Journal of Chromatography, 115 (1975) 222–227

CHROM. 8587

Note

A gas chromatographic assay for probenecid and its metabolites in biological fluids

WALTER D. CONWAY* and SRIKUMARAN MELETHIL**

Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Station B, Box U, Buffalo, N.Y. 14207 (U.S.A.)

(Received July 7th, 1975)

Recent studies have indicated that some metabolites of probenecid possess uricosuric activity[1]. Studies of the metabolic fate of the drug in humans have thus far been conducted using ^{14}C-labelled probenecid[2–4]. Specific assays for non-radioactive probenecid in biological fluids have been reported but none permits measurement of metabolites[5–9]. Separation of the methyl esters of probenecid and its metabolites by GLC on 3% OV-17 has been reported[10], but our recent attempts to develop a quantitative procedure using this liquid phase gave insufficient separation of the methyl esters of the drug metabolites from impurities present in extracts of biological material. A satisfactory resolution of both metabolites and impurities was obtained by chromatography of the propyl esters on 10% OV-1 as described in this paper. Briefly, the method involves direct extraction of the free acids from acidified urine by methylene dichloride or acid hydrolysis of conjugated metabolites prior to extraction, addition of N,N-dibenzyl-(2,5-dimethylbenzenesulfonamide) as an internal standard, followed by gas chromatography using flame ionization detection.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Mk II gas chromatograph fitted with a flame ionization detector and a stainless-steel column (0.125 in. O.D. × 6 ft.) packed with 10% OV-1 on 80–100 mesh Chromosorb W-HP was used. Operating conditions were: column temperature, 250°; injection port temperature, 280°; nitrogen carrier-gas flow-rate, 23 ml/min.; and sensitivity $2.5 \cdot 10^{-11}$ A full scale. Optimum column performance was maintained by occasional injection of approximately 40-μl aliquots of Silyl-8 (Pierce Chemical Co., Rockford, Ill., U.S.A.).

Compounds

Probenecid was supplied by Merck, Sharp and Dohme (West Point, Pa.,

* To whom correspondence and requests for reprints should be addressed.

** Present address: College of Pharmacy and Allied Health Professions, Northeastern University, Boston, Mass. 02115, U.S.A.

U.S.A.). Metabolites were synthesized as reported elsewhere[10,11]. Structures of the metabolites are given in Table I.

N,N-Dibenzyl-(2,5-dimethylbenzenesulfonamide) was the most suitable of a series of substituted benzenesulfonamides tested for use as an internal standard. It was prepared by adding 2,5-dimethylbenzenesulfonyl chloride to two molecular equivalents of dibenzylamine dissolved in pyridine. After 30 min at room temperature, excess 1 *N* hydrochloric acid was added and the precipitated sulfonamide was removed by filtration, washed with water and crystallized from 95% ethanol. Its purity was verified by observation of only a single symmetrical peak when subjected to gas chromatography under the conditions utilized for the assay.

N-Propylnitroguanidine was prepared and converted to N-nitroso-N-propyl-N-nitroguanidine (m.p. 116°)[12,13] which was used without further purification to prepare diazopropane.

Diazopropane was prepared by adding dropwise a solution of 0.9 g of N-nitroso-N-propyl-N-nitroguanidine in 15 ml of diethyl ether to a 50 ml distilling flask containing 5 ml of 50% potassium hydroxide heated at 65° in a water-bath and collecting the distillate in a receiving vessel cooled in ice. When addition of the nitrosoguanidine solution was complete, 10 ml of diethyl ether were added to the distilling flask and distilled to complete the transfer of diazopropane. The distillate contains approximately 0.15 mmoles diazopropane per milliliter of ether. Solvents and other chemicals used were reagent grade.

Calibration plots

Quantities of probenecid and its metabolites, ranging from 25 to 700 μg, were obtained by transferring 25–700-μl aliquots of methanol solutions containing 1 mg/ml of the compounds to small vials and evaporating them to dryness at 50° under a stream of air. Two ml of an ethereal solution containing about 0.3 mmoles of diazopropane was added to the residue. The vials were capped and allowed to stand overnight, after which excess diazopropane and ether were removed under a stream of air at 50°. A 340-μg quantity of internal standard was introduced by adding 200 μl of a solution, prepared by dissolving 17 mg of N,N-dibenzyl-(2,5-dimethylbenzenesulfonamide) in 10 ml of methanol, and removing the solvent in an air stream at 50°. The residue was dissolved in approximately 2 ml of methylene dichloride and approximately 1 μl was introduced into the gas chromatograph. Injections were made with a 10-μl syringe, which was first loaded with 1 μl of diethyl ether adjacent to the plunger and separated from the slug of sample by about 2 μl of air.

Only a single peak was observed for each compound. Plots of amount ratio (amount of sample/amount of internal standard) *versus* peak height ratio (height of sample peak/height of internal standard peak) were linear over the range studied for all compounds except the primary alcohol metabolite. Some tailing was observed for this compound but a linear plot was obtained by using the corresponding area ratios as determined by a planimeter.

Extraction of biological samples

Unconjugated metabolites. A 2-ml aliquot of human urine in a 50-ml centrifuge tube with a PTFE-lined screw cap was acidified with 2 ml of 5 *N* hydrochloric acid and mechanically shaken with 20 ml of methylene dichloride for 30 min. The tube

was centrifuged at 170 *g* for 20 min and the aqueous layer removed by aspiration. A 10-ml aliquot of the methylene dichloride phase was evaporated to dryness in a small vial under an air stream at 50°. The residue was esterified with diazopropane, internal standard was added, and chromatography conducted as described for calibration plots.

When the instrument response to metabolite peaks was greater than full scale and could not be accommodated by a slight change in instrument attenuation, the final volume of methylene dichloride was increased appropriately and an additional quantity of internal standard solution was added to maintain the concentration of internal standard at approximately 170 μg/ml.

When the instrument response fell below about 10% of scale, a second urine sample was processed but a smaller quantity of internal standard (typically 34 μg in 20 μl of methanol) was introduced. The residue after esterification was then taken up in a smaller volume of methylene dichloride (0.2 ml for 34 μg of internal standard), so as to maintain the concentration of internal standard at about 170 μg/ml.

Conjugated metabolites. After addition of the 5 *N* hydrochloric acid as described above, conjugated metabolites were hydrolyzed by heating the samples at 100° for 4 h. Extraction and assay of the hydrolyzed samples as described above provided a measurement of conjugated plus unconjugated metabolites. Conjugated metabolites were then determined by difference.

Recovery experiments

Aliquots of solutions of probenecid and its metabolites in 0.1 *N* sodium hydroxide were added to samples of urine which were then assayed before and after acid hydrolysis of the samples in order to measure the recovery of the compounds as well as their stability under the hydrolytic conditions.

RESULTS AND DISCUSSION

The structures of probenecid and its metabolites and the retention times of their propyl esters on 10% OV-1 are summarized in Table I. A typical chromatogram of the propyl esters of metabolites found in human urine is shown in Fig. 1. Only traces of the primary alcohol metabolite are found in human urine.

A summary of the constants for straight lines fitted to calibration plots by least-square regression analysis are given in Table II. Calibration plots based on peak height ratios were linear for all compounds except the primary alcohol. Better linearity was obtained using area ratios for the primary alcohol, which exhibits some tailing. In each instance, the line intercepts within a negligible distance of the origin and the correlation coefficient is essentially unity.

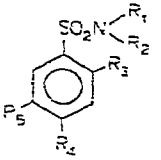
Recoveries of probenecid and its mono-N-propyl, carboxy and secondary alcohol metabolites from human urine are very satisfactory over the range of 10 to 400 μg per 2 ml of urine (Table III). The recovery of the primary alcohol from human urine was not examined because it is not excreted in significant amounts by humans. Its recovery from rat urine, in which appreciable amounts are found, is essentially quantitative.

Statistical analysis of the data in Table III by a one-way analysis of variance for unequal group sizes (*F*-test) indicated that the observed recovery was independent

TABLE I

STRUCTURES FOR THE INTERNAL STANDARD AND METABOLITES OF PROBENECID AND RETENTION TIMES ON 10% OV-1 FOR THE INTERNAL STANDARD AND PROPYL ESTERS OF PROBENECID AND THE METABOLITES

For GLC conditions, see text. Structure:



Abbreviation	*Structure*	*Retention time (min)*
Probenecid	R_1, $R_2 = C_3H_7$; R_3, $R_5 = H$; $R_4 = COOH$	3.9
1° Alcohol	$R_1 = C_3H_7$; $R_2 = CH_2CH_2CH_2OH$; R_3, $R_5 = H$; $R_4 = COOH$	8.0
2° Alcohol	$R_1 = C_3H_7$; $R_2 = CH_2CHOHCH_3$; R_3, $R_5 = H$; $R_4 = COOH$	5.7
Carboxy	$R_1 = C_3H_7$; $R_2 = CH_2CH_2COOH$; R_3, $R_5 = H$; $R_4 = COOH$	11.1
Mono-N-propyl	$R_1 = C_3H_7$-; R_2, R_3, $R_5 = H$; $R_4 = COOH$	3.1
Internal standard	R_1, $R_2 = CH_2$–C_6H_5; R_3, $R_5 = CH_3$; $R_4 = H$	12.8

of the quantity of metabolite extracted over the range of concentration tested ($P > 0.05$) and also that the recoveries remain unchanged after acid treatment (t-test). Since there were no statistically significant differences in recoveries resulting from hydrolytic treatment or from the quantity extracted, an estimate of the overall variation of the analytical technique was obtained by pooling all recoveries for each metabolite (29 individual samples). These values, which range from 3.6 to 6.5%, are summarized in the last line of Table III.

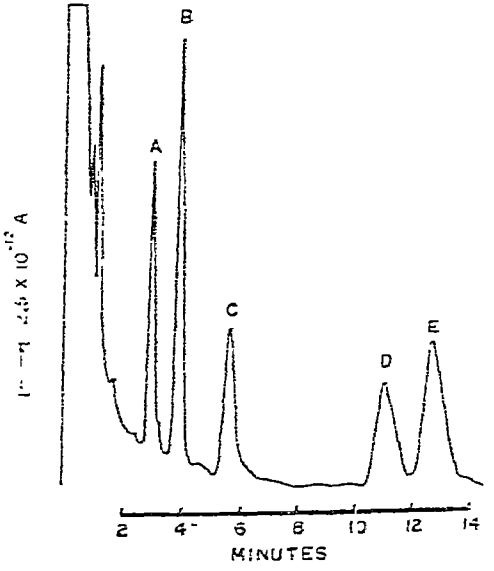


Fig. 1. A typical chromatogram of the propyl esters of probenecid metabolites excreted in human urine: A = mono-N-propyl metabolite; B = probenecid; C = secondary alcohol metabolite; D = carboxylic acid metabolite; E = internal standard, N,N-dibenzyl-(2,5-dimethylbenzenesulfonamide). 10% OV-1 column at 250°, nitrogen carrier gas 23 ml/min.

TABLE II

REGRESSION (m) AND CORRELATION (r) COEFFICIENTS AND Y INTERCEPT (b) FOR CALIBRATION PLOTS OF AMOUNT RATIO (Y) *VERSUS* PEAK HEIGHT RATIO (X) FOR PROPYL ESTERS OF PROBENECID AND ITS METABOLITES

$Y = mX + b$.

Compound	m	b	r
Probenecid	0.35	0.03	1.000
1° Alcohol*	2.84	0.04	0.997
2° Alcohol	0.79	0.01	1.000
Carboxy	1.41	0.02	1.000
Mono-N-propyl	0.45	0.05	0.998

* Coefficients for peak area ratios.

TABLE III

RECOVERY (%) OF PROBENECID AND ITS METABOLITES FROM 2 ml HUMAN URINE

Mean of two or three determinations: E, solution not heated: H, acidified solution heated for 4 h at 100°.

µg added	*Probenecid*		*Mono-N-propyl*		*Carboxy*		*2° Alcohol*		*1° Alcohol**	
	E	*H*	*E*	*H*	*E*	*H*	*E*	*H*	*E*	*H*
10	97.0	—	78.8	—	79.7	—	94.8	—		
25	97.3	—	82.3	—	86.3	—	95.8	—		
50	94.5	98.1	83.0	84.8	87.6	84.4	89.2	96.9		
100	97.9	100.4	84.8	86.5	87.9	88.1	93.5	98.0	99.5	102
200	100.7	99.9	81.1	85.9	82.3	83.7	102.1	94.3		
300	100.6	98.1	82.5	79.7	84.9	78.8	87.0	94.0		
400	98.6	—	85.3	—	88.2	—	88.9	—		
Mean	98.1	99.1	82.4	84.5	85.1	84.3	93.1	96.1		
Pooled mean ± S.D.	98.4 ± 3.6		83.1 ± 4.5		84.8 ± 4.8		94.1 ± 6.5			

* Determined using rat urine; only traces of this metabolite are found in human urine.

Essentially the same recoveries of metabolites have been obtained in analyses of rat bile and rat urine.

This procedure has been utilized in a study of the disposition of probenecid in man, which will be published separately[14]. The method is capable of monitoring concentrations of the drug and its metabolites in urine for at least three days after oral ingestion of an oral dose of 0.5 g of probenecid.

ACKNOWLEDGEMENT

This work was supported in part by General Research Support Grant 5-S01 RR 05454-10 from the National Institutes of Health, Bethesda, Md. 20014, U.S.A.

REFERENCES

1 Z. H. Israili, J. M. Perel, R. F. Cunningham, P. G. Dayton, T. F. Yu, A. B. Gutman, K. R Lond, R. C. Long, Jr. and J. H. Goldstein, *J. Med. Chem.*, 15 (1972) 709.

2 J. M. Perel, R. F. Cunningham, H. M. Fales and P. G. Dayton, *Life Sci.*, 9 (1970) 1337.
3 P. G. Dayton, J. M. Perel, R. F. Cunningham, Z. H. Israili and I. M. Weiner, *Drug Metab. Disp.*, 1 (1973) 742.
4 P. G. Dayton and J. M. Perel, *Ann. N.Y. Acad. Sci.*, 179 (1971) 399.
5 K. Sabih, C. D. Klassen and K. Sabih, *J. Pharm. Sci.*, 60 (1971) 745.
6 E. K. Tillson, G. S. Schuchardt, J. K. Fishman and K. H. Beyer, *J. Pharmacol. Exper. Therap.*, 111 (1954) 385.
7 P. G. Dayton, T. F. Yu, W. Chen, L. Berger, L. A. West and A. B. Gutman, *J. Pharmacol. Exper. Therap.*, 140 (1963) 278.
8 D. Schachter, *J. Clin. Invest.*, 36 (1957) 297.
9 J. M. Perel and P. G. Dayton, *Europ. J. Clin. Pharmacol.*, 3 (1971) 106.
10 A. M. Guarino, W. D. Conway and H. M. Fales, *Europ. J. Pharmacol.*, 8 (1969) 244.
11 W. D. Conway and S. Melethil, *J. Pharm. Sci.*, 63 (1974) 1551.
12 A. F. McKay, *J. Amer. Chem. Soc.*, 71 (1949) 1968.
13 A. F. McKay, W. L. Ott, G. W. Taylor, M. N. Buchanan and J. F. Crooker, *Can. J. Res., Sect. B*, 28 (1950) 683.
14 S. Melethil and W. D. Conway, *J. Pharm. Sci.*, in press.