be the decay of enzymic activity with time or its irreversible inactivation by the substrate. If these were true, reinoculation of plasma with fresh substrate would give decreasing rates



Scheme III—Model of etofibrate plasmolysis.

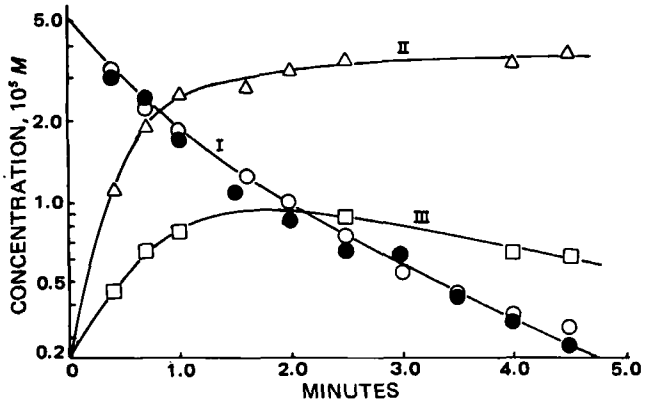


Figure 5—*Semilogarithmic plots of concentrations of I, II, and III with time. Ten minutes after the degradation of 19.77 μg/ml of I in the fresh human plasma at 37.5° (○), 20.17 μg/ml of I (●) was added and its time course monitored.*

or possibly eliminate the biphasic nature. Ten minutes after 19.77 μg/ml of etofibrate was added to fresh human plasma and subsequently degraded at 37.5°, 20.17 μg/ml of etofibrate was again added and its time course monitored (Fig. 5). There was no significant difference between the time courses of the sequential studies of etofibrate degradation in the same human plasma. Both showed terminal half-lives of 1.5 min in this plasma.

Etofibrate showed significantly shorter terminal half-lives of degradation (1.5–1.7 min) in fresh human plasma than in fresh dog plasma (5–10 min). In the several human plasma studies, 50 ± 5% of the degrading etofibrate produced the more stable nicotinate V and the remainder went through the route involving ester III.

2-Hydroxyethyl Nicotinate (V) Stability in Human Plasma—The ester, V, showed apparent first-order degradation in fresh human plasma with half-lives of 45–55 min, which were not significantly affected by concentration in the 2- to 21-μg/ml range (Fig. 6). These half-lives were more shorter than the 12-hr half-life observed in dog plasma (Fig. 3).

2-Hydroxyethyl 2-(4-chlorophenoxy)-2-methylpropionate (III) Stability in Human Plasma—The ester, III, did not degrade by first-order processes at all concentrations in fresh human plasma. Semilogarithmic plots of concentrations with time were not linear; they showed the decreasing convex curve typical of saturable processes (10).

Such saturable processes can be described by:

$$-dC/dt = \frac{\upsilon_{max} C}{K_m + C} \qquad \text{(Eq. 8)}$$

where preliminary estimates of υ_{max} can be made from the initial slope at high concentrations of linear plots of concentration against time (Fig. 7) and the υ_{max} and K_m values can be estimated from the terminal slopes

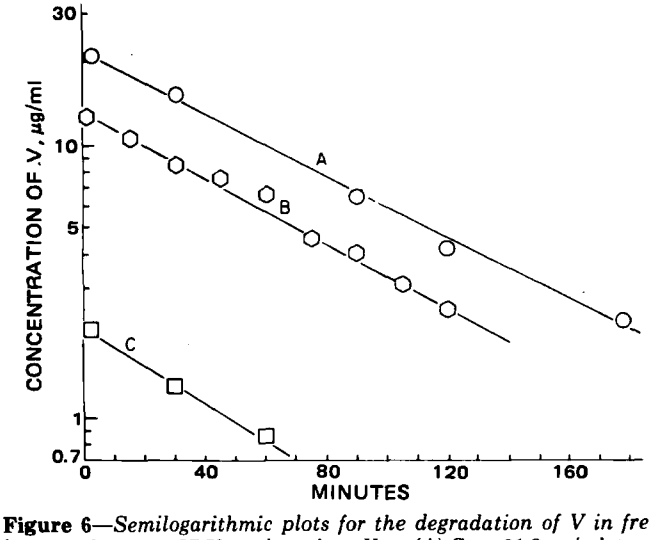


Figure 6—*Semilogarithmic plots for the degradation of V in fresh human plasma at 37.5° against time. Key: (A) C_0 = 21.3 μg/ml, $t_{1/2}$ = 54.5 min; (B) C_0 = 13.4 μg/ml, $t_{1/2}$ = 50.9 min; (C) C_0 = 2.1 μg/ml, $t_{1/2}$ = 45.4 min.*

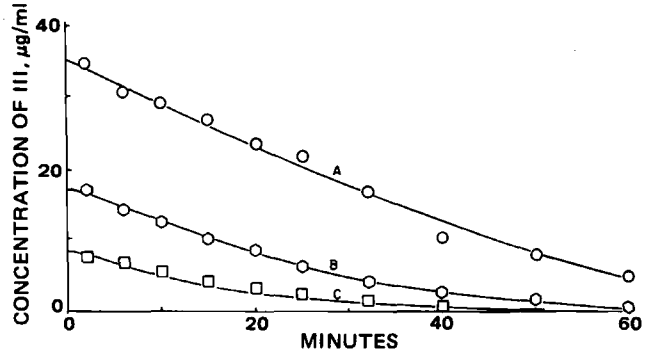


Figure 7—*Plots of concentrations of III with time in fresh human plasma at 37.5° fitted to the Michaelis–Menten equation $-dC/dt = v_{max}C/(K_m + C)$ with v_{max} = 0.77 μg/ml/min and K_m = 8 μg/ml. The initial concentrations of III were 35.2 (A), 17.5 (B), and 8.5 μg/ml (C).*

of ln C against time. The times for various concentrations (C) can be calculated from the integrated form of the Michaelis–Menten equation (Eq. 8):

$$-(K_m \ln C/C_0 + C - C_0)/\upsilon_{max} = t \qquad \text{(Eq. 9)}$$

where C_0 is the initial concentration and C the concentration at any time, t. All of the experimentally observed time courses for 8.5-, 17.5-, and 35.2-μg/ml initial concentrations of III in human plasma could be fitted by Eq. 9 with K_m = 8 μg/ml and υ_{max} = 0.77 μg/ml/min (Fig. 7) to demonstrate the validity of this equation and the postulation of saturable enzymic solvolysis for these initial concentrations. The apparent terminal half-life of III can be calculated from $0.693/(\upsilon_{max}/K_m)$ = 0.693/(0.77/8) = 7.2 min and was close to the half-life for the solvolysis of III in fresh dog plasma.

Clofibrate (VI) Stability in Human Plasma—Clofibrate demonstrated solvolysis in human plasma by a similar saturable enzymic process (Fig. 8), which agreed with the previously indicated saturable kinetics in dog plasma (7). The values of the parameters of Eqs. 8 and 9, υ_{max} = 0.77 μg/ml/min, K_m = 28 μg/ml, $t_{1/2} = 0.693K_m/\upsilon_{max}$ = 25.2 min, fitted the data for plasma level–time studies for initial clofibrate concentrations in human plasma of 125, 55.5, 12, and 6.1 μg/ml (Fig. 8). The identical υ_{max} of 0.77 μg/ml/min for clofibrate and ester III with the different K_m values could indicate solvolyses of these compounds by the same enzyme system, but with different affinities.

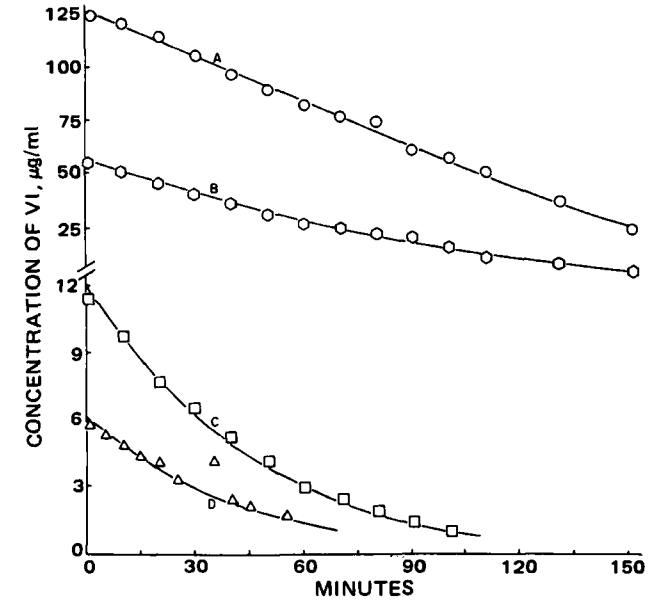


Figure 8—*Plots of VI concentrations with time in fresh human plasma at 37.5° fitted to the Michaelis–Menten equation $-dC/dt = v_{max}C/(K_m + C)$ with v_{max} = 0.77 μg/ml/min and K_m = 28 μg/ml. The initial concentrations were 125 (A), 55.5 (B), 12 (C), and 6.1 μg/ml (D). The apparent half-life of the apparent first-order terminal phase of curve C was 25.9 min.*

Table I—Red Blood Cell–Buffer Partitioning of Etofibrate (I), Clofibric Acid (II), 2-Hydroxyethyl 2-(4-Chlorophenoxy)-2-methylpropionate (III), Nicotinic Acid (IV), and 2-Hydroxyethyl Nicotinate (V)

Compound	Assay Procedures[a]	Tetraethyl Pyrophosphate Concentration, μg/ml[b]	Drug Concentration Range, μg/ml[c]	n	D (Average ± SD)
I	LSC[e]	89.5	0.2–80	6	5.46 ± 0.30
I[d]	Aa	117	1.15–1.265	5	6.03 ± 0.36
III	Aa	117	1.25–22.86	5	3.23 ± 0.22
III[d]	Aa	117	1.36–23.30	5	3.06 ± 0.34
II	Aa	117	1.57–35.38	4	1.98 ± 0.19
II	Aa	0	1.55 ± 35.22	5	2.21 ± 0.23
II[d]	Aa	117	1.56–29.98	5	2.30 ± 0.20
IV	Bd	0	2.10–50.48	5	0.97 ± 0.21
IV	Bd	117	1.66–47.34	5	1.25 ± 0.44
IV[d]	Bd	117	2.24–49.09	5	1.00 ± 0.13
V	Bd	117	2.53–35.05	4	1.09 ± 0.41
V[d]	Bd	117	1.39–35.13	5	1.07 ± 0.29

[a] Capital letter designates the HPLC system; small letter designates the method of preparation for analysis as given in *Experimental*. [b] Concentration of tetraethyl pyrophosphate in the RBC suspension. [c] Drug concentration in buffer after equilibration with red blood cells. [d] Studies effected by simultaneous spiking with I, II, III, IV, and V. [e] Radiolabeled etofibrate was studied and assayed by liquid scintillation counting (LSC).

Inhibition of Clofibric Acid Ester Plasmolysis—The plasmolysis of heroin had been inhibited by the addition of 100 μg/ml of tetraethyl pyrophosphate, a compound that did not hemolyze red blood cells when added to blood nor denature proteins (11). This enzyme inhibitor was also used to inhibit the hydrolysis of clofibric acid esters in human blood and plasma (Fig. 9). The plasmolyses of etofibrate (I), (curves A and B), ester III, (curve C), and clofibrate (VI) (curves D and E) were suppressed for at least 1 hr by adding tetraethyl pyrophosphate to blood or plasma, in contrast with the ready solvolyses of these compounds in uninhibited human plasma (I, curve H; III, curve F; VI, curve G). The use of tetraethyl pyrophosphate as a plasmolysis inhibitor thus provided a method to ascertain the red blood cell partitioning and plasma protein binding of these highly unstable clofibric acid esters in human plasma.

Tetraethyl pyrophosphate showed different patterns in dog and human plasma. Tetraethyl pyrophosphate (137.5 μg/ml) in fresh dog plasma suppressed the solvolysis of I for only 10–12 min (Fig. 10, curves A, B, and E), in contrast with the inhibition in fresh human plasma for at least 130 min (Fig. 10, curve D). Increased (7.5×) tetraethyl pyrophosphate concentrations (963.3 μg/ml) did not even double the time of complete plasmolysis inhibition (Fig. 10, curve C). The solvolysis after 22 min increased to an apparent half-life of 58.2 min, similar to the half-life observed with 128.9 μg/ml of tetraethyl pyrophosphate.

Red Blood Cell–Buffer Partitioning—*Etofibrate and its Solvolytic Products*—The red blood cell–plasma partition coefficient of a drug (*D*), on the presumption that only unbound drug in the plasma can diffuse into the red blood cells, can be defined (12) as:

$$D = [A_{RBC}]/[A_p^u] = \{[A_B]/[A_p^u](1 - H) - [A_p^b]/[A_p^u] - 1\}(1 - H)/H \quad \text{(Eq. 10)}$$

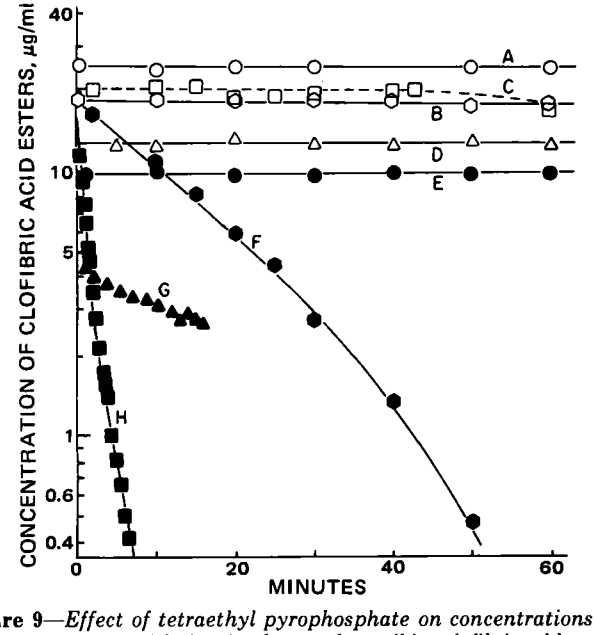


Figure 9—*Effect of tetraethyl pyrophosphate on concentrations of clofibric acid esters with time in plasma after spiking clofibric acid esters in fresh human blood or plasma. Key: (A) fresh human whole blood containing 102.4 μg/ml of tetraethyl pyrophosphate spiked with 18.24 μg/ml of I; (B) fresh human plasma containing 102.4 μg/ml of tetraethyl pyrophosphate spiked with 18.24 μg/ml of I; (C) fresh human plasma containing 47.2 μg/ml of tetraethyl pyrophosphate spiked with 19.84 μg/ml of III; (D) fresh human whole blood containing 99.5 μg/ml of tetraethyl pyrophosphate spiked with 12.65 μg/ml of VI; (E) fresh human plasma containing 101.4 μg/ml tetraethyl pyrophosphate spiked with 9.9 μg/ml of VI; (F) III degradation (C_0 = 16.5 μg/ml) in uninhibited human plasma; (G) VI degradation (C_0 = 4.5 μg/ml) in uninhibited human plasma; (H) I degradation (C_0 = 18.24 μg/ml) in uninhibited human plasma.*

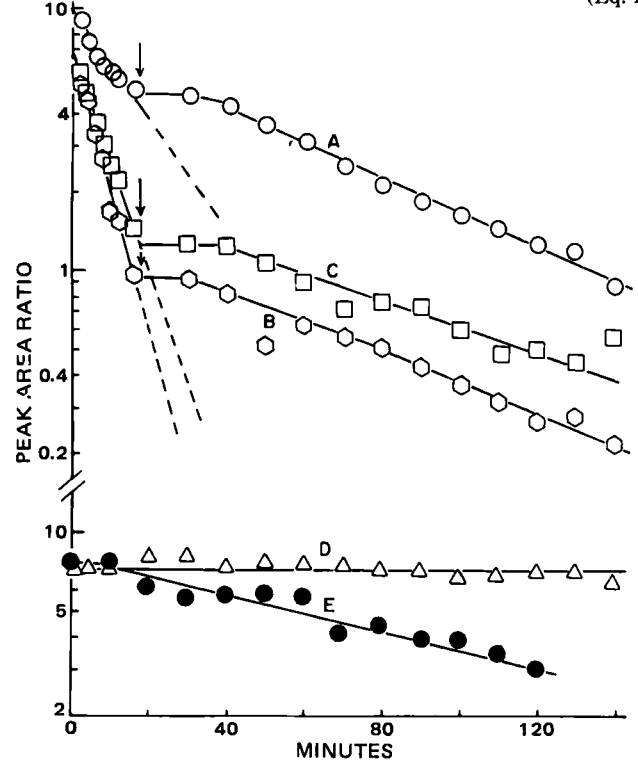


Figure 10—*Effect of tetraethyl pyrophosphate on the solvolysis of I. Key: (A) One-day-old dog plasma spiked with 30.3 μg/ml of I at room temperatuare. After 18 min (arrow) 128.9 μg/ml of tetraethyl pyrophosphate was added. (B) Fresh dog plasma spiked with 20.2 μg/ml of I at 38.5°. After 18 min (arrow) 128.9 μg/ml of tetraethyl pyrophosphate was added, and the test tube was kept at room temperature. (C) Identical to B, except 963.3 μg/ml of tetraethyl pyrophosphate was added. (D) Fresh human plasma containing 137.5 μg/ml of tetraethyl pyrophosphate spiked with 20 μg/ml of I. (E) Identical to D, except dog plasma was used.*

Table II—Human Plasma Protein Binding of Clofibric Acid (II), the 2-Hydroxyethyl Ester (III), Nicotinic Acid (IV), and the 2-Hydroxyethyl Ester[a] (V) by the Ultrafiltration Method

Compound	C_p, μg/ml[b]	C_{pw}, μg/ml[c]	Percent Bound	Average Percent Bound ± *SD* (*SEM*)
II	19.71	0.30	98.48	
	45.18	1.38	96.95	97.20 ± 1.18 (0.68)
	101.97	3.92	96.16	
III	4.96	0.91	81.65	
	10.24	1.59	84.47	
	20.21	2.97	85.30	83.17 ± 1.65 (0.74)
	48.28	8.33	82.75	
	105.16	19.25	81.69	
V	4.66	3.98	14.59	
	9.20	9.08	1.30	
	18.86	17.55	6.95	6.0 ± 6.3 (3.2)
	47.29	46.75	1.14	
IV	6.42	5.93	7.63	
	13.57	11.13	17.98	
	30.84	26.72	13.36	12.6 ± 4.3 (2.2)
	79.32	70.30	11.37	

[a] Tetraethyl pyrophosphate (95.7 μg/ml) was added to plasma before spiking with drug. [b] Drug concentration in plasma before filtration. [c] Drug concentration in ultrafiltrate (plasma water).

where $[A_{RBC}]$ is the total drug concentration in the red blood cells; $[A_p]$, $[A_p^u]$, and $[A_p^b]$ are the respective drug concentrations in plasma, plasma water (*i.e.*, unbound to plasma proteins), and bound to plasma proteins; $[A_B]$ is the total concentration of a drug in whole blood or red blood cell suspension; and H is the hematocrit. The value of $[A_p^b]$ is zero in red blood cell–buffer suspensions.

Etofibrate, clofibrate, and its solvolytic products showed rapid equilibration between buffer and human red blood cells. The apparent partition coefficients, D, did not change with time after the 3 min needed for mixing and subsequent centrifugation. There was no significant concentration dependency of the partition coefficient in the studied concentration ranges (Table I). There was no significant difference between the partition coefficients for the nonsolvolyzable clofibric and nicotinic acids with or without the presence of the enzyme inhibitor tetraethyl pyrophosphate, indicating the noninterference of this compound with the partitioning. The partition coefficients for etofibrate, clofibric acid, and ester III were much greater than unity (Table I), demonstrating specific binding to erythrocytes, greatly in excess of that which would be anticipated from simple volume partitioning.

Clofibrate—The red blood cell–buffer partition coefficient, D, for clofibrate was concentration dependent. It decreased with increasing clofibrate concentration, indicating saturable partitioning into the red blood cells, from 7.8 at 10 μg/ml to 1 at 50 μg/ml.

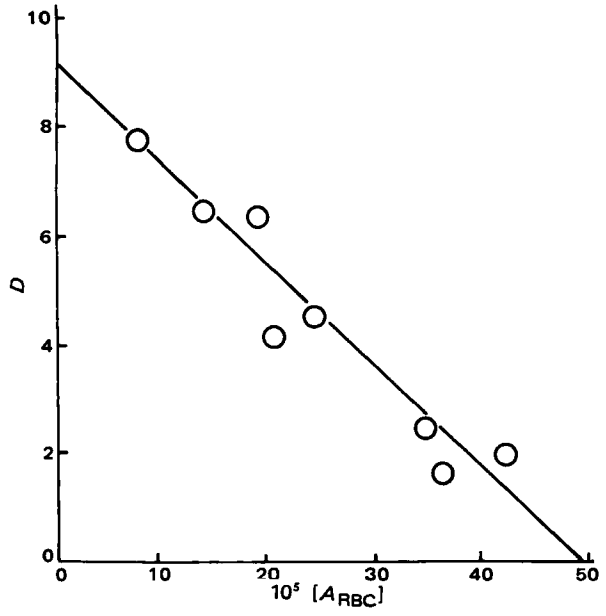


Figure 1—*Modified Scatchard plot for the presumed saturable binding or partitioning (D) of clofibrate to red blood cells from the buffer suspension against the clofibrate concentration in the buffer removed by binding to the red blood cells, [A_{RBC}]. The fitted line was calculated by least-square regression:* $D = 9.185 - 1.92 \times 10^4 [A_{RBC}]$.

The interaction between a ligand and a class of receptors can be formulated as (12):

$$n[A_p^u] + [R] \overset{K}{\rightleftharpoons} [A_nR] \qquad \text{(Eq. 11)}$$

where $[A_p^u]$ is the concentration of free or unbound drug in the buffer phase of the suspension, $[A_{RBC}] = [A_p] - [A_p^u]$ is the concentration of bound ligand to the receptor (erythrocyte) where $[A_p]$ would be the concentration of drug in the buffer phase if there were no binding to the receptor, K is the association constant for the binding of a molecule of drug to one of the equivalent n binding sites of the receptor, and $[R]$ is the concentration of the receptors.

The modified Scatchard equation can be derived on the premise of only one class of n equivalent binding sites (13):

$$D = \frac{[A_R]}{[A_p^u]} = nK[R] - K[A_{RBC}] \qquad \text{(Eq. 12)}$$

and shows that plots of the apparent partition coefficient (D) against the drug concentration in the continuous phase that had been removed to bind the receptors should be linear if there were only one class of n equivalent binding sites per receptor (red blood cell) so that saturable binding was manifested. Such a plot is given in Fig. 11 for a hematocrit of 0.24 at room temperature. The negative of the slope is $K = 1.92 \times 10^4$ M^{-1}. The intercept, $nK[R]$, is 9.185; the number of binding sites $n[R]$ on the postulation of a volume of a red blood cell of 90 μm^3 and 1.11×10^{10} cells/ml of packed red blood cells permits the calculation of the number of binding sites per red blood cell as 4.3×10^{-17} moles/single red blood cell.

Plasma Protein Binding by Ultrafiltration—When isotonic phosphate buffer, pH 7.4, containing 10 μg/ml of etofibrate, clofibrate, or their potential solvolytic products was ultrafiltered, nicotinate V and the clofibric and nicotinic acids showed no significant binding to the filter membranes. The concentration in the first and successive 0.5 ml of filtrate was the same as the initial concentration in the buffer to be filtered (Fig. 12). Etofibrate bound strongly to the membrane. Although the concentration in successive 0.5 ml filtrates of the 5-ml aliquot showed increasing concentrations, a maximum of only 39.8% of the filtered concentration could be recovered in the last 0.5 ml of ultrafiltrate (Fig. 12A). Although the ultrafiltrate showed increased concentrations of etofibrate if the membrane prefiltered 3 ml of 100 μg/ml etofibrate, the concentration recovery was still incomplete (Fig. 12B). Further attempts were made to saturate the membrane with prefiltration of larger concentrations and volumes of etofibrate solutions. However, the variability among successive filtrates and filtrations was too great. The cone filter[10] was chosen rather than the molecular filters[11] for the ultrafiltration studies because it showed the lowest binding for the investigated compounds, particularly for the ester III.

The percentages of drug bound to human plasma proteins by this ul-

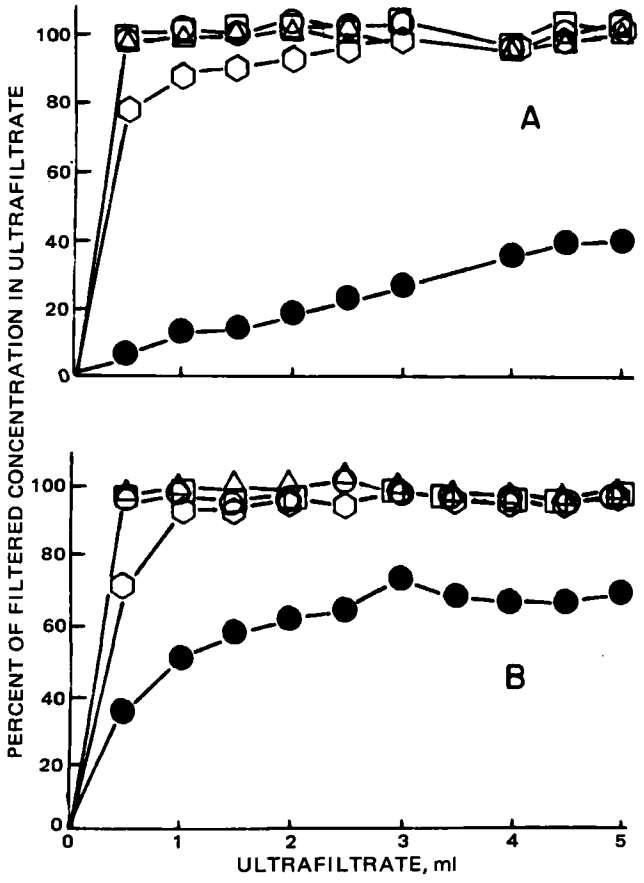


Figure 12—*Percent recovery of ultrafiltrate concentrations of I (●), II (□), III (○), IV (△), and V (○) in 5 ml of pH 7.4 isotonic phosphate buffer in each successive 0.5-ml filtrate. Key: (A) Each drug was filtered separately without membrane pretreatment. (B) The membranes were pretreated by prefiltering 2 ml of buffer containing 100 μg/ml of compound. The excess was rinsed with isotonic buffer.*

trafiltration technique for those compounds that do not show excessive binding to the ultrafiltration membranes are given in Table II. There was no apparent concentration dependence for the protein binding of clofibric acid (II) and ester III, with 97.2 ± 1.2 and 83.2 ± 1.7%, respectively. The results from protein binding studies of nicotinic acid (IV) and the nicotinate V had greater variability, 6.0 ± 6.3 and 12.6 ± 4.3%, respectively. If the study at the lower concentration of 5.9 μg/ml were omitted, the ester

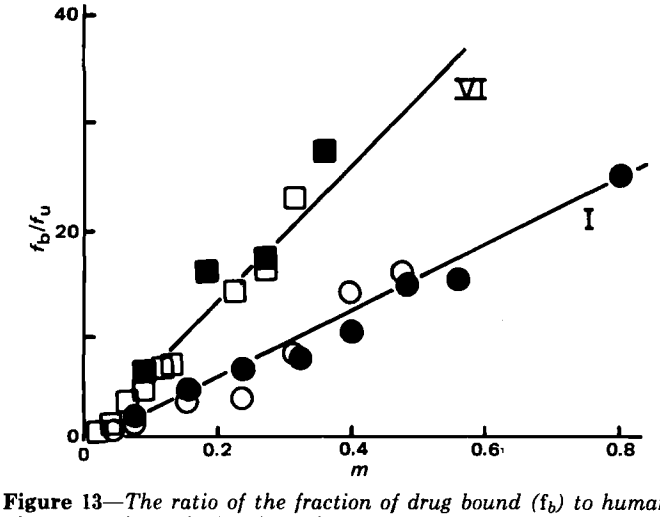


Figure 13—*The ratio of the fraction of drug bound (f_b) to human plasma proteins to the fraction unbound (f_u) as a function of the fraction (m) of normal plasma in pseudoplasma. The intercepts are zero. The slopes are 30 for I at 0.2 μg/ml (●) and 80 μg/ml (○) and 60 for VI at 13.9 μg/ml (■) and 108.4 μg/ml (□).*

Table III—Percent Plasma Protein Binding (100*f*) of Etofibrate and Clofibrate by the Method of Erythrocyte Suspension in Variable Plasma Concentrations, *H* = 0.25

	μg/ml[a]	$S \pm SEM_S$[b]	Percent Bound $(100f) \pm SEM$[c]
Etofibrate[d]	0.212	30.03 ± 1.04	96.78 ± 0.11
	2.19	30.52 ± 1.14	96.83 ± 0.12
	8.12	32.56 ± 0.97	97.03 ± 0.09
	15.8	30.60 ± 1.70	96.84 ± 0.17
	39.2	32.04 ± 1.48	96.97 ± 0.14
	79.7	30.9 ± 2.73	96.87 ± 0.27
Average ± *SD*			96.89 ± 0.09
Clofibrate[e]	13.6	57.32 ± 5.20	98.29 ± 0.16
	13.9	58.87 ± 3.42	98.33 ± 0.10
	27.1	53.51 ± 1.19	98.16 ± 0.04
	54.2	48.13 ± 1.86	97.96 ± 0.08
	108.4	62.1 ± 11.6	98.42 ± 0.03
Average ± *SD*			98.23 ± 0.18

[a] Total plasma concentration of drug before partitioning into red blood cells. [b] Slope of the plot of $f/(1-f)$ *versus* m. [c] $f = S/(1+S)$; *SEM* of $100f$ is $100\{|(S + SEM_S)/(1 + S + SEM_S)] - [(S - SEM_S)/(1 + S - SEM_S)]|/2$. [d] Radioactivity of labeled compound was assayed in red blood cell-equilibrated plasma. The tetraethyl pyrophosphate concentration was 89.5 μg/ml in the red blood cell suspension and 99.45 μg/ml in the plasma. [e] HPLC assay C-d of red blood cell-equilibrated plasma was used. The tetraethyl pyrophosphate concentration was 117 μg/ml in the red blood cell suspension and 115.84 μg/ml in the plasma.

V showed a possible concentration ratio dependence of protein binding.

Human Plasma Protein Binding of Etofibrate and Clofibrate by the Method of Variable Plasma Concentrations in Erythrocyte Suspensions—The high binding of etofibrate and clofibrate to ultrafiltration membranes prevented their use in the determination of plasma protein binding. The instability of these compounds in plasma 1 hr after enzyme inhibition of plasma with tetraethyl pyrophosphate prevented the use of such traditional methods as ultracentrifugation and equilibrium dialysis to determine plasma protein binding. Tetraethyl pyrophosphate undergoes degradation by solvolysis with time.

A method that uses the partitioning of plasma-unbound drug into erythrocytes with varying plasma concentrations to determine plasma protein binding was developed previously by Garrett and Hunt (14) and applied to the nonsaturable protein binding of tetrahydrocannabinol. The method was also applied to the determination of the plasma protein binding of papaverine (15) and methadone (16). In the latter case it was clearly shown that protein binding determined by this technique gave the same results (66% plasma protein binding) as the more conventional ultracentrifugation method. Synthetic blood samples are prepared with known hematocrit, varying fractions of plasma and plasma water (or pH 7.4 phosphate buffer), and known concentrations of drug. The drug-equilibrated erythrocytes are separated from the pseudoblood samples by centrifugation and the pseudoplasma samples are analyzed for their concentrations.

The total amount of drug, A_{tot}, added to pseudoplasma was confirmed by assay and washed red blood cells were added to give an hematocrit of H. From the assayed pseudoplasma concentrations $[A_p]$ of volume $V_p = V_{tot}(1 - H)$, centrifugally separated from a pseudoblood of hematocrit H and volume V_{tot}, the amount of drug partitioned into the red blood cells, A_{RBC}, was calculated:

$$A_{RBC} = A_{tot} - V_p[A_p] \qquad \text{(Eq. 13)}$$

where $[A_{RBC}] = A_{RBC}/V_{RBC}$ and $V_{RBC} = HV_{tot}$. Thus, the fraction (f) of drug in plasma that is unbound to plasma proteins can be calculated from the assayed, separated plasma concentration of drug, $[A_p]$, by:

$$f = 1 - \frac{[A_p^u]}{[A_p]} = 1 - \frac{[A_{RBC}]}{D[A_p]} = 1 - \frac{(A_{tot} - V_p[A_p])}{DHV_{tot}[A_p]} \qquad \text{(Eq. 14)}$$

where D is the partition coefficient for red blood cell–plasma water (or buffer) (Eq. 10). In the special case for clofibrate this partition coefficient was a function of the red blood cell concentration:

$$D = 9.185 - 1.92 \times 10^{-4}[A_{RBC}] \qquad \text{(Eq. 15)}$$

The method is extremely sensitive and particularly useful when both the plasma protein binding and red blood cell–plasma water partition coefficient are high.

In the cases of both etofibrate and clofibrate, plots of the fraction of drug bound to plasma protein, against the fraction of plasma, m, in

pseudoplasma were superimposable for all plasma and unbound drug in plasma concentrations [A_p^u]. Plots (Fig. 13) of $f/(1-f)$ against m are linear for all [A_p^u] and pass through the origin with slopes of S. The fraction of drug bound to 100% plasma when $m = 1$ can then be calculated (Table III). The human plasma protein binding of etofibrate is 96.89 ± 0.09% (*SD*) for plasma concentrations in the 0.2- to 80-μg/ml range. The human plasma protein binding of clofibrate is 98.23 ± 0.18% (*SD*) for plasma concentrations in the 14- to 108-μg/ml range.

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Pharmacokinetic Model for Diazepam and its Major Metabolite Desmethyldiazepam Following Diazepam Administration

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Abstract □ A five-compartment open model was used to simulate the blood concentration profiles of diazepam and its metabolite, desmethyldiazepam, following single- and multiple-dose administrations of diazepam. The parameter estimates for diazepam were previously reported literature values. The parameter estimates for the metabolite were calculated from literature values of blood concentrations of desmethyldiazepam following the administration of clorazepate. The five-compartment open model suggests that ~50% of the administered diazepam is biotransformed to desmethyldiazepam, and that the elimination profile of the metabolite is not altered by the presence of the drug. The model may also be readily adapted to predict the concentrations of diazepam and desmethyldiazepam in cerebrospinal fluid following the administration of diazepam by simply correcting the blood or plasma concentrations of the drug and metabolite for the degree of plasma protein binding.

Keyphrases □ Diazepam—desmethyldiazepam, pharmacokinetics, single- and multiple-dose administrations, five-compartment open model, blood, CSF □ Desmethyldiazepam—diazepam, pharmacokinetics, single- and multiple-dose administrations, five-compartment open model, blood, CSF □ Pharmacokinetics—diazepam, desmethyldiazepam, single- and multiple-dose administrations, five-compartment open model, blood, CSF

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one) is effective in the symptomatic relief of tension and anxiety, as well as for the relief of skeletal muscle spasms (1–5). Desmethyldiazepam, the major metabolite of diazepam, is the pharmacologically active metabolite of the prodrugs clorazepate and prazepam, which are also used as anxiolytic agents (6–8).

Diazepam pharmacokinetics have been described previously by a three-compartment model by Kaplan *et al.* (6) and Moolenaar *et al.* (9). Several investigators have described the pharmacokinetic profile of desmethyldiazepam following oral administration of the prodrug clorazepate in terms of a two-compartment open model (8, 10–12). The present report combines the three-compartment open model for diazepam and the two-compartment open model for desmethyldiazepam to define the pharmacokinetic profiles of diazepam and its metabolite following single- and multiple-dose administrations of the drug.

EXPERIMENTAL

Clinical Protocol—The data used in this study were previously reported by Kaplan *et al.* (study A) (6). Briefly, four healthy male volunteers, ages 25–43, each received single 10-mg doses of diazepam administered intravenously and orally 1 week apart. Commencing 1 week thereafter, each subject received a 10-mg oral dose of the drug every 24 hr for 15 days. The subjects were fasted for 7 hr prior to receiving the single-dose administrations, and food was withheld for 1 hr postadministration.

Blood specimens were obtained 1, 2.5, 5, 10, 20, 30, and 45 min and at 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 30, and 48 hr following intravenous administration, and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 30, and 48 hr following the single-dose oral administration. Following chronic administration, blood samples were obtained 0 (predose), 0.5, 1, 2, 4, and 24 hr postadministration on day 1; 1, 2, and 24 hr postadministration on days 2–11; 1 and 2 hr postadministration on day 12 and at 0 (predose), 1, 2, 4, 6, 8, 12, 24, 30, 36, 48, 72, 96, 120, 144, 168, 192, and 216 hr postadministration on day 15.

Analytical Method—Blood specimens were analyzed for diazepam and desmethyldiazepam by the electron-capture GLC procedure of de Silva and Puglisi (13), with a sensitivity of 10 and 20 ng/ml, respectively, for each component using a 1-ml specimen.

Pharmacokinetic Model—The five-compartment open model used to describe the pharmacokinetic profiles of diazepam and its major me-

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