Determination of clofibrate in biological fluids by thin-layer and gas–liquid chromatography

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Abstract A specific and sensitive method is described for the detection of clofibrate in biological fluids. The drug is separated from associated fatty acids by thin-layer chromatography and the methyl ester is quantified by gas–liquid chromatography. Recovery is excellent, and any small losses are corrected with an internal recovery standard. Although more time-consuming than other available techniques, the method offers advantages for accurate studies of clofibrate metabolism.

Supplementary key words p-chlorophenoxyisobutyric acid · internal standards · tritiated clofibrate

A method is described for the isolation and quantification of clofibrate as the free acid (p-chlorophenoxyisobutyric acid, CPIB) in plasma, urine, bile, and feces, employing thin-layer and gas–liquid chromatography.

Two procedures for the determination of clofibrate in biological fluids have already been published. The method of Thorp (1) utilizes spectrophotometry, whereas that of Horning et al. (2) involves gas–liquid chromatography (GLC); recoveries by the former method are known to be incomplete for plasma, and in the latter report the question is not addressed. The method developed in this laboratory utilizes thin-layer chromatography (TLC) to separate clofibrate from other fatty acids and depends for its quantitative aspects on the use of an internal recovery standard, p-chlorophenoxypropionic acid (CPP), and a GLC standard, arachidic acid (20:0). Quantitation is by GLC of the methyl esters of CPIB, CPP, and 20:0, with detection by hydrogen flame ionization. The method is specific, sensitive, and reproducible, and overall recoveries of clofibrate from biological materials are high (>93% for plasma and urine and >84% for bile and feces).

MATERIALS AND METHODS

Standards

The internal recovery standard CPP was obtained from Pfaltz and Bauer, Inc., Flushing, N.Y. The acid was methylated with 5% HCl–methanol, and the methyl ester was purified by TLC, using silica gel H and benzene.

The standard solution of MeCPP contained 260 µg/200 µl.

The GLC standard, arachidic acid methyl ester (Me 20:0), was obtained from Schwarz/Mann, Orangeburg, N.Y. The standard solution contained 140 µg/200 µl.

Thin-layer chromatography was carried out on 0.5-mm layers of silica gel H (EM Reagent, distributed by Brinkmann Instruments, Inc., Westbury, N.Y.) on 20 × 20 cm plates. Plates were uniformly developed in toluene–hexane 80:20, solutes were visualized with iodine vapor, and recoveries were made by vacuum aspiration according to Goldrick and Hirsch (3), followed by elution with diethyl ether.

Gas–liquid chromatography was carried out with an instrument equipped with a hydrogen flame ionization detector (F & M biomedical gas chromatograph, model 400, Avondale, Pa.). A 6-ft column, 4 mm ID, was packed with 10% EGSS-X on Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Nitrogen was used as carrier gas at a flow rate of 24 ml/min with an inlet pressure of 40 psi. Temperatures were as follows: column, 180°C; flash heater, 220°C; and detector, 240°C. An electronic integrator was used for quantitation of peak areas (digital readout model CRS-10, Infotronics, Houston, Texas).

Radioactive clofibrate. Randomly tritiated clofibrate was generously supplied by Sandoz Pharmaceuticals, Hanover, N.J. (Dr. Alan Timms). It was purified by TLC, using silica gel H and benzene. For oral administration to patients, radioactive clofibrate was dissolved in a small volume of ethanol and mixed with fruit juice.

Radioactivity measurements were performed with a Packard Tri-Carb liquid scintillation spectrometer (model 3380). Counting efficiencies were approximately 40% for ^3H and 52% for ^{14}C. Measurements were done in PPO–POPOP–toluene solution prepared from Liquifluor (Pilot Chemicals, Watertown, Mass.). Direct radioactivity counting of plasma (250 μl) and urine (0.5 ml) was carried out using Aquasol (New England Nuclear, Boston, Mass.). Measurements of radioactivity in bile and feces were made only after extraction. Quenching corrections were performed automatically by an Absolute Activity Analyzer (Packard Instruments, model 544).

Procedure

Saponification. 1 ml of plasma, urine, or bile or 1 g of feces was mixed with 10 ml of methanol in a 125-ml glass-stoppered bottle, and 200 μl of the internal recovery standard MeCPP was added. This mixture was refluxed for 1 hr with 1 ml of 10 N NaOH in order to hydrolyze any conjugated CPIB that might be present.

Extraction. After cooling, the mixture was acidified with 1 ml of concentrated HCl. 20 ml of chloroform and 30 ml of chloroform–methanol 2:1 were then added successively, and the mixture was shaken for 1 min; 10 ml of water was added and the shaking was continued for 1 min. After centrifugation (5 min at 1000 g) the lower phase containing CPIB and CPP was transferred to a 500-ml round-bottomed flask. 20 ml of chloroform was added to the upper phase, and the procedure was repeated twice in order to assure a quantitative transfer. The combined lower phases were evaporated to dryness with a rotary evaporator.

Methylation. 5 ml of 5% HCl–methanol was added to the dried residue and the mixture was left overnight; the methyl esters were concentrated by rotary evaporation. Because of the volatility of MeCPIB and MeCPP, the evaporation must be carried out at room temperature; *care must be taken to stop the evaporation as soon as the residue appears dry.*

Thin-layer chromatography. MeCPIB, MeCPP, and the other methyl esters were quantitatively transferred with chloroform–methanol 2:1 to TLC plates. Because of the large amount of fatty acids in plasma, bile, and feces, one TLC plate is required for each sample in order to obtain a clean separation of the MeCPP and MeCPIB from the other fatty acids. A standard mixture of MeCPIB and MeCPP was applied at one side of the plate. MeCPIB and MeCPP chromatograph together ($R_F = 0.41$), separated from other fatty acids ($R_F > 0.47$, **Table 1**). The portion of the plate corresponding to the standard mixture was recovered, and solutes were eluted into a 100-ml round-bottomed flask. At this stage, 200 μl of Me 20:0, the GLC internal standard, was added. The mixture was again cautiously evaporated to dryness and redissolved in

Me CPP Me 20:0

Me CPIB

Detector response

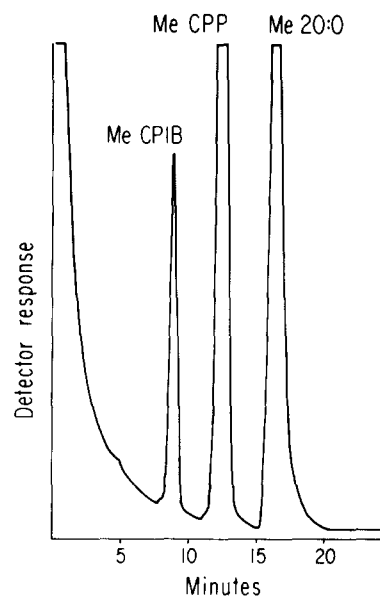5   10   15   20

Minutes

Fig. 1. Gas–liquid chromatographic analysis of the methyl esters of CPIB, CPP, and 20:0.

1 ml of methanol. Radioactivity was counted on a 0.5-ml aliquot, when appropriate, and the remainder was utilized for GLC.

Gas–liquid chromatography. 5 μl of the sample was used for GLC. The first peak in the chromatogram (**Fig. 1**) is that of MeCPIB, with a retention time relative to Me 20:0 of 0.55, and the internal recovery standard MeCPP appears at a relative retention time of 0.75.

Table 2 relates the hydrogen flame ionization responses of MeCPIB, MeCPP, and Me 20:0. The area response of MeCPIB is almost identical with that of MeCPP. However, the area response of MeCPP is only 63% of that of Me 20:0, so that an appropriate correction factor is required when calculating MeCPP recovery rela-

TABLE 1. R_F values and relative retention times of methyl esters of plasma fatty acids, CPIB, CPP, and 20:0

Compound	TLC R_F Value[a]	GLC Relative Retention Time[b]
Me 14:0	0.50	0.20
Me 16:0	0.50	0.35
Me 16:1	0.50	0.43
Me 18:0	0.53	0.58
Me 18:1	0.50	0.71
Me 18:2	0.47	0.91
MeCPIB	0.41	0.55
MeCPP	0.41	0.75
Me 20:0	0.50	1.00

[a] R_F values (relative to the solvent front) were obtained by chromatographing the samples on silica gel H plates in toluene–hexane 80:20.

[b] GLC retention times are relative to Me 20:0, using 10% EGSS-X on Gas-Chrom Q.

TABLE 2. Hydrogen flame ionization responses of MeCPIB, MeCPP, and Me 20:0

GLC injection mixture (μg)		MeCPIB Observed (μg)	Hydrogen flame ionization response of MeCPIB relative to MeCPP (%)
MeCPIB	MeCPP		
339.9	297.4	343.6	101
283.2	297.4	285	101
226.6	297.4	225	99
170	297.4	166.4	98
113.3	297.4	108.7	96
56.7	297.4	56.9	100

GLC injection mixture (μg)		MeCPP Observed (μg)	Hydrogen flame ionization response of MeCPP relative to Me 20:0 (%)
MeCPP	Me 20:0		
446.2	145.8	281	63
390	145.8	261.3	67
260	145.8	156	61
195	145.8	122.9	61
149	145.8	90.8	63
130	145.8	81.9	63

TABLE 3. Recovery of MeCPIB and MeCPP after evaporation to dryness of a mixture under differing conditions[a]

Conditions of Evaporation	% Recovery		MeCPIB/ MeCPP
	MeCPIB	MeCPP	
Vacuum at 25°C	100	97	1.03
	101	100	1.01
Vacuum at 40°C	97	93	1.03
	79	72	1.09
Vacuum at 50°C	73	70	1.03
Nitrogen stream at 25°C	63	61	1.05
	87	86	1.01
Nitrogen stream at 40°C	18	20	0.98
	15	17	0.88

[a] Recoveries were computed by GLC, using Me 20:0 as recovery standard.

tive to that of Me 20:0. This factor has been verified by several other experiments in which the proportions of the three constituents were widely varied.

Calculation. The amount of CPIB in the sample is calculated in terms of the CPP recovery standard as follows: μg of CPIB in 1 ml of plasma = (MeCPIB area response/MeCPP area response) × 260. The recovery of MeCPIB (and/or MeCPP) is calculated as follows, utilizing the correction factor of 100/63: % recovery of MeCPIB (= % recovery of MeCPP) = (MeCPP area response/Me 20:0 area response) × 140 × (100/63) × (100/260).

RESULTS

Suitability of the internal recovery standard

Losses of clofibrate occur at various stages in this procedure, necessitating the use of the overall recovery standard CPP. Up to the methylation step the drug is in the form of its free acid (mp 120°C), which is much less likely to be lost during the extraction procedure than is the more volatile MeCPIB (bp 138°C at 18 mmHg) during and after TLC. Using plasma containing ^{3}H-labeled CPIB, losses amounted to 1.2% up to the TLC step. During and after TLC, losses of both MeCPIB and MeCPP occur by evaporation but, as seen in **Table 3**, these losses are almost identical: rotary evaporation at 25°C is clearly preferable to use of a nitrogen stream at that temperature.

Specificity

During TLC, MeCPIB is usually cleanly separated from the other fatty acids present in plasma, bile, and feces. Occasionally, however, small amounts of fatty acids are eluted with MeCPIB and MeCPP and appear in the

final sample analyzed by GLC. The GLC peaks of Me 14:0, Me 16:0, and Me 16:1 appear before the peak for MeCPIB and in no way interfere with its quantification. However, the relative retention time of Me 18:0 (0.58) is so close to that of MeCPIB (0.55) that isolation of MeCPIB from fatty acid methyl esters by TLC must be complete in order to accurately estimate CPIB. Therefore, one should adhere to the operational rule that, when the GLC pattern indicates a significant contamination of the pattern by fatty acid methyl esters, the sample should be subjected to repeat TLC in which only the trailing part containing the drug is recovered. Significant contamination is indicated by the finding of measurable peaks other than those of the methyl esters of CPIB, CPP, and 20:0.

Sensitivity

Assuming 80% recovery and the utilization of 5 μl of sample for GLC, the method as described detects levels of clofibrate less than 10 μg per ml of plasma, urine, or bile or per g of feces. If smaller quantities of the drug are anticipated, decreasing the volume of methanol added at the end of the procedure to 0.5 ml will increase detection sensitivity to less than 5 μg/ml. If radioactivity counting is not required, solution of the final mixture of methyl esters can be carried out with as little as 10 μl of methanol, in which case the sensitivity is increased to 0.1 μg/ml.

Recovery and precision

To determine the actual recovery of clofibrate from biological fluids (uncorrected by use of the internal recovery standard CPP), two approaches were used. The first involved the addition of unlabeled clofibrate in the form of the free acid CPIB to plasma and urine. The results shown in **Table 4** indicate that the recovery from plasma was 94% and from urine 93%. In similar tests, recoveries were 85% from feces (range 80–91%) and 84% from bile (range 83–85%).

In the second approach, plasma and urine from a subject given ^{3}H-labeled clofibrate by mouth was used.

TABLE 4. Recovery experiments

	Recovery of unlabeled CPIB from plasma			
CPIB added to 1 ml of plasma, μg	53.68	107.36	161	214.72
% Recovery of added CPIB, mean ± SD (n)	96 ± 11 (4)	96 ± 9 (4)	94 ± 7 (4)	91 ± 6 (4)
		Mean recovery 94%		
Coefficient of variation, %	11	9	7	7
	Recovery of unlabeled CPIB from urine			
CPIB added to 1 ml of urine, μg	53.68	107.36	214.72	322
% Recovery of added CPIB, mean ± SD (n)	85 ± 3 (4)	91 ± 2 (4)	96 ± 4 (4)	98 ± 7 (4)
		Mean recovery 93%		
Coefficient of variation, %	4	2	4	7

The recovery of clofibrate was calculated by comparing the total counts in plasma and urine with those obtained after extraction of the drug by the procedure described above. Recovery of labeled clofibrate from plasma was 92 ± 6% and from urine 91 ± 4%.

Reproducibility of the method was assessed by running eight sets of replicate samples. Table 4 shows that the mean coefficient of variation was 6%, with a range of 2–11%.

DISCUSSION

The method described can be utilized for accurate and specific detection of clofibrate levels in plasma, urine, bile, and feces.[3] We have not applied it for analyses in other tissues.

In patients on long-term clofibrate therapy (daily oral dose of 2 g), considerable amounts of the drug are present in the urine and substantial quantities in the feces. Fasting plasma levels of clofibrate are usually greater than 100 μg/ml, and fasting biliary levels are greater than 50 μg/ml (4). As the method can detect clofibrate levels as low as 0.1 μg/ml, its sensitivity appears to be satisfactory for further pharmacokinetic studies.

Recovery of clofibrate is greater than 90% from plasma and urine and greater than 80% from bile and feces. But whatever the losses, the properties of the internal recovery standard MeCPP are such that a precise correction can be made in the final calculation of MeCPIB. This feature of the method suggests that "shortcuts" can be usefully applied, such as a single extraction of the acidified saponification mixture with chloroform–methanol 2:1 (88% recovery from plasma) or a single transfer of the methyl ester mixture to the TLC plate (79% recovery from plasma). Although final yields are reduced, the internal recovery standard compensates adequately. More rapid methods of methylation also may be useful.

The assay of clofibrate in biological fluids as carried out by the spectrophotometric technique devised by Thorp (1)

is stated to give excellent recoveries from urine but only 68% recoveries from serum and tissues. We have verified that the Thorp method is nearly quantitative for urine, but in our hands the values for plasma are 40–45% of those determined by the present procedure. Thus, the method described in this report improves on recovery from plasma, introduces a reliable internal recovery standard to correct for losses during the procedure, and depends for actual assay on the more specific method of GLC. One operator can complete the analysis of 32 samples in 5· days by this approach. The less specific spectrophotometric method of Thorp (1) allows a fivefold greater accumulation of data and hence is to be preferred as a screening procedure. The present method, however, offers advantages for accurate studies of drug metabolism. ▪

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[3] Adipose tissue has also been examined, but to date no clofibrate has been found in the tissue from treated patients.