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A RAPID GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLOROPHENOXYISOBUTYRIC ACID IN PLASMA AND URINE

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SUMMARY

A rapid gas chromatographic method is described for the determination of chlorophenoxyisobutyric acid (the active metabolite of clofibrate) in plasma and urine. The assay involves an extraction into toluene and back-extraction of the chlorophenoxyisobutyric acid and the internal standard (2-naphthoic acid) into the methylating reagent (trimethylanilinium hydroxide). Concentrations of 1 μg/ml in plasma and urine can easily be measured; the precision of the method is $3.3 \pm 0.7\%$ for plasma and $2.7 \pm 0.4\%$ for urine. There is no interference from endogenous compounds or from drugs commonly prescribed together with clofibrate.

INTRODUCTION

Clofibrate, the ethyl ester of 2-(4-chlorophenoxy)-2-methylpropionic acid, has been found effectively to lower plasma cholesterol and triglyceride levels in man[1–8]. Although its effectiveness is generally accepted as due to decreased lipoprotein production, its exact mode of action is still not clear[9]. Pharmacokinetic data on clofibrate are still scarce at present[10–13], and the relationship between the plasma concentration during chronic treatment and the hypolipidaemic effect of clofibrate has been neither established nor rejected.

Clofibrate is immediately and quantitatively hydrolyzed *in vivo* to *p*-chlorophenoxyisobutyric acid (CPIB). The methods currently available for determining CPIB in biological fluids are often based on spectrophotometry[14,15] and apparently lack specificity. Gas chromatographic (GC) methods recently described are tedious, since an additional thin-layer separation is involved[16], or several extraction steps are needed in order adequately to purify the sample for GC[17]; the method of Berlin also requires synthesis of an internal standard that is not available from commercial sources.

We have recently described a GC method for the determination of CPIB in plasma and urine[18] in which the parent compound, clofibrate, was used as internal standard. This seemed to be justified, as unchanged clofibrate itself was not detectable

at any time in plasma. The following disadvantages, however, made the assay unsuitable for application to large numbers of samples for routine or research purposes: (1) the internal standard, clofibrate, could only be added after extraction, evaporation and derivatization of the compound to be assayed (CPIB) and thus did not represent a true internal standard; (2) clofibrate is an ester and is difficult to handle, being easily subject to hydrolysis during the assay procedure; (3) the coefficient of variation was only 9.0%, mainly because of (1) and (2) above, and (4) the assay was time-consuming (only about 20 samples could be analysed in a working day).

This paper describes a simple, rapid and sensitive GC method for the determination of CPIB in plasma and urine; 2-naphthoic acid is used as internal standard, and flash-heater methylation is employed.

EXPERIMENTAL

Reagents

2-Naphthoic acid (purum) was obtained from Fluka (Buchs, Switzerland); the internal standard solution was prepared by dissolving 10 mg of 2-naphthoic acid in 500 ml of toluene. *p*-Chlorophenoxyisobutyric acid was a gift from Deutsche ICI (Plankstadt, G.F.R.). Trimethylanilinium hydroxide (TMA-OH) was prepared according to Brochmann-Hanssen and Oke[19]; 0.4 *M* TMA-OH in methanol was diluted with an equal volume of water before use. All solvents used were of reagent grade.

Gas chromatography

A Hewlett-Packard Model 5750 gas chromatograph was used, equipped with a flame ionization detector; the silanised glass column (6 ft. × 4 mm I.D.) was packed with 3% of SE-30 on 80–100 mesh Gas-Chrom Q and was operated at 150°, with a carrier gas (nitrogen) flow-rate of 25 ml/min; the injection-port temperature was 290°. The detector was operated at 270° with a hydrogen flow-rate of 20 ml/min and an oxygen flow-rate of 200 ml/min. Under these conditions, the retention times were 1.5 min for CPIB and 2.5 min for the internal standard.

Procedure

Venous blood (6 ml) from patients receiving clofibrate was drawn into heparinized tubes, and the plasma was separated by centrifugation. To 1.0 ml of plasma in a 15-ml glass tube were added 1.0 ml of 0.4 *M* hydrochloric acid and 6 ml of toluene containing 120 μg of the internal standard; the tube was then shaken for 5 min and centrifuged for 3 min at 4000 *g*. A 5-ml portion of the organic phase was transferred to a pointed centrifuge tube, TMA-OH (50 μl) was added, and the mixture was extracted on a vortex-mixer for 1 min. After brief centrifugation, 1 μl of the aqueous layer was injected directly into the gas chromatograph.

The determination of urinary CPIB was carried out essentially as described for plasma. For analysis of the glucuronide metabolite of CPIB in urine, the sample was diluted 1:10 with 0.2 *M* sodium acetate buffer of pH 5.0, and 4 ml of the diluted sample were incubated overnight with 2000 Fishman units of a glucuronidase–arylsulphatase preparation from *Helix pomatia* (Boehringer, Mannheim, G.F.R.).

When determining plasma concentrations of less than 10 μg of CPIB per ml,

the concentration of internal standard added was reduced to one tenth of that originally used.

Standard graphs for the determination of CPIB in plasma and urine were prepared by assaying a series of blank plasmas and urines, to which known amounts of CPIB had been added, in the way described above. The ratio of the peak height for CPIB to that for the internal standard was calculated and plotted against the known concentration.

RESULTS AND DISCUSSION

Figs. 1 and 2 show calibration graphs for determining CPIB in plasma and urine, respectively, over the concentration range 10–200 μg/ml. Although these con-

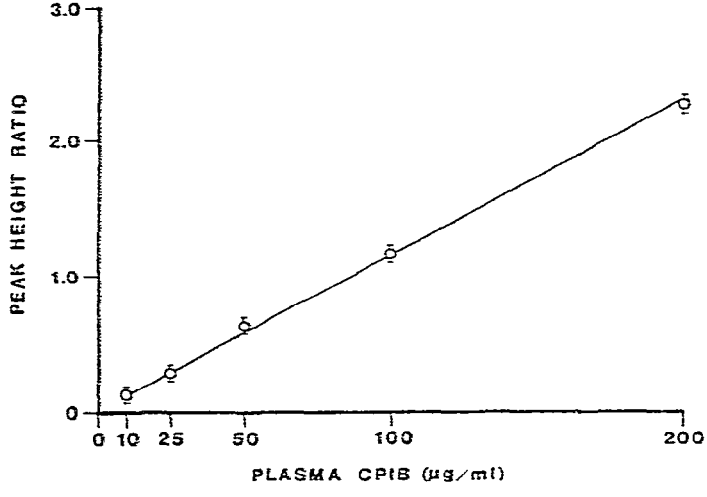

Fig. 1. Calibration graph for CPIB following extraction from plasma.

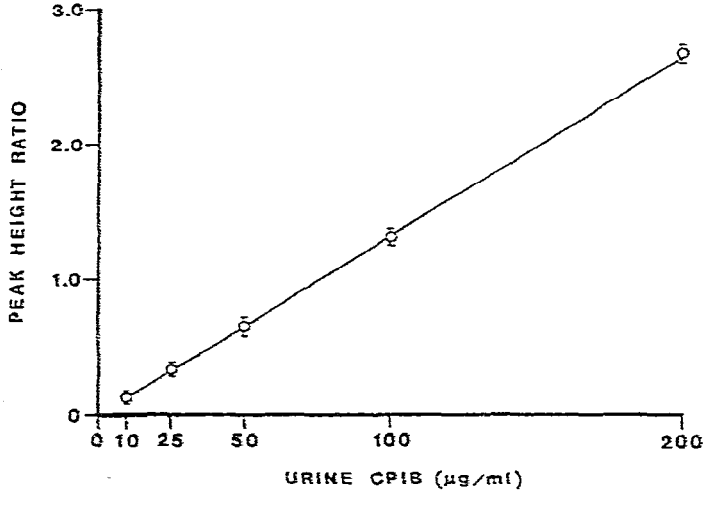

Fig. 2. Calibration graph for CPIB following extraction from urine.

centrations cover the range to be expected when clofibrate is administered chronically[15] in the doses usually recommended[11], it might be desirable in the course of pharmacokinetic studies to measure concentrations as low as 1 μg/ml. For this purpose, the amount of internal standard added is cut down to one tenth (12 μg) and the ana-

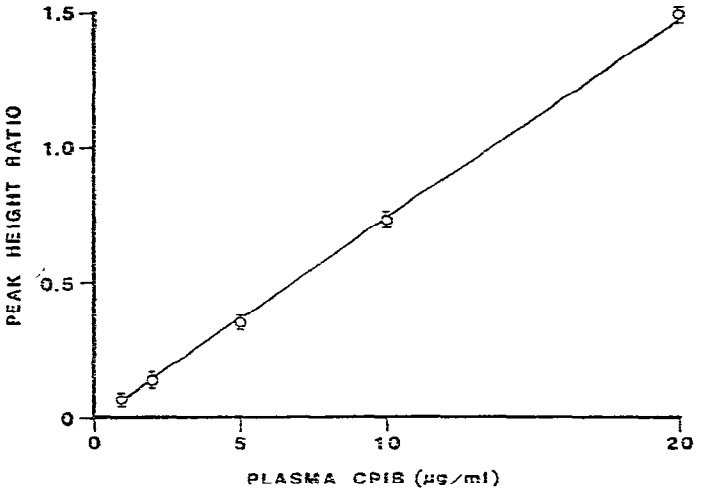

Fig. 3. Calibration graph for plasma CPIB in the concentration range 1–20 μg/ml.

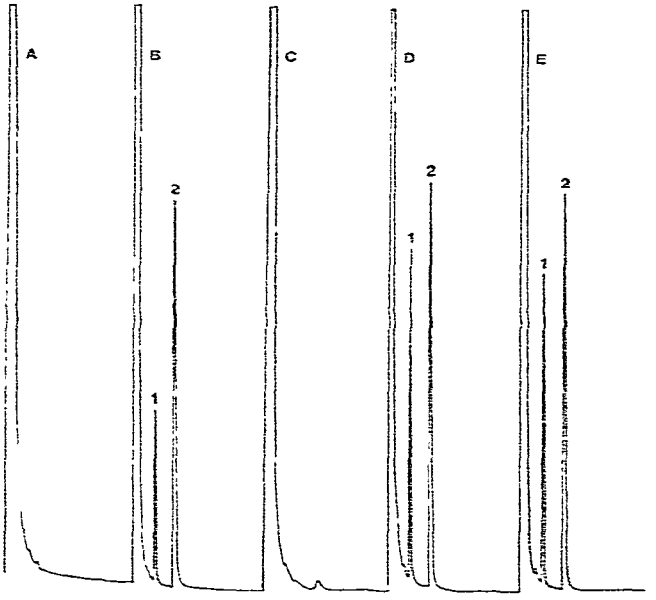

Fig. 4. Gas chromatograms for: (A) material extracted from blank plasma; (B) material extracted from blank plasma to which CPIB (50 μg/ml) and internal standard (120 μg) have been added; (C) material extracted from blank urine; (D) material extracted from blank urine to which CPIB (80 μg/ml) and internal standard (120 μg) have been added; (E) material extracted from plasma obtained from a patient receiving clofibrate. The peaks indicated are: (1) CPIB; (2) internal standard (2-naphthoic acid).

lytical procedure is applied without further modification (see Fig. 3). All standard graphs were rectilinear over the concentration ranges tested.

A typical chromatogram is shown in Fig. 4, from which it can be seen that no interfering peaks are produced by co-extracted endogenous compounds. The chromatogram (E) of a sample from a patient suffering from hyperlipoproteinaemia shows no additional peaks due to free fatty acids that have been encountered in one other method[20].

The coefficient of variation was determined by assaying six samples at each of the concentrations 10, 20, 50, 100 and 200 μg/ml in both plasma and urine. The coefficient of variation was 3.3% for plasma and 2.7% for urine, *i.e.*, reproducibility was good.

The one extraction into toluene proved to be sufficient to obtain good recovery; the recovery was 71 $\pm$ 2% (n = 10) from plasma and 91 $\pm$ 2% (n = 10) from urine.

The specificity of the method was assessed by assaying plasma and urine samples from patients taking other drugs. Butylbiguanide, digoxin, ethacrinic acid, furosemide, glibenclamide, α-methyldopa, oxifedrine, reserpine and spironolactone were tested and did not interfere with the assay when administered in normal therapeutic doses.

With the method presented here, plasma and urine samples from patients receiving clofibrate can be assayed rapidly and with good accuracy. Twenty samples can easily be analysed in 2 h.

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