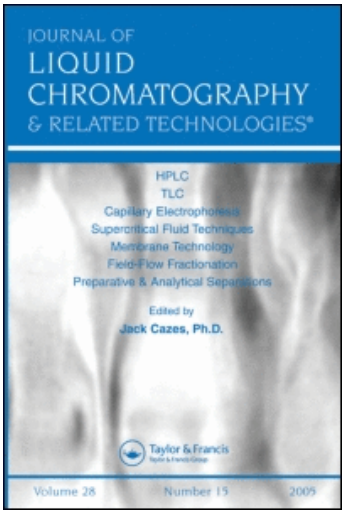

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SIMPLIFIED HPLC METHOD WITH SPECTROPHOTOMETRIC DETECTION FOR THE ASSAY OF CLOFIBRIC ACID IN RAT PLASMA

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ABSTRACT

A simple and rapid reversed-phase HPLC method for the assay of clofibric acid in rat plasma is presented. After a simultaneous deproteinization-extraction step of a 200 μL plasma sample with a solution of propyl paraben in acetonitrile, the clear extract was injected onto a Microsorb-MV C18 chromatographic column and eluted with 1% acetic acid in acetonitrile-water (45:55) at the rate of 0.8 mL/min. At the detection wavelength of 230 nm, clofibric acid and propyl paraben, the internal standard, eluted at 7.0 min and 5.5 min, respectively. Peak responses were linearly related to concentrations of clofibric acid in the range 1.5-30 μg/mL, with a minimum detectable concentration of analyte of 75 ng on-column. Recoveries of clofibric acid from plasma samples spiked at 1.5-30 μg/mL levels of analyte were >94% (range 94.6-99%). The proposed method was easily applied to the determination of the plasma plasma levels of clofibric acid derived from the oral and intraperitoneal administrations of clofibrate as a liquisolid compact and as the contents of a commercial soft gelatin capsule to rats.

INTRODUCTION

Liquisolid compacts are dry, nonadherent, free-flowing and readily compressible powder formulations of liquid and solid poorly water-soluble drugs, obtained by the simple admixture of

the drug or its solution in nonvolatile, high boiling point solvents with suitable powder excipients (1,2). Interest on liquisolid compacts has been fostered by reports indicating that use of this type of formulation will result in the improvement of the *in vitro* dissolution profile of certain liquid and solid lipophilic drugs (3,4).

As part of a study intended to evaluate the efficiency of liquisolid compacts as a means of systemically delivering lipophilic liquid drugs, this laboratory decided to compare the plasma levels of the antilipidemic agent clofibrate after its oral and intraperitoneal administration as a liquisolid compact formulation and as the liquid contents of a commercial soft gelatin capsule to rats. For this reason, an analytical method was required to determine the temporal changes in ensuing plasma drug levels.

Following its oral administration to humans and animals, clofibrate undergoes rapid and quantitative hydrolysis by intestinal, plasma and tissue esterases (5-9). As a result, analytical methods for clofibrate only measure the active metabolite p-chlorophenoxyisobutyric acid (clofibric acid), and not the parent molecule (9-27). In this regard, the analysis of clofibric acid in biological samples has been accomplished largely through the use of spectrophotometry (9-11), gas chromatography (GC) (12-18) and high-performance liquid chromatography (19-27). In addition to being nonspecific, spectrophotometric methods are found to yield low recovery values after extraction of the sample with a water-immiscible organic solvent (9,10) or to require a preliminary chemical modification of the analyte (5). In contrast, GC methods are specific and accurate, but they usually include a lengthy and often tedious sample extraction and purification (12-14) as well as a derivatization step (12-16,18).

In comparison to GC methods, the use of HPLC approaches circumvents the need for a preliminary sample derivatization and, in addition, offer the advantages of greater simplicity and flexibility of experimental conditions. However, some of the proposed HPLC methods are prone to potential sample losses in the course of the transferring of highly volatile solvents used for extracting clofibric acid from the biological matrix (20,24,27) or of a multistep sample purification procedure (19). In other cases, the method necessitates an ion-pair reagent (27), special detection conditions (26) or a very large volume of sample (20,27).

The purpose of this report is to describe a reversed-phase HPLC method with UV detection that will permit the analysis of clofibric acid in rat plasma in a rapid and straightforward manner, without the need for transfer steps or extraction with a highly volatile solvent, and using a small plasma sample. This method is well suited for studies evaluating the systemic delivery of drugs from solid dosage forms such as liquisolid compact formulations in animal models.

EXPERIMENTAL

Materials and Reagents

Clofibric acid, clofibrate, and propyl paraben were obtained from Sigma Chemical Company, St. Louis, MO, and were used as received. Clofibric acid was also prepared by saponification of clofibrate using previously described conditions (13) and recrystallization from diluted ethanol. Analytical reagent grade glacial acetic acid, and HPLC grade acetonitrile and water were from J.T. Baker Inc., Phillipsburg, NJ. The internal standard solution was prepared by dissolving propyl paraben in acetonitrile to a concentration of 1 μg/mL. This solution was stable indefinitely. A stock solution of clofibric acid was prepared by dissolving this compound in a few drops of ethanol, and diluting to volume with water to a concentration of 120 μg/mL.

Apparatus and Chromatographic Conditions

The chromatographic system consisted of LC-10 pump, LC-90 spectrophotometric detector (Perkin-Elmer), and ChromJet recording integrator (Spectra- Physics). Samples were introduced through a Model 7125 injector fitted with a 50 μL sample loop (Rheodyne).

Separations were performed on a Microsorb-MV C18, 5 μm, 15 cm x 4.6 mm i.d. column (Rainin), protected with an Adsorbosil C18 guard column (Alltech). Elutions were carried out at ambient temperature with 1% acetic acid in acetonitrile-water (45:55), filtered and degassed prior to use. The flow rate was 0.8 mL/min, and the detection wavelength was 230 nm and 0.05 AUFS.

Method Validation

The linearity of the method was investigated by diluting the stock solution of clofibric acid (120 μg/mL) either with water or drug-free rat plasma to concentrations in the range 1.5-30 μg/mL, and putting these solutions through the proposed assay method. A calibration curve was constructed by plotting the ratio of the peak heights of clofibric acid to internal standard against the concentration of clofibric acid added.

Analyte recovery was determined by comparing the peak heights of clofibric to internal standard for standard preparations in plasma against the same preparations in water. Within-day reproducibility was assessed by analyzing a set of samples containing various concentrations of clofibric acid in plasma at six different times. Day-to-day reproducibility was studied by analyzing the same sets of spiked plasma samples over three consecutive days.

Animal Studies

Sprague-Dawley rats, 225-250 g in weight and fasted overnight, were divided into groups of 4 rats each. To one group, a solution of clofibrate liquisolid compact in 50% ethanol

was administered by the oral route using a curved oral-feeding needle; to another group, the same solution was administered intraperitoneally. These experiments were repeated with the contents of a commercial soft gelatin capsule (Atromid-S®) previously diluted with 50% ethanol to the same drug concentration as the liquisolid compact formulation. All treatments were carried out using a 20 mg/kg dose. Blood samples were collected periodically by the orbital sinus technique into polypropylene test tubes that contained a small amount of disodium EDTA. After mixing with the aid of a vortex mixer, the blood samples were centrifuged at 5000 rpm for 10 min to separate the corresponding plasmas, which were immediately transferred with Pasteur pipets to clean test tubes and kept at 4°C pending their analysis.

Assay Method

To a 13 x 100 mm screw-cap culture tube, 200 μL of plasma sample and 200 μL of internal standard solution were added in succession (to samples estimated to contain between 15 and 60 μg/mL of clofibric acid, 25 μL of acetonitrile was also added). After gentle shaking, the mixture was vortexed for 30 seconds and next centrifuged at 5000 rpm for 10 min. An aliquot of the clear supernatant was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Because of its pKa of 3, clofibric acid is expected to be present at the physiological pH of the plasma almost entirely ionized (5). For this reason, this compound is generally eluted using mobile phases composed of various ratios of acenotrile-water or methanol-water and to which either a buffer or an organic acid has been added to lessen tailing and improve resolution from other sample peaks. In the proposed method, acetonitrile and acetic were selected over methanol and buffers to minimize column inlet backpressure. In addition, acetic acid was found more convenient to a buffer since it can be added to the mobile directly, thus simplifying the preparation of the mobile phase. The concentration of acetic acid added was selected after determining its effect on both resolution R (calculated from $2[t_1-t_2]/w_1+w_2$) between the internal standard peak and analyte peak and on peak shapes.

Chromatograms of a rat plasma sample free of clofibric acid and of a plasma sample containing clofibric acid and propyl paraben, the internal standard, are shown in Figures 1(a) and 1(b), respectively. A resolution >2.0 between the two compounds was obtained in < 8 minutes. Plasma components coextracted with the analyte did not interfere with the analysis. Using the recommended detection conditions, the limit of detection of clofibric acid was about 75 ng on column. A greater sensitivity has been achieved at a detection wavelength below 230 nm (22,23). However, the addition of acetic acid to the mobile phase lead to detector overload due to

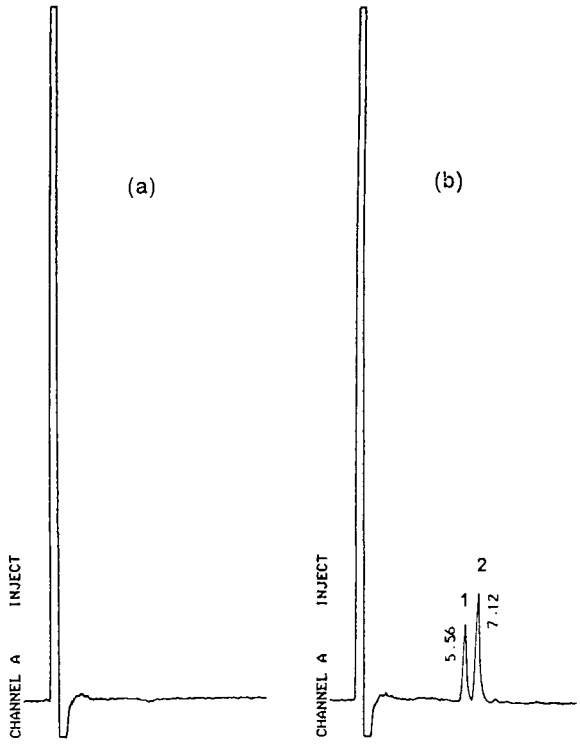

Fig. 1 Typical chromatograms of plasma samples obtained from: (a) a rat before clofibrate administration, and (b) a rat that had received a 20 mg/kg oral dose of clofibrate. Key: 1, propyl paraben, the internal standard; 2, clofibric acid.

significant solvent self-absorption at wavelengths below 230 nm. A way to obviate this problem may be to replace the acetic acid by a less absorbing acid such as phosphoric acid. Furthermore, acetonitrile deproteinization of plasma samples and centrifugation prior to injection of the supernatant has been criticized as giving lower sensitivity than methods employing liquid-liquid extraction (26); but this detail is contingent to the dilution ratio of the sample with deproteinizing solvent, which in the proposed method was kept sufficiently low (i.e., 1:1) so as to permit accurate assay of clofibric acid in rat plasma at concentrations as low as 1.5 μg/mL. Nevertheless, for samples suspected of containing clofibric acid in concentrations ranging from 15-60 μg/mL, it is advisable to increase the volume of the test sample by adding acetonitrile (ca.

Table 1

Data for recovery of clofibric acid from rat plasma by proposed HPLC method

Amount added, μg/mL	No. of samples	Recovery	
		Mean ± SD, %	RSD, %
1.5	6	98.2 ± 0.01	0.01
1.875	6	98.3 ± 0.01	0.01
3.75	6	95.2 ± 0.05	0.05
7.5	6	95.6 ± 0.05	0.05
15.0	6	94.9 ± 0.03	0.03
30.0	6	95.6 ± 0.05	0.05

Table 2

Interday and intraday reproducibility of peak height ratio data for proposed HPLC method[a]

Concentration, μg/mL	No. of samples	Intraday		Interday, 3 days	
		Mean ± SD	RSD, %	Mean ± SD	RSD, %
1.875	3	0.29 ± 0.01	3.45	0.28 ± 0.01	3.35
3.75	3	0.56 ± 0.03	3.35	0.58 ± 0.02	3.80
7.5	3	0.72 ± 0.01	1.39	0.73 ± 0.01	3.39
15.0	3	1.41 ± 0.03	2.13	1.48 ± 0.04	2.37
30.0	3	2.86 ± 0.08	2.80	2.93 ± 0.10	3.41

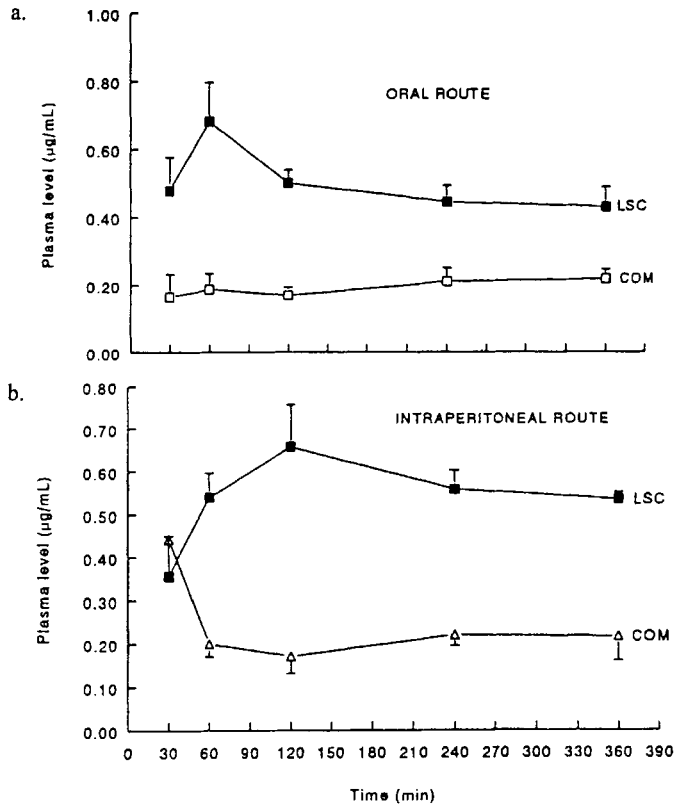

Figure 2. Temporal changes of the plasma levels of clofibric acid in rats receiving either a commercial soft gelatin capsule (COM) or a liquisolid compact (LSC) of clofibrate (20 mg/kg) by the oral and the intraperitoneal routes. Vertical lines represent the SEM for 5 rats.

25 µL) to ensure more quantitative recoveries of analyte. Alternatively, one could decrease the volume of internal standard solution; or evaporate the sample to dryness and reconstitute the residue in a as small as possible volume of mobile phase or of a solvent compatible with the mobile phase (20,22,24).

Results of a recovery study from spiked plasma, performed at levels of clofibric acid between 1.5 and 30 µg/mL are summarized in Table 1. In general recoveries at all levels were better than 94% of the amount added, and the pooled RSD was 0.03%.

The calibration curve was linear from 1.5 to 30 µg/mL (n = 4), with the line equation being $y = 0.17x + 0.03$ ($r = 0.999$), where y is the ratio of the peak height of clofibric acid to

Table 3

Retention times relative to clofibric acid of related arylphenoxyisobutyric acid compounds

Compound	Solvent A[a]	Solvent B[b]
Clofibric acid	1.00	1.00
Bezafibrate	1.03	1.25
Fenofibric acid	2.26	1.58
Gemfibrozil	4.16	2.46
Clofibrate	4.45	2.75
Fenofibrate	10.39	6.67

[a]Solvent A: 1% acetic acid in acetonitrile-water (45:55), 0.8 mL/min; retention time of clofibric acid = 7.52 min.
[b]Solvent B: 1% acetic acid in acetonitrile-water (55:45), 0.8 mL/min; retention time of clofibric acid = 4.10 min.

internal standard, x is the concentration of clofibric acid (μg/mL), and r is the correlation coefficient.

Results of intraday and interday precision studies for various levels of clofibric acid, in terms of mean ± SD and RSD of peak height ratios, are summarized in Table 2.

Figures 2(a) and 2(b) depict the time-related changes in plasma levels of clofibric acid that follow oral and intraperitoneal administrations, respectively, of a clofibrate liquisolid compact and the contents of a commercial soft-gelatin capsule. It is quite evident that the liquisolid compact formulation yields significantly higher plasma drug levels than the commercial product at all experimental intervals. These results parallel those observed *in vitro* and indicating that the release of clofibrate from a liquisolid compact is greater than from a commercial soft gelatin capsule (4).

As shown in Table 3, the recommended experimental conditions will also permit the differentiation of clofibric acid from clofibrate, the parent compound, and from other related aryloxyisobutyric acid compounds. In those instances where the metabolite is the compound present in the plasma, the precursor elutes much later, especially in the case of fenofibrate.

In summary, the proposed HPLC method should be useful for the assay of clofibric acid in the large number of plasma samples that one encounters in the course of pharmacokinetic studies of clofibrate in small animal models. It is simple in term of experimental steps, economic in terms of reagents and sample requirements, and valid in terms of linearity, accuracy, reproducibility and specificity.

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