Analysis of Folinic Acid in Human Serum Using High-Performance Liquid Chromatography with Amperometric Detection

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Abstract □ An assay for the separation and quantification of folinic acid in serum was developed using high-performance liquid chromatography with electrochemical detection. Folinic acid was extracted from serum using a C18 minicolumn treated with dibasic ammonium phosphate. The drug was eluted from this column with methanol, which was evaporated under a nitrogen stream at 50°. The mobile phase, pH 3.5 ammonium phosphate buffer–methanol–acetonitrile (93:4:3), was pumped at a flow rate of 3.0 ml/min. The recovery of folinic acid added to human serum was 101.11 ± 8.5% (mean ± *SD*). A plot of folinic acid peak height as a function of concentration was linear over the range of 2.5×10^{-7} to 2.5×10^{-6} *M*. Neither methotrexate nor other reduced folates interfered with the analysis of folinic acid. Sample preparation and analysis can be completed within 2 min of sample collection.

Keyphrases □ Folinic acid—analysis by high-performance liquid chromatography, amperometric detection, human serum □ High-performance liquid chromatography—amperometric detection, folinic acid and other folates, human serum

Folinic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)-methyl]amino]benzoyl]-L-glutamic acid, is a reduced folate used to prevent or reduce the toxicity associated with high-dose methotrexate therapy for the treatment of neoplastic disease (1). The optimization of folinic acid dosage regimens necessitates an understanding of its disposition; however, studies characterizing the pharmacokinetics of this compound have been hampered by the lack of a rapid, sensitive, and specific assay method. Previously reported methods for folinic acid quantification include microbiological assay (2), radiochemical assay (3), radioimmunology (4), and spectrophotometry (5). The application of these techniques to pharmacokinetic studies is somewhat limited since spectrophotometric methods are not sufficiently sensitive, differential microbiological assays are generally not precise, and radiochemical and radioimmunologic techniques may suffer from a lack of specificity.

A high-performance liquid chromatographic (HPLC) assay employing electrochemical detection, suitable for the routine analysis of folinic acid, has been designed and is free from interference from folic acid, dihydrofolic acid, 5-methyl tetrahydrofolic acid, and methotrexate. This assay has been applied to the quantification of folinic acid in human serum.

EXPERIMENTAL

Reagents—Folinic acid[1], 5-methyltetrahydrofolic acid[2], dibasic ammonium phosphate[3], methanol[4], acetonitrile[4], EDTA[5] (disodium ethylenediaminetetraacetic acid), and phosphoric acid[3] were used as received.

HPLC System—A liquid chromatograph consisting of a solvent delivery system[6], a fixed-loop injector[7] with a 100-μl loop, a microparticulate radially compressed reverse-phase column[8], an electronic noise filter[9], and an electrochemical detector with a 5-mm diameter glassy carbon electrode[10] was used. The temperature of the column was adjusted by placing the column in a constant-temperature oven[11]. Data were collected and analyzed using an electronic integrator/recorder[12].

The mobile phase consisted of 0.5 *M* ammonium phosphate buffer–methanol–acetonitrile (93:4:3) with pH adjusted to either 3.5 or 5.5. The pH of the mobile phase used for characterization of the system was 5.5; the pH was decreased to 3.5 when serum samples were analyzed, to increase resolution. EDTA (10 m*M*) was added to decrease background electrical noise. The mobile phase was prepared daily, filtered, and deaerated before use. Double-distilled, deionized water was used for the preparation of all solutions.

Electrochemical Characteristics of the Folate Analogues—The oxidation characteristics of folinic acid were evaluated using the mobile phase at a pH of 5.5 and a flow rate of 3.0 ml/min. The concentration of folinic acid was 1.0×10^{-6} *M*. Applied potentials were adjusted between +0.3 and +0.9 V. A 30-min equilibration period was necessary following each change in applied potential to reestablish a suitable baseline.

Extraction—Ascorbic acid (1 mg/ml) was added to all serum samples immediately after collection to prevent drug degradation. Folinic acid was extracted from serum using reverse-phase C18 disposable cartridges[13]. The cartridges were activated using 10 ml of methanol followed by 10 ml of 5 m*M* ammonium phosphate buffer, pH 5.5. One milliliter of serum was then pushed through the cartridge followed by 3 ml of 5 m*M* ammonium phosphate buffer, pH 5.5. The effluent was discarded. The outlet end of the cartridge was then connected to a vacuum and the cartridge was dried for 5 min, after which time the drug was eluted with 2 ml of methanol. The eluant was collected and evaporated at 50° under a nitrogen stream in a sample concentrator[14]. The evaporated samples were stored at −4°; samples were reconstituted in 500 μl of mobile phase before being assayed.

Recovery Studies—The recovery of folinic acid in serum was determined by comparison of peak heights of drug prepared in mobile phase to peak heights obtained after extraction from serum. A standard curve was constructed by plotting peak height as a function of concentration for extracted serum samples containing known amounts of folinic acid over the range of 2.5×10^{-7} to 2.5×10^{-6} *M*. The data were analyzed using a linear regression program on a programmable calculator. Concentrations of all samples were calculated using the resulting regression coefficients.

RESULTS AND DISCUSSION

Figure 1 is a plot of detector response (log peak height) as a function of applied potential for folinic acid. The threshold for the oxidation of this compound was +0.4 V. An increase in applied potential resulted in an increase in generated current until a plateau was reached at +0.7 V. It was not possible to characterize response at >+0.9 V due to an extremely high background current, presumably caused by the oxidation of components in the mobile phase.

[1] Lederle Laboratories, Pearl River, N.Y.
[2] Sigma Chemical, St. Louis, Mo.
[3] Fisher Scientific, Fair Lawn, N.J.
[4] Waters Associates, Milford, Mass.
[5] Allied Chemical, Morristown, N.J.
[6] Model 6000A; Waters Associates, Milford, Mass.
[7] Model 7120; Rheodyne Inc., Cotati, Calif.
[8] RCSS C18 Column; Waters Associates, Milford, Mass.
[9] Model SC102; Foxboro Instruments, New Haven, Conn.
[10] Model LC-2A; BioAnalytical Systems, West Lafayette, Ind.
[11] Model 15; Thelco, Inc., New York, N.Y.
[12] Model 720; Waters Associates, Milford, Mass.
[13] C^{18} SEP-PAK; Waters Associates, Milford, Mass.
[14] Model 190; Fisher Scientific, Fair Lawn, N.J.

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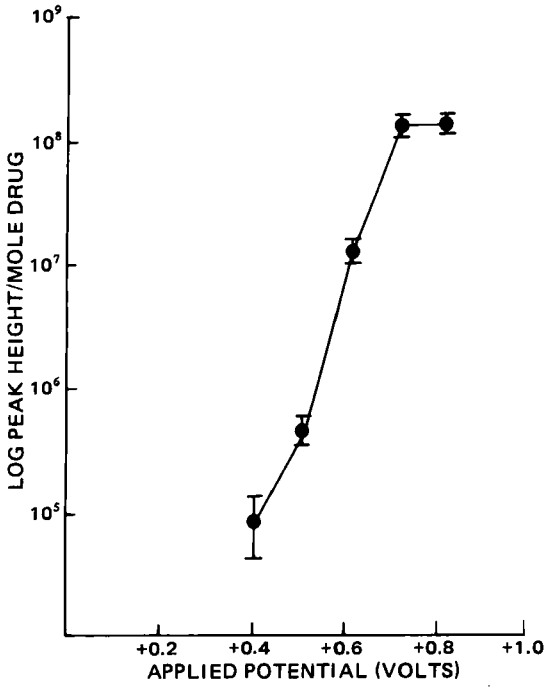

Figure 1—*Detector response as a function of applied potential for folinic acid.*

High electrolyte concentrations are necessary in mobile phases when electrochemical detection is used in order to reduce resistance and increase the current at the electrode surface. Varying concentrations of electrolyte may also affect the diffusion coefficient of the electroactive species, limiting the current at the electrode. The effect of varying ammonium phosphate concentrations, at a constant pH of 5.5, on detector response (Fig. 2) was determined for folinic acid. There was a constant increase in response with increasing salt concentration until a plateau was reached at 0.01 *M*.

The effect of buffer concentration on the retention of folinic acid was also characterized. Figure 3 describes the retention time for folinic acid as a function of ammonium phosphate concentration (pH 5.5). It is evident that as buffer concentration increases, there is a corresponding increase in retention time.

The general effect of mobile phase velocity on the diffusion layer thickness within the electrochemical cell and the resulting effect on current yield has been reported (6). The optimal flow rate, in terms of detector response, was determined by plotting response (measured as peak height) as a function of flow rate (Fig. 4) for folinic acid. It is apparent that the peak height decreases with increasing flow rate between 1.0 and 7.0 ml/min.

Investigation of the effect of flow rate on retention (Fig. 5) showed that significant deviations from linearity occur as flow rate increases. Both the capacity and selectivity of the radially compressed column decreased with increases in flow rate. A log–log transformation (Fig. 5 inset) of the data, however, was linear. These findings are in general agreement with studies of flow control in HPLC reported by Schrinker (7).

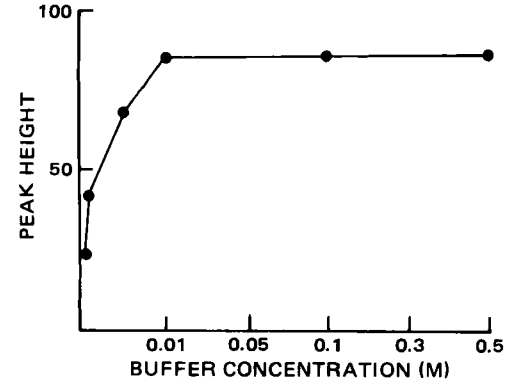

Figure 2—*Detector response as a function of mobile phase salt concentration (pH 5.5).*

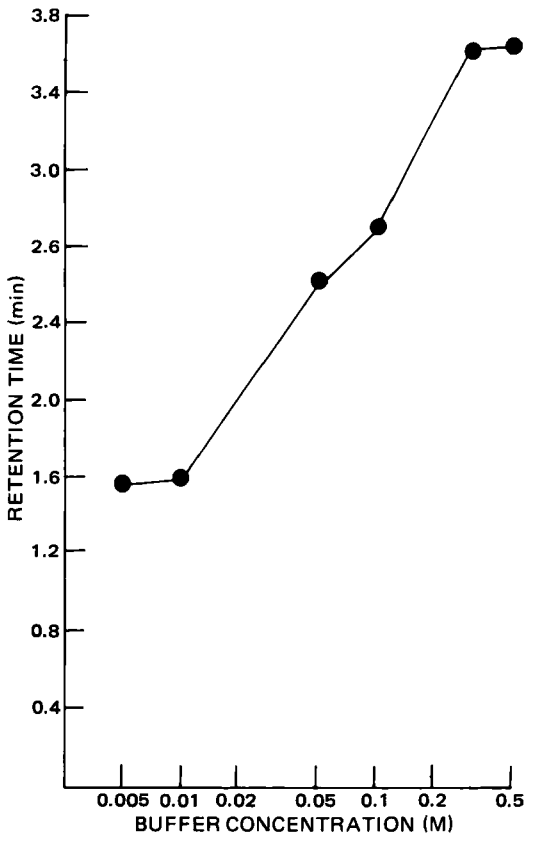

Figure 3—*Effect of buffer concentration on folinic acid retention.*

It has been reported by Gilpin and Sisco (8) that both normal and bonded phases may be affected by temperature, and that variations in capacity >25% have been reported with temperature changes of ≤1°. Because of the high efficiency and reactivity associated with radially compressed columns, the effect of temperature on the retention time of folinic acid was investigated. Table I reports the retention times for folinic acid over a range of temperatures between 20° and 50°. A decrease in retention time (capacity) with increasing temperature was observed; however, the resolution of the system actually improved at higher temperatures. This can best be explained by the decrease in folinic acid peak width.

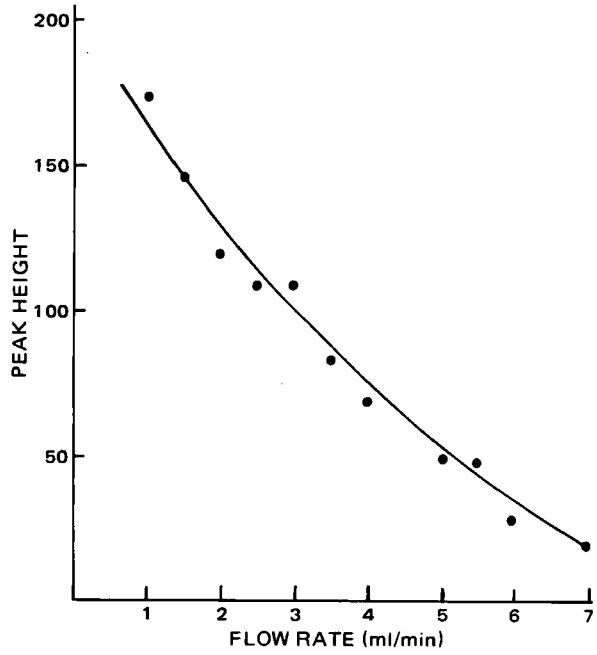

Figure 4—*Detector response to folinic acid as a function of mobile phase flow rate.*

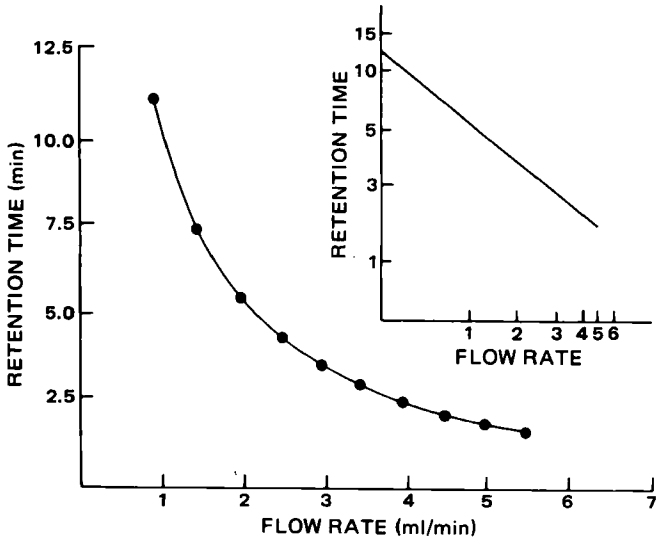

Figure 5—*Retention time as a function of flow rate for folinic acid. The inset is the log–log transformation of the graph.*

Temperature fluctuations were found to affect daily chromatographic results. Ambient temperatures varied as much as 10° during the course of the day, causing as much as 20% changes in observed retention times for folate compounds using the same mobile phase. The installation of a constant-temperature oven circumvented the effect of temperature changes on the system. Column temperatures were held constant at 40°, and the retention time was found to be reproducible within the 5% limits of the system.

Figure 6 is a plot of the retention of folinic acid as a function of pH. The mobile phase flow rate was 3.0 ml/min, and the ammonium phosphate concentration was 0.5 *M*. It was evident from examination of this figure that pH has a significant effect on folinic acid retention.

The extraction and recovery of folinic acid added to pooled human serum was virtually complete. The recovery of drug over the concentration range of 2.5×10^{-7} to 2.5×10^{-6} *M* ranged from 94 to 117% with a mean value of 101%. Folinic acid-spiked serum samples were found to be stable for 2 months when stored at −4°. However, after extraction, samples were only stable for 2 days. A plot of calculated folinic acid concentration as a function of actual concentration in spiked serum

Table I—Effect of Temperature on Folinic Acid Retention

Temperature	Retention Time, min
20°	4.60
30°	4.13
40°	3.50
50°	2.68

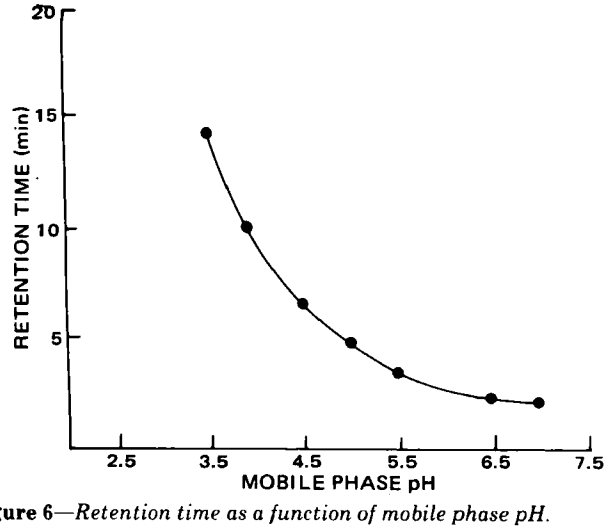

Figure 6—*Retention time as a function of mobile phase pH.*

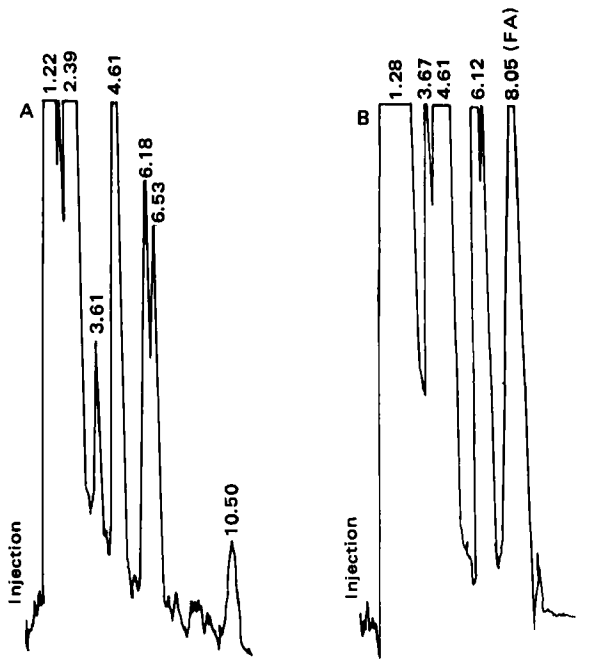

Figure 7—*Chromatograms from subject KB using chromatographic conditions described in the text. Numbers above peaks represent retention time in minutes. Key: (A) serum blank; (B) serum sample collected 5 min after intravenous injection of 10 mg of folinic acid; (FA) folinic acid (concentration 7.3×10^{-6} M).*

samples was linear ($r = 0.99$; slope = 1.02). The slope was not significantly different from 1.0 (Student's t test, $p > 0.05$) and the intercept was not significantly different from 0 (Student's t test, $p > 0.05$). To improve the resolution between folinic acid and other eluting components of the sample matrix, it was necessary to decrease the pH of the mobile phase from 5.5 to 3.5. The retention time for folinic acid under these conditions was 8.05 min. The compound was completely resolved from neighboring peaks at 6.53 and 10.50 min in all samples. The column capacity for folinic acid was 7.13; the efficiency of the column was calculated to be 530 plates. Figure 7A is a representative chromatogram of a drug-free serum sample; Fig. 7B shows a chromatogram obtained from a serum sample collected 5 min after the injection of 10 mg iv of folinic acid to the same 80-kg human volunteer.

Folic acid, dihydrofolic acid, and 5-methyltetrahydrofolic acid analyzed under these conditions had retention times of 4–6 min; therefore, these compounds did not interfere with folinic acid. Detection of methotrexate under these chromatographic conditions requires an applied potential of >+0.9 V. Therefore, the presence of methotrexate in serum samples obtained from patients receiving high-dose methotrexate therapy should not interfere with the analysis of folinic acid.

Figure 8 is a semilogarithmic plot of the data obtained following rapid

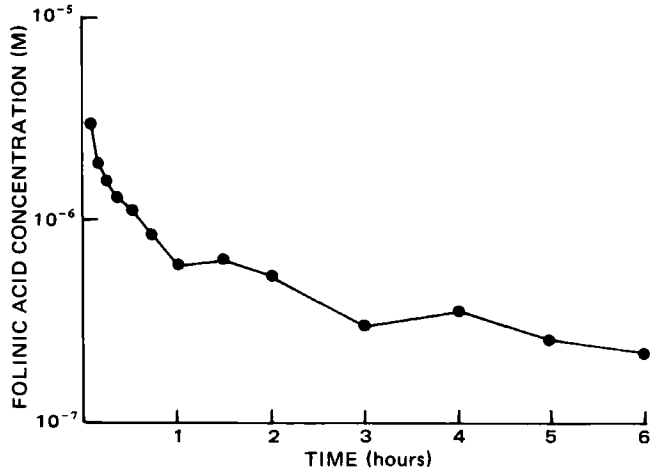

Figure 8—*Folinic acid concentration as a function of time following the intravenous administration of 10 mg of drug.*

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.

REFERENCES

(1) R. K. Blakely, "The Biochemistry of Folic Acid and Related Pteridines," A. Neuberger and E. L. Tatum, Eds., North Holland Publishing, Amsterdam, 1969.

(2) B. M. Mehta, W. H. Conti, and H. Gisolfi, *Cancer Treat. Rep.*, **62**, 345 (1978).

(3) S. P. Rothenberg, M. Da Costa, and Z. Rosenberg, *Anal. Biochem.*, **93**, 483 (1979).

(4) V. Raso, *Cancer Treat. Rep.*, **61**, 585 (1977).

(5) S. K. Chapman, B. C. Greene, and R. R. Streiff, *J. Chromatogr.*, **145**, 302 (1978).

(6) P. T. Kissinger, *Anal. Chem.*, **49**, 447a (1977).

(7) H. Shrinker, *Am. Lab.*, **10**, 111 (1978).

(8) R. K. Gilpin and W. R. Sisco, *J. Chromatogr.*, **194**, 285 (1980).

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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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