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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ANALYSIS OF DRUGS IN BIOLOGICAL FLUIDS

IV. DETERMINATION OF CLOFIBRINIC ACID

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SUMMARY

A rapid, sensitive and specific high-pressure liquid chromatographic method is described for the quantitative analysis of clofibrinic acid in plasma, saliva and urine. In contrast to previously reported gas–liquid chromatographic methods, which require derivatization of clofibrinic acid before chromatography, the present method involves a simple two-step extraction procedure and chromatographic determination of the underivatized clofibrinic acid. Concentrations between 1.0 and 25.0 μg per sample can be measured with a coefficient of variation from 1 to 6%.

INTRODUCTION

A number of methods have been reported for the analysis of clofibrinic acid, the active metabolite of the hypolipidaemic drug clofibrate, in plasma and urine (Table I). The most widely used method has been a spectrophotometric assay[1,2] that involves solvent extraction of clofibrinic acid from acidified plasma or urine, and subsequent measurement of the ultraviolet absorbance at 226 nm. Although this method is rapid and convenient, it suffers from the lack of specificity inherent in spectrophotometric assays.

Several gas–liquid chromatographic (GLC) methods have been described for the determination of clofibrinic acid. Some of these methods have time-consuming preparative steps before GLC, such as column[3] or thin-layer chromatography[4,5]; another involves several extraction steps[6]. Such techniques of sample preparation are time-consuming and render these methods unsuitable for use with large numbers of samples. Four additional GLC methods[7–10] have a number of common features;

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TABLE I

COMPARISON OF METHODS OF ANALYSIS FOR CLOFIBRINIC ACID IN BIOLOGICAL FLUIDS

References	*Analytical method*	*Sample*	*Sensitivity (μg/ml)*	*Reproducibility (coefficient of variation)*	*Comments**
1, 2	Spectrophotometric	Plasma, urine	Not given	Not given	Lacks specificity
3	Column chromatography–GLC	Serum	Not given	Not given	Few details; prior chromatography
4	Thin-layer chromatography–GLC	Plasma, urine, bile, faeces	0.1	6.4% (Replicates between 53.68 and 322 μg/ml)	Prior thin-layer chromatography
5	Thin-layer chromatography–GLC	Plasma	Not given	Not given (correlation coefficient 0.999 for calibration curve)	No internal standard; prior thin-layer chromatography
6	GLC	Plasma	1	4.8% (replicates at 2.5, 10.1 and 50.5 μg/ml)	Multiple extractions; diazomethane used***
7	GLC	Serum	Not given	Not given	Few details
8	GLC	Plasma	0.25	Not given (correlation coefficient 0.999 for calibration curve)	Diazomethane used***
9	GLC	Plasma, urine	3	9%**	Time consuming
10	GLC	Plasma, urine	1	10% (Replicates at 1 μg/ml)	Diazomethane used***
11	GLC	Plasma, urine	1	3.0% (Calibration curves of 10–200 μg/ml from plasma and urina	Two-step extraction
This paper	HPLC	Plasma, urine, saliva	0.5	4.9% (Calibration curves of 1–25 μg/sample from plasma, urine and saliva	Two-step extraction without derivatization

* All the GLC methods include a derivatization step.
** Quoted in ref. 11.
*** See Results and discussion.

each involves solvent extraction, evaporation and derivatization before GLC. The most recently reported GLC method[11] appears to be rapid and convenient.

This paper reports the application of high-pressure liquid chromatography (HPLC) to the analysis of clofibrinic acid in plasma, saliva and urine. The method

is rapid, specific, sensitive and accurate, and, in contrast to GLC methods, does not involve derivatization.

EXPERIMENTAL

Reagents and materials

Clofibrinic acid [2-(4-chlorophenoxy)-2-methylpropionic acid] was a gift from Ayerst Laboratories (Montreal, Canada) and 2-(4-chloro-3-methylphenoxy)-2-methylpropionic acid, the internal standard, was a gift from Astra Pharmaceuticals (Södertälja, Sweden). The acetonitrile was of "distilled in glass" quality and was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.); all other solvents and reagents were of reagent grade.

Sample preparation

A schematic representation of the procedure is shown in Fig. 1. Plasma (0.1–1.0 ml), saliva (1.0 ml) or urine diluted 1:100 with distilled water (1.0 ml), is placed in a PTFE-lined screw-capped culture tube, and 100 μl of internal standard solution (containing 6.7 μg of the internal standard), 0.5 ml of 0.5 *N* sulphuric acid and 5 ml of toluene are added. The samples are extracted by mixing (using a Labquake® automatic shaker) for 10 min, followed by centrifugation at 1200 *g* for 10 min to separate the organic and aqueous phases. The lower aqueous phase is frozen by immersing the tube in a dry ice–acetone bath, and the organic phase is poured into another tube, which has an elongated cone (capacity approx. 50 μl) at its base. Then 50 μl of 0.2 *N* NaOH are added, and the mixture is extracted on a Vortex mixer for 2 min. After brief centrifugation, the aqueous phase is drawn into a syringe that already contains 10 μl of a solution of 5% glacial acetic acid in water, and this mixture is injected into the chromatograph.

For the analysis of the glucuronide conjugate of clofibrinic acid in urine, 5 ml

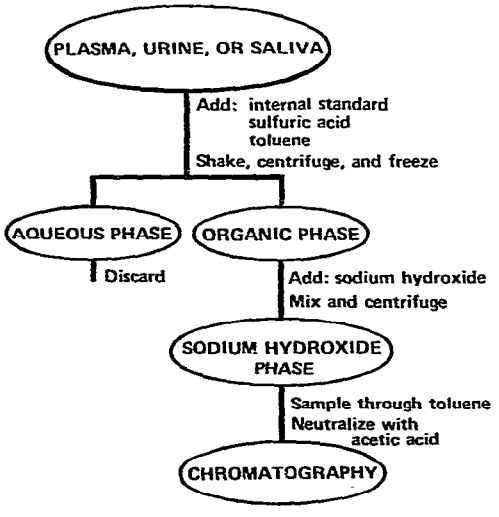

Fig. 1. Flow diagram of sample preparation.

of 6 *N* hydrochloric acid were added to each sample, and the solutions were heated at 98° for 30 min before the extraction. The samples were then cooled and analysed as described above, except that the addition of dilute sulfuric acid in the first step was omitted.

Chromatography

A Varian (model 8500) high-pressure liquid chromatograph fitted with a Varian MicroPak CH-10 reverse-phase column (25 cm × 6.3 mm O.D. × 2.2 mm I.D.) was used for the analysis. The absorbance was measured at 235 nm, with 0.5 absorbance unit full-scale deflection and a slit width of 2 nm, using a Varian Variscan variable-wavelength spectrophotometer. One pump of the dual-pump gradient-elution chromatograph contained acetonitrile and the other 0.5% acetic acid in distilled water; an isocratic 42% acetonitrile mixture of the two solvents was used. With a dual-pump chromatograph, it was convenient to use two pumps operating under isocratic conditions. However, an acceptable alternative was to use the desired mixture of the two solvents in a single pump. The flow-rate of the solvent mixture was 70 ml/h with a column-input pressure of 197 atm (2900 p.s.i.). Chromatograms were recorded on a Varian A-25 dual-pen recorder with 0–50 and 0–200 mV spans.

Calibration and accuracy

Calibration curves were constructed by adding known amounts of clofibrinic acid and internal standard to control plasma, saliva or urine. The peak-height ratio of clofibrinic acid to internal standard was plotted against the amount of clofibrinic acid added. In order to calibrate the method and determine its accuracy for each batch of unknown samples, standards of 1, 2, 5, 10, 15, 20 and 25 μg of clofibrinic acid were added to the control samples, which were assayed concurrently with the unknown samples. The peak-height ratio of each standard was divided by the amount of clofibrinic acid added to give normalized peak-height ratios. The mean normalized peak-height ratio was used to calculate the amount of clofibrinic acid in unknown samples, and the standard deviation of the normalized peak-height ratios was used to determine the accuracy of the method over the range of clofibrinic acid standards employed. The reproducibility of the method was also studied by submitting replicate plasma samples containing 1, 5, 10 and 20 μg of clofibrinic acid to the entire procedure. The effect of sample size on the method was investigated by adding 10 μg of clofibrinic acid to tubes containing different volumes of plasma (between 0.1 and 1.0 ml), which were then assayed for clofibrinic acid. The volumes of the internal-standard solution, sulphuric acid and toluene were kept constant.

To estimate the recovery for the analytical procedure, five control-plasma samples with 10 μg of clofibrinic acid added were analysed, and the mean height of the clofibrinic acid peaks was compared to the mean height of five peaks obtained by injecting 10 μg of clofibrinic acid directly into the chromatograph.

Application of the method to measure plasma and saliva concentrations

A healthy male volunteer received five doses of 1 g of clofibrate (2 capsules of Atromid-S®, Ayerst) every 12 h for 3 days. Samples of venous blood and saliva were collected at 0, 1, 2, 4, 6, 8, 13, 24 and 28 h after administration of the fifth and last dose. The blood (5 ml) was collected in heparinized Venoject® tubes, and, after cen-

trifugation, the plasma was transferred to glass containers, which were stored at −15° until analyzed.

Saliva samples were obtained by having the subject chew on a small PTFE disc; all the saliva produced during approx. 4 min just before blood sampling was collected in a glass vial. The saliva samples were immediately frozen and stored at −15° until analyzed.

RESULTS AND DISCUSSION

In order to be sufficiently volatile for GLC determination, clofibrinic acid must be derivatized, usually by methyl esterification of the carboxyl group. Methods of derivatization in which hydrochloric acid–methanol[4] or boron trifluoride–methanol[5] is used are time consuming, and other methods involve use of toxic and unstable reagents such as ethereal solutions of diazomethane[6,8,10]. The use of HPLC allows the determination of underivatized clofibrinic acid, thus shortening the sample preparation and avoiding use of toxic reagents.

The use of a reverse-phase column permits direct injection of an aqueous solution of the sample on to the column. Because of this, a simple procedure for sample preparation can be used (Fig. 1), which selectively extracts and concentrates acidic compounds. Such a technique is both more rapid and more selective than are those involving evaporation of solvent. The efficiency of this method of extraction is also high, as shown by the observation that 82% ± 8% (S.D.) of the clofibrinic acid added to plasma was actually injected into the chromatograph (see Experimental).

In order to achieve efficient separation of compounds with ionizable functions on reverse-phase columns, either the ionization must be suppressed, or a large counter-ion must be added and the compounds of interest chromatographed as ion-pairs[12]. For carboxylic acids such as clofibrinic acid, it is convenient to suppress ionization by adding acetic acid to the solvent. Acetic acid has appreciable ultraviolet absorbance, and therefore it would be desirable to keep its concentration low in order to reduce background absorbance and thus achieve a high signal-to-noise ratio. However, the need for rapid neutralization of the aqueous sodium hydroxide solution that is injected on to the column with each sample requires the use of an adequate amount of acetic acid. Failure to neutralize the sodium hydroxide gives rise to peak tailing and double peaks and results in deterioration of the column. For these reasons, the sodium hydroxide solution containing the sample is neutralized before injection on to the column, by drawing the sample into a syringe that contains 10 μl of aqueous 5% acetic acid.

Although clofibrinic acid has an absorption peak at 226 nm[1,2], the absorbance is measured at 235 nm in this method. This is because of the high background absorbance encountered at lower wavelengths, primarily a function of the acetic acid concentration. Since it is necessary to suppress the ionization of the clofibrinic acid with an acetic acid concentration in the eluting solvent mixture of about 0.3%, this wavelength was chosen as a compromise between sensitivity and the need to reduce the background absorbance. At 235 nm, good reproducibility is still attainable with 1 μg of clofibrinic acid, and concentrations as low as 0.5 μg/ml can be measured.

None of the plasma, saliva or urine control samples showed peaks interfering with the peaks of clofibrinic acid or the internal standard (Fig. 2a); a typical chro-

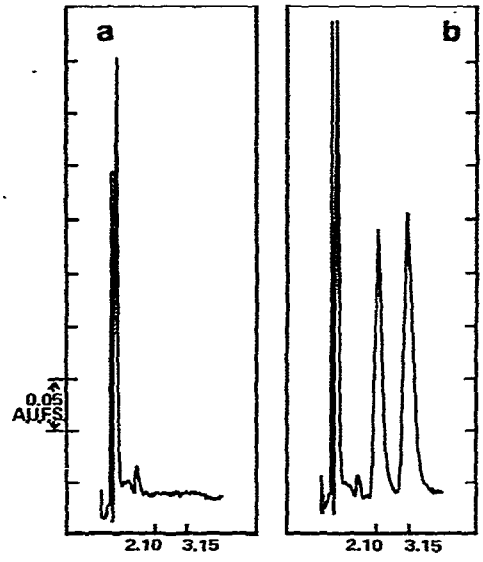

Fig. 2. Chromatograms of (a) control plasma and (b) plasma containing 5.0 μg of clofibrinic acid and the internal standard. The marks on the chromatograms correspond to the retention times of clofibrinic acid (2.10 min) and the internal standard (3.15 min). In order to improve visual clarity, only one tracing of the dual pen recorder is shown.

matogram for a plasma sample is shown in Fig. 2b. With the chromatographic conditions previously described, the retention times for clofibrinic acid and the internal standard are 2.10 and 3.15 min, respectively, allowing a sample injection to be made approximately every 5 min.

Estimates of accuracy for the method are shown in Table II. The average normalized peak-height ratio obtained from calibration curves from plasma, saliva

TABLE II

ESTIMATES OF ACCURACY FOR THE METHOD

Biological fluid	*Concentration, μg/sample*	*Number of samples*	*Mean normalized peak-height ratio*	*Coefficient of variation, %*
Calibration curve data				
Plasma	1–25	7	0.19935	2.9
Plasma	1–25	7	0.19102	5.0
Saliva	1–25	7	0.21027	6.3
Urine (untreated)	1–25	7	0.20114	4.8
Urine (acid hydrolysis)	1–25	7	0.18763	5.4
		Average:	0.19788	4.9
Reproducibility at a given concentration				
Plasma	1	7	0.21764	5.5
Plasma	5	3	0.20048	1.9
Plasma	10	5	0.19532	1.4
Plasma	20	5	0.21475	0.7
		Average:	0.20705	2.4

and urine had a mean coefficient of variation of 4.9% for 5 such calibration curves. This estimate of accuracy covers the entire range of the assay procedure, from 1 to 25 μg of clofibrinic acid per sample. Reproducibility studies on replicates containing 1, 5, 10 or 20 μg of clofibrinic acid per sample had a mean coefficient of variation of 2.4% (Table II). As it may be necessary to use variable volumes of plasma for clofibrinic acid measurements, the effect of plasma volume on the peak-height ratio of clofibrinic acid to internal standard was examined; the peak-height ratios were independent of the volume of plasma used between 0.1 and 1.0 ml.

Clofibrinic acid is excreted in the urine, some 70–95% of the dose being excreted as a glucuronide, and the rest as unconjugated acid[8,9,13]. On a typical dose regimen of 2 g per day, and assuming a daily urine output of 1–2 l, the total concentration in the urine is of the order of 1–2 mg/ml. By using 1 ml of 1:100 dilution of urine, the concentrations of both the unconjugated (untreated sample) and the conjugated clofibrinic acid (acid hydrolysed) in the urine fall within the concentration range of the analysis.

Application of the method to determination of clofibrinic acid in plasma and saliva from a healthy male volunteer is demonstrated in Fig. 3. Although the concentrations in saliva are only about 1% of those in plasma, the method is able to measure clofibrinic acid in both body fluids in a subject receiving a commonly employed dosing regimen. These results suggest the potential usefulness of saliva analysis for monitoring clofibrinic acid concentrations in patients.

The method described here for the quantitative determination of clofibrinic acid in plasma, saliva and urine by HPLC has the advantage over most other published methods of being simple and rapid (Table I). By using the techniques described, 40–50 samples can easily be assayed in a day. The sample preparation is a simple

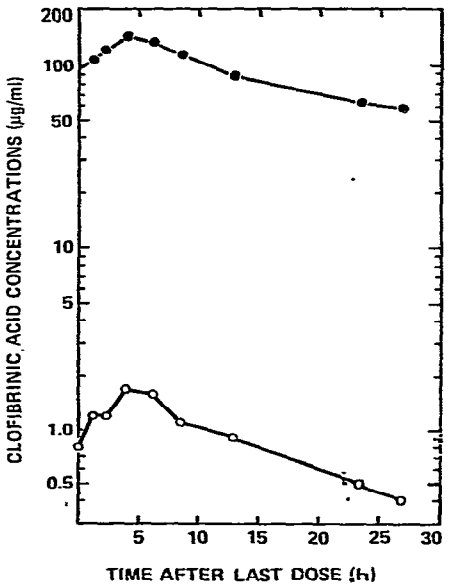

Fig. 3. Semi-logarithmic plot of plasma (●) and saliva (○) concentrations of clofibrinic acid in a healthy volunteer after the last dose of a multiple-dosing regimen of 2 g of clofibrate per day.

two-stage procedure (Fig. 1), which does not require the prior chromatographic preparation[3–5] or multiple extractions[6] described in published GLC methods (Table I). The use of a reverse-phase HPLC system permits the determination of underivatized clofibrinic acid and the internal standard and allows a simple method of sample preparation. Of the previously published methods, only the GLC analysis described by Gugler and Jensen[11] offers similar advantages of convenience (Table I). However, the present HPLC method offers a simpler approach to the analysis of clofibrinic acid than GLC methods by eliminating the need to form volatile derivatives before chromatographic separation.

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