Journal of Chromatography, 106 (1975) 97–102

CHROM. 8055

QUANTITATIVE DETERMINATION OF *p*-CHLOROPHENOXYISOBUTYRIC ACID IN BLOOD PLASMA BY GAS–LIQUID CHROMATOGRAPHY

TUONG CHI CUONG and ANNE TUONG

Department of Drug Metabolism, Castaigne, S.A., 195, route d'Espagne, 31023 Toulouse Cedex (France)

(Received October 16th, 1974)

SUMMARY

A gas–liquid chromatographic method for the determination of *p*-chlorophenoxyisobutyric (CPIB) acid in blood plasma is described. The substance is extracted from acidified plasma into benzene, the extract is evaporated to dryness and the residue is methylated with an alcohol-free solution of diazomethane and submitted to chromatography on a glass column packed with 3% OV-17. Data on the recovery, reproducibility and sensitivity of the method are given. CPIB acid plasma levels in rats after acute and prolonged treatments with the aluminium salt of CPIB acid (Atherolip) are also presented.

INTRODUCTION

The derivatives of *p*-chlorophenoxyisobutyric (CPIB) acid are widely used in the medical field for treatment of hyperlipidaemia. Their effect in reducing plasma lipid levels (triglycerides and cholesterol) has been demonstrated[1,2], but metabolic studies suffered from a lack of a specific method for the quantitation of CPIB acid in biological fluids.

To our knowledge, all of the described methods, except one, involved ultraviolet absorption measurements of CPIB acid itself[3–6] or of its hydrolysis product, *p*-chlorophenol[7], and differed only in the extraction solvent used: isooctane–ethanol[3,4], methylene chloride[5], heptane–isoamyl alcohol followed by re-extraction into phosphate buffer[6], and chloroform[7].

A gas chromatographic method for the determination of CPIB ethyl ester in biological fluids was developed by Silvestri[8]. The procedure relied on extraction of the ester from acidified plasma into diethyl ether–light petroleum. After washing, drying over sodium sulphate and evaporation of the solvent, the residue was subjected to chromatographic analysis on a 5% butanediol succinate column. So far as CPIB acid itself was concerned, it was indicated briefly that the residue was esterified after adsorption on Amberlite IRA-400 resin by treatment with dry hydrochloric acid in methanol. Chromatograms of the chemicals but not of biological extracts were given. As the main plasma metabolite of all known CPIB acid derivatives is the acid itself,

it is of prime importance that, after methylation, no biological impurities are eluted with the same retention time as CPIB methyl ester.

This paper describes a convenient procedure for the determination of CPIB acid that is specific and sensitive enough for use in pharmacokinetic studies.

EXPERIMENTAL

Reagents and materials

All the reagents used were of analytical grade: perchloric acid, benzene and *n*-hexane (Merck, Darmstadt, G.F.R.); octadecane (Fluka, Buchs, Switzerland); N-methyl-N-nitroso-*p*-toluenesulphonamide (Diazald; Aldrich, Milwaukee, Wisc., U.S.A.); 2-(2-ethoxyethoxy)ethanol, potassium hydroxide and diethyl ether (Prolabo, Paris, France) were used for the preparation of diazomethane.

Octadecane in *n*-hexane (100 mg/l and 250 mg/l) was used as external standard. Diazomethane was prepared freshly every 2 days and kept refrigerated.

Apparatus

A Hewlett-Packard Model 5751G gas chromatograph fitted with a flame ionization detector (FID) and a Varian Aerograph Model A-25 1-mV recorder were used. The electrometer output was recently connected to a laboratory data system (Hewlett-Packard Model 3352B, consisting of an HP 2100 A computer, 16-bit word, 12K of core memory and an HP 2752A teleprinter) through an A/D HP 18652A converter. All gases were purified on molecular sieve gas filters (Hydro-Purge, Applied Science Labs., State College, Pa., U.S.A.). Diverter valves (Loenco, Altadena, Calif., U.S.A.) mounted on all gas inlet lines to the detector facilitated the control of the fluid flows. A coiled glass column, 6 ft. × 2 mm I.D., packed with 3% (w/w) OV-17 on Chromosorb W HP, 100–120 mesh, was conditioned overnight at 250° with a low helium flow-rate (5–7 ml/min).

The chromatographic conditions were as follows: injector temperature, 180°; detector temperature, 210°; column initial temperature, 150° for 6 min, then programmed to 195° at 10°/min and held at the final temperature for 5 min, before recycle. The flow-rates were: helium carrier gas, 25 ml/min; helium auxiliary gas, 35 ml/min; air, 400 ml/min; hydrogen, 40 ml/min.

Extraction procedure

A volume of 1.0 ml or less of plasma was diluted with distilled water to a final volume of 2.0 ml. The mixture was acidified with 1.0 ml of 0.4 *M* perchloric acid and extracted with 5.0 ml of benzene by gentle shaking for 15 min in an Omni-Shaker (Buchler Instruments, Fort Lee, N.J., U.S.A.) at 20 cycles/min. After centrifugation for 15 min at 1000 *g* (Wifug Type X-1 centrifuge, Wifug, Stockholm, Sweden), 4.0 ml of the organic phase were transferred into a tapered 7-ml sample vial and evaporated to dryness under a stream of nitrogen in a water-bath at 40°.

Methylation procedure

An alcohol-free ethereal solution of diazomethane was prepared according to De Boer[9], but scaled down ten-fold. This methylating agent was added dropwise to the above dry residue until a persistent yellow colour was obtained. The mixture was

kept for 10 min in an ice-bath. The solvent and the excess of diazomethane were then carefully removed under vacuum, while the sample vial was cooled to −10° in an ice–salt mixture. The residue was immediately dissolved in an appropriate volume of octadecane and 1–4 μl of the solution were injected into the chromatograph by means of a 10-μl microsyringe (Terumo, Tokyo, Japan).

Calculations

Manual method. A calibration graph was constructed from chromatograms of plasma spiked with known amounts of CPIB acid (Fig. 1) by plotting the peak height ratio between CPIB methyl ester and octadecane against the weight ratio between CPIB acid and octadecane (Fig. 2). Values for unknown plasma concentrations of CPIB acid were calculated by using the slope of the calibration graph.

Computer method. As the chromatograph was connected to the laboratory data system, construction of a calibration graph was no longer necessary except for testing visually the linearity of the relationship of the area ratio to weight ratio.

The average response factor of CPIB methyl ester to standard was determined by the computer after multiple injections of known amounts of CPIB acid (methylated) and octadecane. Different parameters for the integration and peak identification, defined by the operator, were then programmed into the system. Immediately after completion of the allowed time of analysis, the report was printed out in the specified format.

RESULTS AND DISCUSSION

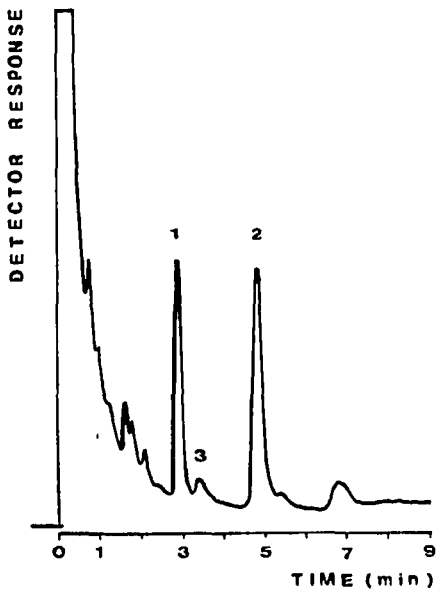
Methodology

The method involved a unique extraction with 5 ml of solvent per millilitre of plasma. No washing of the organic phase or drying over sodium sulphate was needed, careful centrifugation and pipetting being used instead.

The retention times of CPIB methyl ester and of octadecane (external standard) were 3 and 5 min, respectively (Fig. 1). Although the analysis was complete within 6 min, a cycle time of about 16 min with temperature programming was necessary for purging high-boiling substances of plasma from the column.

Fig. 2 indicates a linear relationship between the peak height ratio between CPIB methyl ester and octadecane and the weight ratio between CPIB acid and octadecane. The equation for the regression line of best fit was $y = 0.6997x - 0.0954$ (correlation coefficient 0.999). The results in Table I show the apparent recovery in a 4.0-ml aliquot from 5.0 ml of extraction solvent to be nearly 70% for both series of experiments employing different calculation methods; however, with the computer system better reproducibility and linearity were obtained.

The sensitivity of the FID towards pure CPIB methyl ester was 1 ng, but with plasma extracts the limit of detection increased considerably to 50 ng injected, *i.e.*, 250 ng/ml in plasma. An unknown component in the blank plasma, eluted as peak number 3 just behind CPIB methyl ester (Fig. 3), prevented better sensitivity from being attained. However, if the need arose, higher sensitivity could be achieved by introducing a purification step before the methylation procedure by whirling the dry residue with 1.0 ml of methanol and 3.0 ml of *n*-hexane on a vortex mixer (Scientific Industries, Springfield, Mass., U.S.A.) for 1 min, discarding the hexane phase,



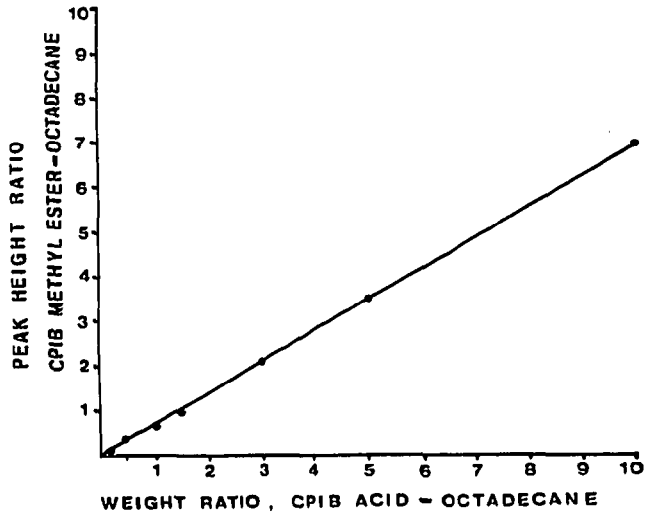


Fig. 1. Gas chromatogram of a plasma extract containing CPIB methyl ester (1) and octadecane (2). Peak (3) is impurity from plasma.

Fig. 2. Calibration graph constructed from analyses of plasma samples with known weight ratios of CPIB acid to octadecane (manual method of calculation).

evaporating the methanol phase to dryness under a stream of nitrogen, and then methylating as previously described under *Methylation procedure*.

This modification was very efficient in removing the interfering peak number 3, but also impaired the recovery of CPIB acid, which decreased from 70% to 35%. However, the overall sensitivity of detection was increased to 5 ng injected, corresponding to 50 ng/ml in plasma.

TABLE I

RECOVERY OF CPIB ACID FROM PLASMA

*Amount added** (μg)	*Manual method by peak height*		*Computer method by area*	
	*Amount found (μg)***	*Recovery (%)****	*Amount found (μg)***	*Recovery (%)****
0.4	—	—	0.28	69.50 ± 1.95
0.6	0.38	63.88 ± 4.20	0.41	67.95 ± 1.93
1.0	0.68	68.00 ± 2.90	0.69	69.63 ± 1.30
2.0	1.38	69.00 ± 2.29	1.39	69.58 ± 0.67
5.0	3.52	70.42 ± 2.23	3.50	70.00 ± 1.59
10.0	6.80	68.00 ± 1.61	6.91	69.11 ± 1.03
15.0	10.17	67.80 ± 2.14	10.50	69.35 ± 1.60
20.0	13.81	69.03 ± 2.30	13.83	69.15 ± 0.94
30.0	20.92	69.73 ± 2.01	20.99	69.90 ± 1.67
50.0	34.94	69.88 ± 1.51	—	—
100.0	69.90	69.90 ± 1.49	—	—

* The plasma volume was 1 ml in all instances.
** Mean of three determinations.
*** Average percentage recovery ± standard deviation.

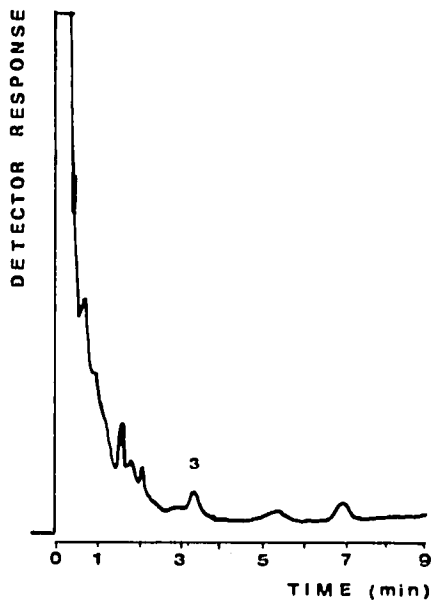


Fig. 3. Gas chromatogram of a blank plasma extract. Peak number 3 is impurity from plasma.

Animal experiments

During toxicological experiments, rats received the aluminium salt of CPIB (Atherolip, Laboratoire Solac, Toulouse, France). The acute doses were 360 and 450 mg/kg with and without concomitant treatment with vitamin B_6 as indicated in Table II. The details of the long-term administration are given in the footnotes to Table III. Blood samples were collected on heparin at 2 h and/or 5 h after dosing. In all instances, 0.1 ml of plasma was used for analysis.

Table II shows that the addition of vitamin B_6 did not have a clear influence upon the absorption of Atherolip.

TABLE II

CPIB ACID PLASMA LEVEL IN MALE RATS AFTER ORAL ADMINISTRATION OF ATHEROLIP WITH OR WITHOUT ADDITION OF VITAMIN B_6

The plasma volume analyzed was 0.1 ml in all instances.

*Rat weight (g)**	*Dose (mg/kg)*		*CPIB acid plasma concentration (μg/ml)***	
	Atherolip	*Vitamin B_6*	*2 h*	*5 h*
172.7 ± 8.2	360	—	156.9 ± 17.7 (10)	141.8 ± 38.5 (10)
170.7 ± 9.7	360	8	146.5 ± 43.0 (10)	136.0 ± 17.5 (10)
174.9 ± 12.8	450	—		171.9 ± 15.6 (10)
171.2 ± 11.9	450	10		195.2 ± 30.8 (10)

* Mean weight ± standard deviation.

** Mean concentration ± standard deviation (number of animals given in parentheses).

At 5 h, the overall mean plasma level was about 139 μg/ml for a dose of 360 mg/kg of Atherolip and attained 183 μg/ml for the 450 mg/kg dose.

After prolonged administration, again no influence of vitamin B_6 occurred, but female rats displayed a significantly lower CPIB acid plasma concentration (481 μg/ml) than male rats (724 μg/ml), 2 h after the last dose of 450 mg/kg of Atherolip.

TABLE III

CPIB ACID PLASMA LEVEL IN RATS AFTER PROLONGED ORAL ADMINISTRATION OF ATHEROLIP WITH OR WITHOUT ADDITION OF VITAMIN B_6

The plasma volume analyzed was 0.1 ml in all instances. Animals were treated during 7 weeks, 5 days/week, the first 2 weeks with 450 mg/kg·day of Atherolip with or without 10 mg/kg·day of vitamin B_6, the last 5 weeks with 1350 mg/kg·day of Atherolip with or without 10 mg/kg·day of vitamin B_6, before receiving the last dose.

Rat		*Last dose (mg/kg)*		*CPIB acid plasma concentration (μg/ml)** at 2 h*
Sex	*Weight (g)**	*Atherolip*	*Vitamin B_6*	
Male	318.5 ± 41.4	450	—	728.1 ± 193.0 (13)
	297.8 ± 57.2	450	10	719.9 ± 226.1 (8)
Female	244.4 ± 15.2	450	—	476.1 ± 193.1 (13)
	219.4 ± 8.9	450	10	486.1 ± 159.0 (8)

* Mean weight ± standard deviation.

** Mean concentration ± standard deviation (number of animals given in parentheses).

ACKNOWLEDGEMENT

The authors are grateful to Mrs. Eveline Salles for providing skilful technical assistance.

REFERENCES

1 E. Edmond, J. Daver, M. Daver, J. Blanchard, M. Ricoeur and C. C. Tuong, *Thérapeutique*, 46 (1970) 55.
2 D. Steinberg, in R. J. Jones (Editor), *Atherosclerosis: Proceedings of the 2nd International Symposium*, Springer, New York, 1970, p. 500.
3 D. B. Hunninghake and D. L. Azarnoff, *Metabolism*, 17 (1968) 588.
4 A. M. Barrett and J. M. Thorp, *Brit. J. Pharmacol. Chemother.*, 32 (1968) 381.
5 L. Almirante, L. Bruseghini and L. Fazi, *Boll. Chim. Farm.*, 108 (1969) 292.
6 E. L. Tolman, H. M. Tepperman and J. Tepperman, *Amer. J. Physiol.*, 218 (1970) 1313.
7 J. M. Thorp, *Lancet*, *i* (1962) 1323.
8 S. Silvestri, *Farmaco, Ed. Prat.*, 25 (1970) 197.
9 T. J. de Boer, *Rec. Trav. Chim. Pays-Bas*, 73 (1954) 229.