Journal of Chromatography, 223 (1981) 147—154
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 788

THE SIMULTANEOUS ANALYSIS OF CLOFIBRIC ACID AND PROBENECID AND THE DIRECT ANALYSIS OF CLOFIBRIC ACID GLUCURONIDE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J. ROBBERT VEENENDAAL and PETER J. MEFFIN*

Department of Clinical Pharmacology, The Flinders University of South Australia, Bedford Park, South Australia 5042 (Australia)

(First received August 28th, 1980; revised manuscript received November 10th, 1980)

SUMMARY

A rapid and selective high-performance liquid chromatographic method for the simultaneous quantitative analysis of clofibric acid and probenecid in plasma and urine and for the direct analysis of clofibric acid glucuronide in plasma is described. Both methods involve direct injection of deproteinised body fluids. Concentrations of as low as 10 mg/l of clofibric acid and probenecid and 1.5 mg/l of clofibric acid glucuronide can be measured by the analysis. The coefficient of variance for these methods ranges from 1—7%.

INTRODUCTION

It has been recently reported in man that probenecid and naproxen co-administration produces an elevation in plasma naproxen concentration via a proposed mechanism involving the inhibition of glucuronide conjugation [1]. Like naproxen, clofibric acid, the active metabolite of clofibrate, is cleared predominantly as a glucuronide conjugate [2]. A study was instituted with clofibrate and probenecid to further examine the inhibition of glucuronide conjugation by probenecid, the results of which will be reported elsewhere [3]. To facilitate this study a method has been developed which enables the concentrations of clofibric acid and probenecid to be measured simultaneously in the same plasma or urine sample. In order to detect small concentrations of clofibric acid glucuronide (CAG) in plasma in the presence of high clofibric acid concentrations, a direct method has been developed for CAG which does not require its chemical or enzymatic hydrolysis to clofibric acid. This approach overcomes the problems of sensitivity and specificity inherent in indirect methods based on hydrolysis.

0378-4347/81/0000—0000/$02.50

EXPERIMENTAL

Materials

The sodium salt of clofibric acid, 2-(*p*-chlorophenoxy)-2-methylpropionic acid, was supplied by Imperial Chemical Industries (Melbourne, Australia). Probenecid, β-glucuronidase type VII from *E. coli*, phenolphthalein glucuronide and D-saccharic acid 1,4-lactone were purchased from Sigma (St. Louis, MO, U.S.A.). Flurbiprofen [2-fluoro-α-methyl(1,1′-biphenyl)-4-acetic acid] was supplied by the Boots Company (Sydney, Australia). The acetonitrile was chromatographic grade (Unichrome 210, Ajax Chemicals, Sydney, Australia). All other chemicals were of analytical grade.

Clofibric acid and probenecid analysis in plasma or urine

Plasma or urine, 0.1 ml, was added to a 1.5-ml capacity disposable centrifuge tube containing 0.250 ml of a 30 mg/l solution of flurbiprofen in acetonitrile as internal standard. The tubes were capped, shaken on a vortex mixer for 30 sec and centrifuged for 2 min at 10,000 *g*. A 0.02—0.05 ml aliquot of the supernatant was sampled with a 0.1-ml syringe and injected into the high-performance liquid chromatograph (Waters Model 6000A) via a Waters Model U6K injector (Waters Assoc., Milford, MA, U.S.A.). A 10-μm particle size reversed-phase column (Waters μBondapak C_{18}) was used for the analysis with a mobile phase of acetonitrile—glacial acetic acid—water (450 : 5 : 545) and a flow-rate of 2 ml/min. A variable-wavelength detector (Waters Model 450) operated at 235 nm was used to monitor the column effluent. The detector output was measured using a dual-pen recorder with 10 and 50 mV voltage spans. The detector was usually operated at 0.04 a.u.f.s. deflection.

Calibration curves were constructed by adding known amounts of both clofibric acid (as the sodium salt) and probenecid to plasma or urine to produce concentrations of 10, 20, 50, 100, 150 and 200 mg/l. These samples were analysed and peak height ratios of both clofibric acid and probenecid to the internal standard were plotted versus the concentration. The peak height ratios were divided by the concentration of clofibric acid or probenecid and multiplied by 100 to give a normalised peak height ratio for 100 mg/l. The mean normalised peak height ratio was used to determine the concentrations of clofibric acid or probenecid present in unknown samples. The coefficient of variation (C.V.) of the normalised peak height ratios provides estimates of the accuracy of the method over the range of the assay. The reproducibility of the method was also determined by analysing replicate samples containing 20 and 50 mg/l of clofibric acid and probenecid in plasma and urine. The specificity of the method was assessed by determining retention times of various drugs under the chromatographic conditions described.

Clofibric acid analysis after hydrolysis

β-Glucuronidase hydrolysis. One ml of plasma, or urine diluted with water, was added to an 8-ml capacity culture tube fitted with a PTFE-lined screw cap. To the tube was added 1 ml of 0.075 *M* phosphate buffer (pH 6.8) containing approximately 2000 Fishman units of β-glucuronidase activity. The tubes were mixed and incubated for 2 h at 37°C in a water bath. Preliminary experiments

had demonstrated that no significant increase in clofibric acid concentration occurred after 1 h under the conditions described. The concentrations of clofibric acid liberated by the enzyme were measured in a 0.1-ml sample, as described for the analysis in plasma and urine.

The method was calibrated and an estimate of its accuracy was obtained by adding known amounts of clofibric acid to plasma and urine to produce concentrations in the range of 20—200 mg/l. The calibration procedure used was that described for the analysis of clofibric acid and probenecid in plasma and urine.

Acid hydrolysis. Urine (1 ml) was hydrolysed in 3 *N* hydrochloric acid (final concentration) for 30 min at 100°C. On cooling, 0.5 ml of 10 *N* sodium hydroxide was added and 0.1 ml of the solution was analysed as described for clofibric acid and probenecid in urine or plasma. The method was calibrated over the range of 250—1500 mg/l by adding known amounts of clofibric acid to urine. The calibration procedure was as described for the analysis of clofibric acid and probenecid in plasma and urine.

Isolation and characterization of clofibric acid glucuronide

Urine samples were collected from a volunteer over 8-h periods, prior to (control) and after the ingestion of 1 g of clofibrate (CAG-containing urine). Samples were examined using the chromatographic conditions previously described except that the mobile phase was acetonitrile—0.01 *M* sodium citrate buffer, pH 3.0 (25:75). This chromatographic analysis was carried out for both control and CAG-containing urine, prior to and after treatment with β-glucuronidase, as described for CAG analysis by β-glucuronidase hydrolysis. Treatment with β-glucuronidase was also carried out in the presence of 30 mg/ml of D-saccharic acid 1,4-lactone.

Control and CAG-containing urine (100 ml) were adjusted to pH 2.0 with dilute sulphuric acid and extracted with 2 × 200 ml of ethyl acetate. The organic phase was dried with anhydrous calcium chloride, filtered and the ethyl acetate was removed at 45°C under reduced pressure. The residue was taken up in 5 ml of 0.01 *M* sodium citrate buffer (pH 3.0) and the solution was filtered. The ethyl acetate extracts were subjected to the same treatments and chromatographic analyses as the control and CAG-containing urine samples from which they were derived.

On the basis of the above treatments (see Results and discussion), a chromatographic peak was identified which corresponded to CAG. The column effluent corresponding to this peak was collected following a series of 0.3—0.5 ml injections of the CAG-containing extract. The acetonitrile content of this purified fraction was removed under reduced pressure at 76°C. The purified CAG fraction in citrate buffer was again subjected to the treatments and chromatographic analyses already described for urine and the ethyl acetate extract. In addition, the UV absorption spectrum of the CAG fraction was determined and compared with that of clofibric acid.

Direct analysis of clofibric acid glucuronide in plasma

Plasma containing CAG (0.1 ml) was added to a 1.5-ml capacity disposable centrifuge tube containing the internal standard, 0.2 ml of a 7 mg/l solution of

phenolphthalein glucuronide in 10% aqueous trichloroacetic acid. The samples were then treated as described for the analysis of clofibrate and probenecid in plasma or urine. The chromatographic conditions were those used in the isolation and characterisation of CAG. The method was calibrated by adding known amounts of chromatographically pure CAG to plasma (0.1 ml of CAG fraction in buffer to 10 ml of plasma) to produce concentrations of 1.5, 3.0, 7.5, 30, 60, 120 and 240 mg/l of CAG. The calibration procedures were those described for the analysis of clofibric acid and probenecid in plasma or urine.

RESULTS AND DISCUSSION

Clofibric acid and probenecid analysis in plasma or urine

The retention times of clofibric acid, probenecid and the internal standard were 4.6, 5.8, and 8.2 min, respectively, under the conditions described. A chromatogram of a plasma sample containing 20 mg/l of clofibric acid and probenecid is shown in Fig. 1a, while Fig. 1b shows the chromatogram of a control plasma sample. There was an absence of interfering peaks in control plasma and urine from five subjects examined. Samples containing salicylate, paracetamol, ibuprofen and naproxen also showed that these compounds do not interfere with the assay.

The reproducibility of the method over the range calibrated and at two fixed concentrations is shown in Table I. In the range of 10—200 mg/l the C.V. for

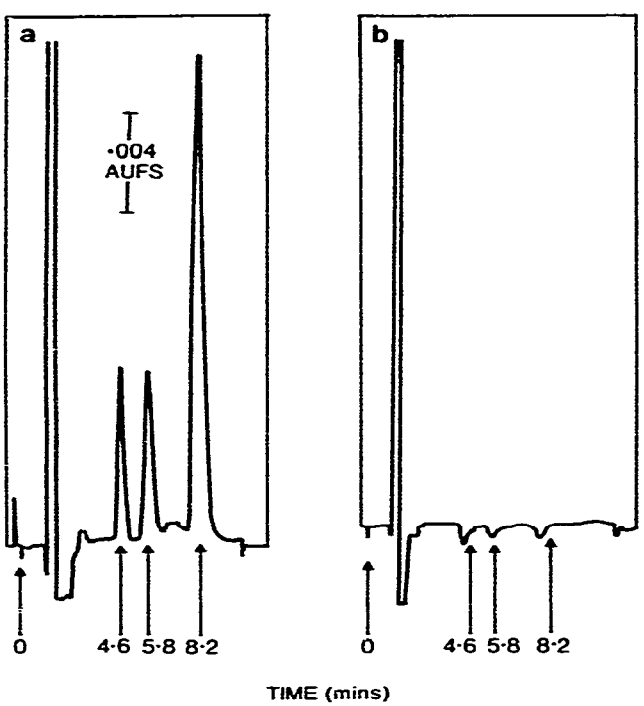

Fig. 1. (a) Chromatogram of plasma containing 20 mg/l of clofibric acid and probenecid with retention times of 4.6 and 5.8 min respectively; that of the internal standard, flurbiprofen (30 mg/l) is 8.2 min. (b) Chromatogram of blank plasma.

TABLE I

CALIBRATION DATA

	Concentration (mg/l)	Mean C.V. of normalised peak height ratio (%)	
		Clofibric acid	Probenecid
Plasma (N = 9)	10—200	3.8	4.7
Urine (N = 2)	10—200	1.9	2.1
Reproducibility			
Plasma (n = 4)	20	3.5	3.0
	150	2.8	2.9
Urine (n = 4)	20	0.9	6.1
	150	1.5	2.2

the normalised peak height ratio for either clofibric acid or probenecid is approximately 5% for plasma and 2% for urine. The reproducibility of replicates at either 20 or 150 mg/l is similar to that seen over the entire range of the method, the C.V. ranging from approximately 1 to 6%.

A number of high-performance liquid chromatographic methods have been reported in the literature for clofibrate [4—6] and probenecid [7, 8] in biological samples. Previously reported methods have used solvent extraction and evaporation or double extraction methods of sample preparation, whereas we use a more rapid and convenient approach of protein precipitation and direct injection of the resulting supernatant.

The reproducibility of the reported methods is similar to the present analysis. The sensitivity of our method is less than that reported by others. Common doses of clofibrate are 0.5 or 1 g twice daily which result in plasma clofibric acid concentrations of 65—100 mg/l [9]. Similarly, probenecid is commonly taken in doses of 0.5—1 g twice daily, resulting in plasma concentrations of 100—200 mg/l [9]. The present method which measures clofibric acid and probenecid concentrations down to 10 mg/l is more than sufficiently sensitive for multiple dosing studies and also satisfactory for single dose studies with the usual doses. The comparative lack of sensitivity of the present method is compensated for by the small sample volume required (0.1 ml) and the ease of sample preparation.

Clofibric acid glucuronide by hydrolysis

Chromatograms from plasma and urine samples treated with either β-glucuronidase or acid were essentially similar to those obtained from untreated samples, such as that shown in Fig. 1. No peaks arose from these treatments which interfered with those of clofibric acid or the internal standard. Reproducibility data for the method are shown in Table II. In the range of 20—200 mg/l, the C.V. of the normalised peak height ratio for the enzymatic method is less than 5%. The range (250—1500 mg/l) over which the method was established for the acid treatment, reflects the high concentrations of clofibric acid found in

TABLE II

CALIBRATION DATA FOR CLOFIBRIC ACID AFTER HYDROLYSIS

	Concentration (mg/l)	C.V. of normalised peak height ratio (%)
β-Glucuronidase hydrolysed plasma	20—200	4.8
β-Glucuronidase hydrolysed urine	20—200	2.1
Acid hydrolysed urine	250—1500	3.9

urine after acid hydrolysis rather than any limits of sensitivity. Over this range, the reproducibility of the acid hydrolysis method was comparable to that of the enzymatic method (Table II).

Purification of clofibric acid glucuronide

Chromatograms of CAG-containing urine showed a peak at 6.5 min which was not present in control urine and which was not due to clofibric acid which had a retention time of 20 min under these conditions. The peak at 6.5 min was not present in CAG urine which had been treated with β-glucuronidase, but was present in β-glucuronidase-treated urine which also contained D-saccharic acid 1,4-lactone, a specific inhibitor of the enzyme [10]. Treatment of CAG-containing urine with β-glucuronidase not only abolished to peak at 6.5 min but also produced a corresponding increase in the peak due to clofibric acid. A similar examination of the ethyl acetate extract of control and CAG-containing urine gave the same pattern of results as observed in urine.

Fig. 2a shows the chromatogram of the purified CAG fraction prior to treatment with β-glucuronidase. The absence of a peak at 20 min indicates that this fraction is uncontaminated with clofibric acid. Fig. 2b shows the chromatogram of the sample shown in Fig. 2a, after it has been treated with β-glucuronidase. All but approximately 5% of the peak at 6.5 min is removed by this treatment, and there is a corresponding increase in the clofibric acid peak at 20 min.

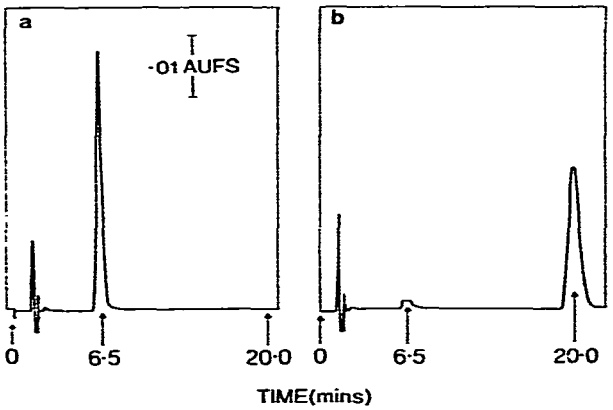

Fig. 2. (a) Chromatogram of purified extract of clofibric acid glucuronide which shows a single peak at 6.5 min. (b) Chromatogram of the same sample after β-glucuronidase hydrolysis; the peak due to clofibric acid glucuronide at 6.5 min has been replaced by a peak at 20 min due to clofibric acid.

No peaks other than that due to clofibric acid resulted from this treatment. The presence of D-saccharic acid 1,4-lactone during β-glucuronidase treatment prevented a reduction in the peak at 6.5 min and the appearance of a peak due to clofibric acid.

The UV absorbance spectrum of the peak at 6.5 min exhibited maxima at 227 nm and 277 nm, as did an authentic sample of clofibric acid. The ratio of the absorbance at 227 nm to that at 277 nm was 0.095 for the peak at 6.5 min and 0.088 for clofibric acid.

Assuming that conjugation of clofibric acid with glucuronic acid does not alter its UV absorbance characteristics, there was a stoichiometric relationship between the amount of CAG present in the purified extract and the amount of clofibric acid measured after enzymatic hydrolysis. All of the above data are consistent with the peak at 6.5 min of the purified fraction being due to CAG. Furthermore, this fraction is essentially pure by enzymatic, chromatographic and UV absorbance criteria.

Direct analysis of clofibric acid glucuronide in plasma

Under the chromatographic conditions described, the retention times of CAG and the internal standard (phenolphthalein glucuronide) were 10.5 and 8.1 min respectively (Fig. 3). Although some samples showed small peaks

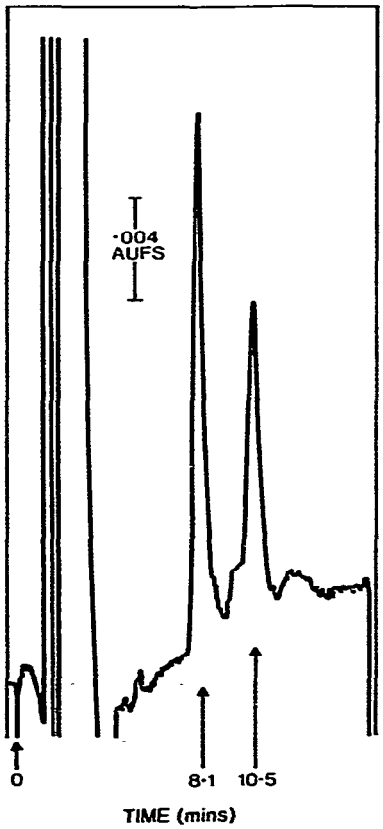

Fig. 3. Chromatogram of plasma containing the internal standard phenolphthalein glucuronide and 15 mg/l clofibric acid glucuronide (retention times 8.1 and 10.5 min, respectively).

which were not completely resolved from the CAG peak (Fig. 3), the presence of these peaks did not seriously reduce the accuracy of the method. The calibration curve for the direct analysis of CAG in plasma was linear. In the range of 1.5—250 mg/l, the C.V. of the normalised peak height ratio was 7.3% and the regression coefficient was 0.9988. A disadvantage of this approach is that under the chromatographic conditions required to separate CAG from interfering peaks in plasma, slowly eluting peaks occur, including that due to clofibric acid, which permit relatively few (10—15) samples to be assayed in a day.

The usual methods by which glucuronides are measured require an estimate of the aglycone to be made before and after enzymatic or chemical hydrolysis. The difference of these two measurements is then taken as an estimate of the glucuronide present. This approach has two major disadvantages. The method becomes less accurate as the ratio of aglycone to conjugate increases and such an approach might fail to detect small concentrations of CAG in plasma in the presence of high clofibric acid concentrations. The second disadvantage of indirect methods concerns specificity. On the one hand, it has been reported that β-glucuronidase treatment fails to completely hydrolyse the glucuronides of clofibric acid and some other aglycones [11]. Chemical methods which may liberate greater amounts of aglycone from a sample have the disadvantage that it is difficult or impossible to document their specificity. The approach outlined here overcomes both of the above problems, in that it allows the estimate of CAG in the presence of high concentrations of clofibric acid and has well documented specificity.

ACKNOWLEDGEMENTS

This work was supported by the National Health and Medical Research Council of Australia. We wish to thank Imperial Chemical Industries, Australia, for the gift of sodium clofibric acid.

REFERENCES

1 R. Runkel, E. Mroszczak, M. Chaplin, H. Sevelius and E. Segre, Clin. Pharmacol. Ther., 24 (1978) 706.
2 R. Gugler, Clin. Pharmacokinetics, 3 (1978) 425.
3 J.R. Veenendaal, P.M. Brooks and P.J. Meffin, Clin. Pharmacol. Ther., in press.
4 T.D. Bjornsson, T.F. Blaschke and P.J. Meffin, J. Chromatogr., 137 (1977) 145.
5 R.N. Woodhouse, D.G. Cresswell, L.F. Chasseaud and R.R. Brodie, J. Chromatogr., 137 (1977) 218.
6 W.T. Robinson, L. Cosyns and M. Kraml, Clin. Biochem., 11 (1978) 214.
7 R.K. Harle and T. Cowen, Analyst (London), 103 (1978) 492.
8 R.A. Upton, J.N. Buskin, T.W. Guentert, R.L. Williams and S. Riegelman, J. Chromatogr., 190 (1980) 119.
9 F. Bochner, G. Carruthers, J. Kampmann and J. Steiner, Handbook of Clinical Pharmacology, Little, Brown and Co., Boston, MA, 1978, pp. 135 and 261.
10 G.A. Levy, Biochem. J., 52 (1952) 464.
11 E.M. Faed and G. McQueen, Clin. Exp. Pharmacol. Physiol., 5 (1978) 195.