Combined GC and High Resolution Mass Spectrometric Determination of Probenecid

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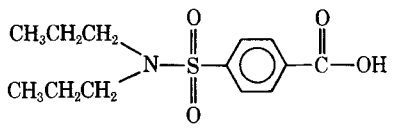
Abstract □ A sensitive GC method for measurement of probenecid in biological fluids is described. This method involves the conversion of probenecid to its methyl ester by treatment with dimethylsulfate under basic conditions. Analysis was performed on Gas Chrom Q coated with DC-200. The major metabolic product of probenecid, the glucuronide conjugate of the unchanged drug, was identified by combined GC and high resolution mass spectrometry following enzymatic hydrolysis.

Keyphrases □ Probenecid determination—biological fluids □ Biological fluids—probenecid determination □ Dimethylsulfate–probenecid reaction—probenecid analysis □ GLC–mass spectroscopy, combined—analysis

Probenecid [(di-*n*-propylsulfamyl)benzoic acid] (I), a sulfonamide derivative, is used as a uricosuric agent in the treatment of gout. It has also been useful in the laboratory for studying the hepatic and renal organic acid secretory mechanisms that excrete drugs and other chemicals. Several spectrophotometric methods have been described (1–4) to determine the levels of the drug in biological fluids. However, most of these methods are neither sensitive nor specific and the "blank" is often quite high.



I

Guarino and Schanker (5), in their study of the biliary excretion of probenecid, pointed out that the drug undergoes metabolic changes and that these metabolites are excreted as the glucuronide.

While this work was in progress, Guarino *et al.* (6) identified the metabolites of probenecid by mass spectrometry. They reported that probenecid was excreted into the bile of rats unchanged to the extent of about 60% and that the rest was excreted as the glucuronide conjugate of the side-chain oxidized products of probenecid and also the dealkylated product. However, the present work indicates that these metabolites are quantitatively minor in relation to the simple probenecid glucuronide.

This report describes a very sensitive, quantitative combined GC–mass spectrometric method for the determination of probenecid in biological fluids.

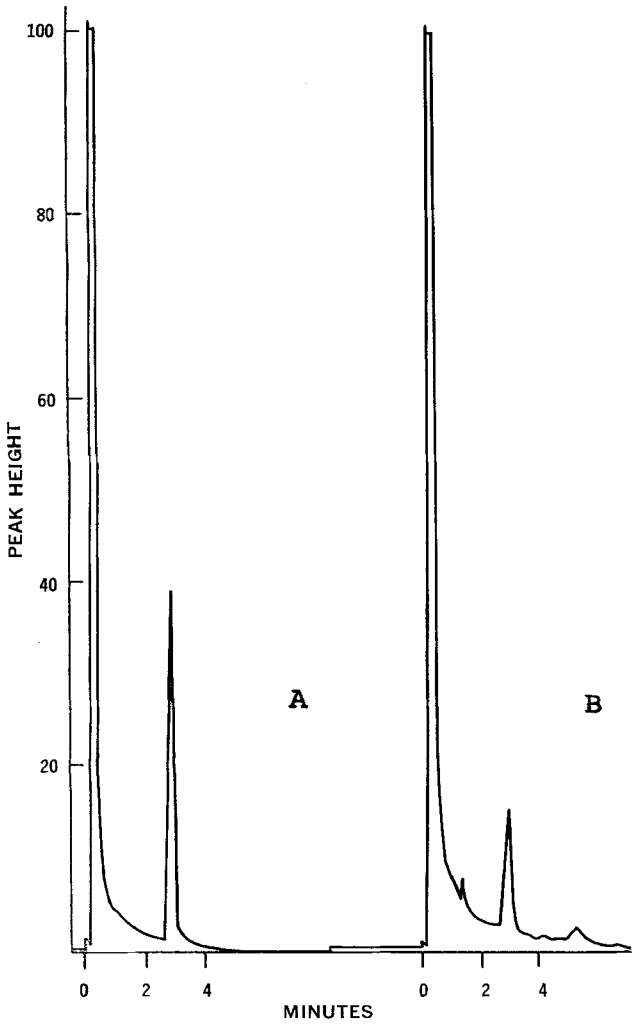


Figure 1—*Gas chromatogram of: (A) pure probenecid, and (B) probenecid extracted from bile treated with β-glucuronidase. Curve A represents 5-μl. injection of 40 mcg. probenecid in 100 μl. chloroform.*

EXPERIMENTAL

Reagents—Probenecid[1] and dimethylsulfate[2] were used. Chloroform and heptane were analytical redistilled reagents. Acetate buffer, 0.2 *N*, pH 5.6, was made by mixing 4.8 ml. of AcOH solution (0.2 *N*; 11.55 ml. in 1000 ml. H_2O) and 45.2 ml. of sodium acetate solution (0.2 *N*; 16.4 g. in 1000 ml. H_2O) and diluting the mixture with water to 100 ml. Methanolic potassium carbonate solution was made by mixing 1 ml. of 5% aqueous solution of K_2CO_3 and 9 ml. of MeOH.

Preparation of Probenecid Methyl Ester—A solution of 0.5 g. of probenecid in 20 ml. methanolic K_2CO_3 was placed in a flask fitted with a magnetic stirrer. Ten milliliters of Me_2SO_4 was added, and the reaction was allowed to proceed for 15 min. at 70°. MeOH was then removed under reduced pressure, and the residue was treated with 20 ml. acetate buffer, pH 5.6. The reaction mixture was then extracted with 50 ml. $CHCl_3$. The chloroform extract was dried (Na_2SO_4), and solvent was removed under reduced pressure. TLC of the residue, using freshly prepared silica gel G plates, gave only one uniform spot, R_f 0.83 with chloroform and 0.17 with benzene. The residue was purified on a silica column. The fraction

[1] Supplied by Merck Institute for Therapeutic Research, West Point, Pa.

[2] Matheson Coleman and Bell, Norwood, Ohio.

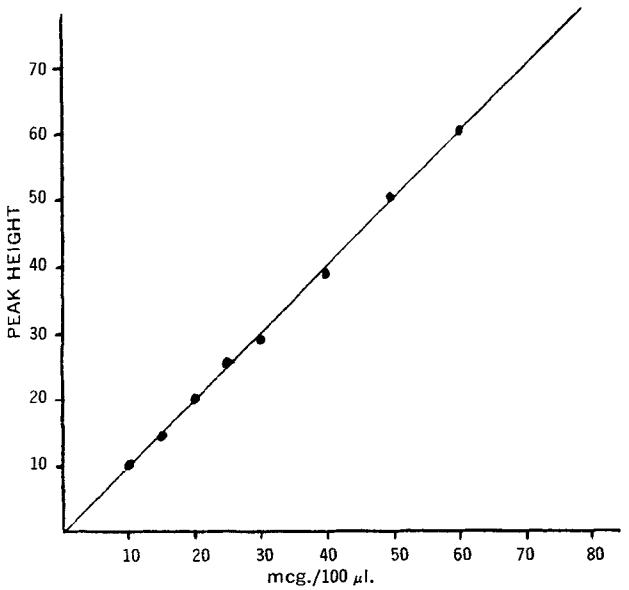


Figure 2—*Relationship between peak height and amount injected. Five microliters injected in each case.*

eluted with $CHCl_3$ was collected and gave a white solid (m.p. 50–51°) upon removal of solvent. The IR spectrum of the product showed no absorption in the hydroxyl region.

Anal.—Calcd. for $C_{14}H_{21}NO_4S$: C, 56.18; H, 7.02; N, 4.68. Found: C, 56.24; H, 7.13; N, 4.56.

The methyl ester of probenecid was also prepared using HF–CH_3OH reagent, but the yield was not quantitative as in the case of the Me_2SO_4–K_2CO_3 method.

GC—An F&M model 5755B gas chromatograph, equipped with a flame-ionization detector, was used for chromatography. The column was 1.4 m., 0.32 cm. (4.5 ft., 0.125 in.) stainless steel tubing packed with Gas Chrom Q, 80–100 mesh, coated with 5% DC-200. The operating temperatures used for analysis of probenecid were: column, 190°; injection port, 310°; and detector, 320°. Helium was used as the carrier gas at a flow rate of 40 ml./min.

Standards—The sodium salt of probenecid was added to water, plasma, urine, or bile samples in varying amounts (2.5–60 mcg./ml.). The resulting mixtures were acidified with 1 ml. 1 *N* HCl and then extracted by shaking with 5 ml. of chloroform for 5 min. The chloroform extracts were taken to dryness under a stream of air, and each residue was treated with 1 ml. of MeOH–K_2CO_3 and 0.1 ml. of Me_2SO_4. The reaction was allowed to proceed for 5 min. in a water bath (70°). Methanol was then removed under a stream of air at the temperature of the water bath, and 1 ml. of pH 5.6 buffer was added to each residue. Extraction of probenecid methyl ester was accomplished by shaking each standard with 10 ml. of chloroform or heptane. The organic layer was separated and dried under a stream of air. GC analysis was carried out by adding 100 μl. of chloroform to each residue and injecting 1–5 μl. onto the column under the conditions cited previously.

Biological System—Simonsen Sprague-Dawley rats (300–350 g.) were anesthetized with pentobarbital sodium (45 mg./kg.). The femoral vein and artery were cannulated with polyethylene tubing (PE-50) for administering the probenecid and collecting blood samples, respectively. The bile duct was cannulated with PE-10 tubing for collection of bile samples.

Probenecid was administered intravenously at a dose of 40 mg./kg. Bile samples were collected from the cannulated bile duct at hourly intervals. Blood samples were obtained from the cannulated femoral artery at 10 min., 1, 2, 3, and 4 hr. after administration of the probenecid; 0.2 ml. plasma or bile was used for the analysis. Heparin was used as the anticoagulant. Urine samples were collected from another group of rats at 6-hr. intervals for 48 hr.

Each sample was acidified with 1 ml. 1 *N* HCl and extracted with 5 ml. $CHCl_3$. The free probenecid partitioned into the $CHCl_3$, and the conjugated metabolite remained in the aqueous phase. The layers were separated, and the aqueous layer from each sample was adjusted to pH 5.0 (1 *N* NaOH and checked with pH meter) and incubated with 2 ml. β-glucuronidase solution (1 mg./ml. in acetate buffer, pH 5.0) overnight. The mixture was acidified and extracted with 5 ml. chloroform. The chloroform extracts (before and after treatment with the enzyme) were taken to dryness separately under a stream of air. Each residue was then treated with 1 ml. MeOH–K_2CO_3 solution followed by 0.1 ml. Me_2SO_4, and the reaction was allowed to proceed for 5 min. in a water bath at 70°. Methanol was then removed under a stream of air, and 1 ml. of pH 5.6 buffer was added followed by 5 ml. $CHCl_3$. Extraction was aided by shaking for 2 min. The chloroform layer was taken to dryness and redissolved in 100 μl. of chloroform. Analysis was carried out by injecting 1–5 μl. of the resulting solutions onto the column under the conditions cited previously.

Mass Spectrometry—The medium resolution mass spectra of probenecid methyl ester and the methylated drug obtained from biological samples were determined on a Finnigan quadrupole mass spectrometer. All samples were introduced to the ionization chamber through a Varian Aerograph model 1740 gas chromatograph. The column used was 3.05 m., 0.32 cm. (10 ft., 0.125 in.) stainless steel tubing packed with Gas Chrom Q coated with 3% OV-1. The oven temperature was programmed from 180 to 250° at 6°/min. Helium was used as the carrier gas, with a flow rate of 30 ml./min.

The high resolution mass spectrum of probenecid methyl ester was obtained using an A.E.I. MS-9 double-focusing mass spectrometer. The MS-9 was coupled to a digital data system[3]; computer processing of the digitalized data was carried out on Battelle's CDC-6400 computer. Samples were introduced by direct probe operating at 120°. The ionizing voltage was 70 ev., the source temperature was 200°, the multiplier setting was 5.0, and the accelerating voltage was −8 kv.

Table I—Recovery of Probenecid from Plasma

Amount Added, mcg./ml.	Percent Recovered
10	98
20	95
40	100
60	93
100	96
200	94

Table II—Plasma Disappearance and Biliary Excretion of Probenecid[a]

10 min.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
Plasma Disappearance of Probenecid, mcg./ml.					
172	109	43.1	3.1	0	0
181	61	6.2	1.2	0	0
206	119	12.5	0.6	0	0
Free Probenecid in Bile, mcg./ml.					
	469	250	156	43.7	6.2
	375	281	148	85.9	12.5
	625	516	148	93.7	7.8
Conjugated Probenecid in Bile, mcg./ml.					
	364	463	55	58.6	11.2
	199	165	311	26.5	12.5
	366	62	60	16.5	12.6
Biliary Flow, μl./min./kg.					
	80	67	59	45	32
	83	71	52	43	38
	68	60	44	31	13

[a] Probenecid given intravenously (40 mg./kg.).

RESULTS AND DISCUSSION

Methylation—The methyl derivative of probenecid was obtained in quantitative yield using Me_2SO_4 as the methylating agent under basic conditions. The methyl derivative of probenecid, however, underwent some hydrolysis on standing for a long time. This was shown by high resolution mass spectrometry.

[3] Designed and built at Battelle.

GC—The gas chromatograms of pure probenecid methyl ester and that obtained from biological material are shown in Figs. 1A and B, respectively. Under the conditions cited in the method, the methyl ester of probenecid had a retention time of 3.0 min. Chromatograms of control samples did not contain any peaks with the retention time of probenecid. Addition of increasing amounts of probenecid to samples of known concentrations of the drug produced an increase in the peak height having the retention time of the drug.

The relationship between peak height and the quantity of the drug injected was linear at optimum instrument sensitivity, as shown in Fig. 2. Duplicate runs were reproducible; for example, with a 50 mcg./ml. solution, the standard error was ±0.23.

The GC method is applicable to biological fluids such as plasma, bile, and urine. The extraction procedure for probenecid from these fluids was adequate and gave 93–100% recovery of added drug (Table I).

Table II demonstrates the application of this GC method for determining the plasma disappearance and biliary excretion of probenecid in rats. Three hours after a 40-mg./kg. dosage of probenecid, essentially all the probenecid had disappeared from the plasma. No conjugated probenecid was detectable in the plasma at any time interval.

The excretion of probenecid into the bile was also measured. Both free and conjugated probenecid were detected in the bile, in contrast to only free probenecid being detected in the plasma. Probenecid is detected in the bile for 5 hr. after the administration of 40 mg./kg. of probenecid, whereas no detectable amounts were found in the plasma after the 3rd hr. This suggests that the probenecid is stored in the liver, as are many compounds that are excreted into the bile. The results indicate that the amount of the free probenecid extracted in the bile represents 55–60% of the total probenecid present in the bile.

Identification of Probenecid Glucuronide—Tillson (1) reported in 1954 that probenecid is excreted both as the unchanged free acid and as its simple acyl glucuronide. However, Guarino and Schanker (5) later reported that the glucuronide is that of metabolically changed probenecid. In a more recent study, Guarino *et al.* (6) demonstrated that probenecid undergoes side-chain W and W-1 oxidation, followed by glucuronide formation and excretion of these glucuronides into the bile. *N*-Dealkylated probenecid was also detected in the bile, but no simple acyl glucuronide was found.

In the present study, free probenecid was removed from the bile of rats administered probenecid by acidifying the bile with 1 *N* HCl and extracting it into chloroform. To make sure that all of the unconjugated probenecid was removed by this extraction, the aqueous layer was adjusted to pH 5.0, allowed to stand overnight, and then reextracted with chloroform after acidification with HCl. GC of this second extract showed no peak corresponding to probenecid. This evidence demonstrated that the drug extracted from bile samples after treatment with β-glucuronidase was due to the presence of the glucuronide conjugate and not due to the presence of unextracted residues of probenecid.

Table III—High Resolution Mass Spectral Data of Probenecid and Its Methyl Ester

m/e	Relative Intensity	*m/e*	Relative Intensity
14	1.7	83	2.6
17	2.6	85	2.3
18	16.0	91	3.2
27	4.6	95	1.3
28	16.8	97	3.9
29	3.6	103	5.0
30	2.4	104	3.9
36	2.6	105	2.0
39	2.4	109	1.2
40	3.4	111	1.5
41	9.5	120	1.5
42	6.6	121	31.7
43	16.5	122	2.9
44	4.2	135	37.2
45	2.5	136	3.5
50	1.7	149	1.0
51	1.4	185	34.2
55	4.7	186	2.5
56	9.9	187	1.8
58	2.0	197	7.3
60	1.2	199	26.2
64	1.1	200	3.4
65	6.3	201	1.9
67	1.3	214	8.8
69	3.2	228	3.8
70	8.3	256	100.0
71	3.8	257	13.5
72	2.2	258	6.0
73	1.4	268	3.3
75	2.9	270	79.2
76	5.4	271	10.2
77	3.6	272	5.0
81	2.2	285	3.5
82	1.2	299	3.5

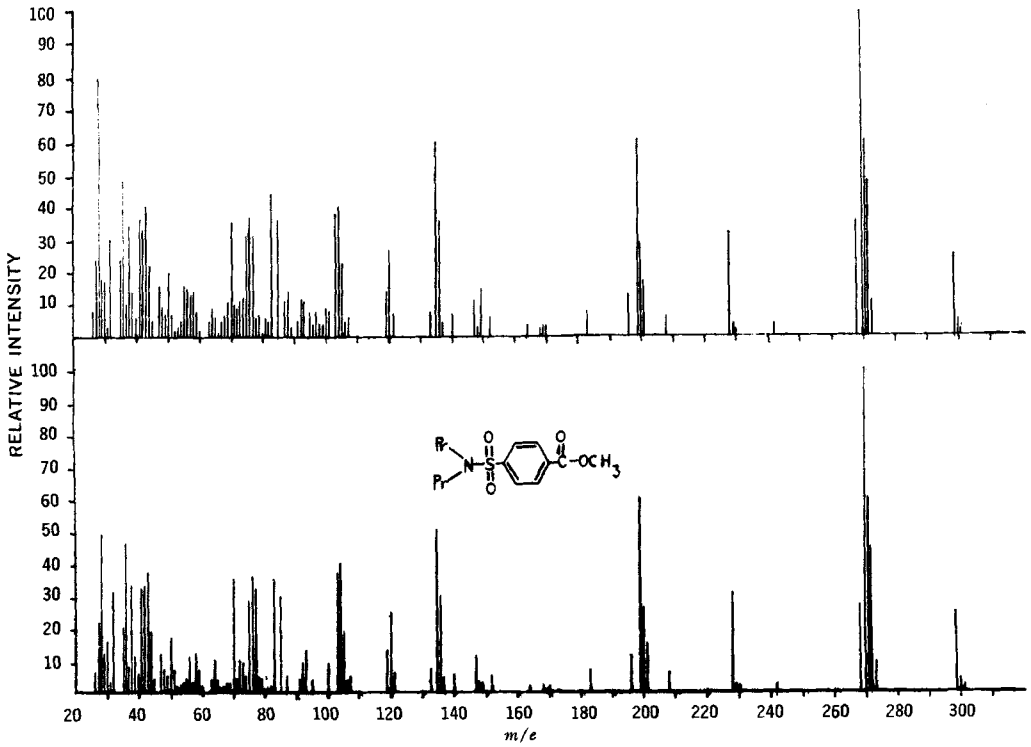


Figure 3—*Mass spectra of probenecid methyl ester and of probenecid metabolite.*

After the bile was treated with β-glucuronidase, additional peaks were observed on the gas chromatogram (Fig. 1). The major peak has the same retention time after enzymatic cleavage as does the parent probenecid (Fig. 1).

Further evidence was obtained that this peak was probenecid by the combined GC–mass spectrometry described previously. To establish firmly the masses of the prominent ions in the mass spectrum of the probenecid methyl ester, a high resolution mass spectrum was obtained (Table III) on the standard sample introduced into the mass spectrometer in the direct probe. The mass spectra of probenecid and that obtained by enzymatic hydrolysis of the glucuronide conjugate were determined by combined GC–mass spectrometry, and both spectra were identical (Fig. 3). The computer printout of probenecid spectrum was identical to that reported by Guarino *et al.* (6). Thus, no attempt will be made to identify fractions or fragmentation pattern.

Therefore, from this study, it would appear that the major metabolic product of probenecid in the rat is the simple acyl glucuronide. The authors do not feel that this is the only metabolite of probenecid, but it is the major metabolite because it is the only easily detectable peak observed by GLC when the probenecid peak remains on scale. However, when a larger quantity of metabolites is placed on the column so that the probenecid peak goes off scale, additional peaks are observed. By examining the data reported by Guarino *et al.* (6), it would appear that their results are similar but that they inadvertently disregarded their major metabolite, the metabolite that was in such great quantity that it went off scale and whose retention time was identical to the simple acyl glucuronide of probenecid. Therefore, it would appear that the major metabolic product of probenecid found in rat bile is the simple acyl glucuronide of probenecid.

CONCLUSION

The GC method described is accurate, sensitive, and specific for the determination of probenecid. The method is applicable to biological fluids such as blood, bile, and urine, using only very small amounts of these fluids.

The major metabolic product of probenecid was identified to be the simple acyl glucuronide conjugate. Conclusive evidence was obtained from the retention time and mass spectrometry following enzymatic hydrolysis. The mass spectra are simple and can be described mainly in terms of bond fission.

REFERENCES

(1) E. K. Tillson, *J. Pharmacol. Exp. Ther.*, **111**, 385(1954).

(2) K. H. Beyer, *Arch. Int. Pharmacodyn. Ther.*, **48**, 97(1954).

(3) I. M. Weiner and J. A. Washington, *Johns Hopkins Hosp. Bull.*, **106**, 333(1960).

(4) P. G. Dayton, J. F. Yü, W. Chen, L. Burger, L. A. West, and A. B. Lutma, *J. Pharmacol. Exp. Ther.*, **159**, 278(1963).

(5) A. M. Guarino and L. S. Schanker, *ibid.*, **164**, 387(1968).

(6) A. M. Guarino, W. D. Conway, and H. M. Fales, *Eur. J. Pharmacol.*, **8**, 244(1969).

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DRUG STANDARDS

Determination of Chloride in Aluminum Hydroxide Gels by Use of a Chloride-Selective Electrode

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Abstract □ The determination of the chloride content of aluminum hydroxide wet gels and dried powders is important for the control of both in-process materials and finished products. The application of the chloride-selective electrode was studied, and the response was found to be Nernstian in slurries containing 0.010–4.0% Cl and 1.00–2.50% in Al_2O_3 over the pH range 5.7–7.8. The modest equipment costs and operator skill required and the rapidity commend the method.

Keyphrases □ Chloride determination—aluminum hydroxide gels □ Chloride-selective electrode—chloride determination in aluminum hydroxide gels □ pH, aluminum hydroxide content, effects—chloride-selective electrode response

Aluminum hydroxide, as a wet gel or dried powder, is a major antacid and is manufactured by the controlled neutralization of an acidic solution of an aluminum salt, commonly aluminum chloride. The product is freed of chloride by persistent washing. Consequently, the determination of residual chloride is important both for in-process control and as a specification for the finished pharmaceutical grade product.

Chloride is conventionally determined in the products by a Volhard titration or by a turbidity comparison. In the Volhard titration the product is dissolved in nitric acid, a known volume of a standard silver nitrate solution is added, and the silver chloride precipitate is rendered inactive by addition and shaking with nitrobenzene. The excess of silver ion in the supernatant is titrated with a standard thiocyanate solution after addition of iron (III) nitrate as the indicator.

Ion-selective electrodes offer exciting possibilities for applied analysis, especially where many samples of a