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# Excretion of Probenecid and Its Metabolites in Bile and Urine of Rats

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**Abstract** □ The metabolites of probenecid excreted in the bile of rats with and without ligation of the renal pedicles were investigated using a new GLC assay procedure. Within 8 hr after administration, 63.8% of a 40-mg/kg iv dose of probenecid was accounted for in the bile of normal, anesthetized rats. The metabolites found (as percent of dose) include probenecid (10.0%), probenecid glucuronide (15.7%), glucuronide of the *N*-2-hydroxypropyl derivative (20.3%), glucuronide of the *N*-3-hydroxypropyl derivative (14.2%), and the unconjugated *N*-2-carboxyethyl derivative (3.6%). Ligation of the renal pedicles increased the excretion of each metabolite, raising the total recovery to 86.6%. These oxidized metabolites, but not probenecid or its glucuronide, were excreted in urine in unconjugated form (3–5% each). The unconjugated mono-*N*-depropylated metabolite accounted for 11.2% of the dose in the urine of normal, unanesthetized rats but was not found in the bile of the anesthetized animals.

**Keyphrases** □ Probenecid—metabolites, excretion in bile and urine of rats with and without renal pedicle ligation, GLC analysis □ Metabolism—probenecid, metabolites excreted in bile and urine of rats with and without renal pedicle ligation, GLC analysis □ GLC—analysis, probenecid metabolites, rats

The metabolic fate of probenecid, which was introduced as a uricosuric agent in 1950, has only recently been elucidated in humans and rats (1, 2). One major metabolite in rat bile was suggested to be the ether glucuronide of *p*-(*N*-propyl-*N*-2-hydroxypropylsulfamoyl)benzoic acid (2). In contrast, a more recent study (3) claimed the major metabolite to be the acyl glucuronide of probenecid. The later conclusion was based on the observation that the peak for probenecid was the only one readily detected by GC of the aglycones released by enzymatic hydrolysis of bile samples from rats given probenecid, 40 mg/kg iv. In the first study (2), the renal pedicles of the animals were ligated; in the later study (3), such ligation was not indicated.

The lack of agreement on the relative amounts of the various metabolites excreted probably arises from the need for a reliable quantitative assay for probenecid and its metabolites in biological fluids. A quantitative procedure based on GLC of the methyl esters for the determination of probenecid and two of its oxidation products is presented. An assay based on the propyl esters, which can be used for all known metabolites of probenecid, will be more completely described in a subsequent publication. These assays were used to measure the excretion of probenecid and its metabolites in the urine and bile of rats and to study the effect of renal ligation on the disposition of the drug and its metabolites in bile.

## EXPERIMENTAL

**Instrumentation**—A gas chromatograph[1], equipped with a flame-ionization detector and a 2.8-mm × 2-m (0.125-in. × 6-ft) stainless steel column packed with 10% OV-1 on 80–100-mesh Chromosorb W-HP, was used. Operating parameters were: column temperature, 225°; injection port, 280°; nitrogen carrier gas flow rate, 23 ml/min; and sensitivity, $2.5 \times 10^{-11}$ amp full scale.

**Procedure**—To determine free metabolites, biological samples (1 ml of urine or 0.2 g of bile plus 0.8 ml of water) in 50-ml centrifuge tubes were acidified with 1 ml of 5 *N* HCl and extracted by shaking for 30 min with 20 ml of methylene dichloride. A 10-ml aliquot of the methylene dichloride extract was evaporated to dryness on a water bath (50°) under a stream of air. All subsequent evaporations were conducted in the same way. The residue was dissolved in 0.5 ml of methanol and treated with 2 ml of an ether solution containing approximately 0.3 mmole of diazomethane. The samples were kept at room temperature for 1 hr, after which excess diazomethane was removed by evaporation. The residue was taken up in approximately 2 ml of ether, and a 25–50-μl aliquot containing 75–150 μg of *N,N*-dibenzylbenzenesulfonamide in methanol was added as an internal standard. The ether was evaporated and the residue was taken up in 0.25–0.50 ml of methylene chloride. Approximately a 1-μl aliquot was injected into the gas chromatograph. The quantity of internal standard and the final volume of methylene chloride were chosen so as to keep the GC response within the established range of linearity. Reproducibility was significantly enhanced by making the injection with a 10-μl syringe first loaded with 1 μl of ether adjacent to the plunger and separated from the sample by about 2 μl of air.

[1] Perkin-Elmer Mk II.

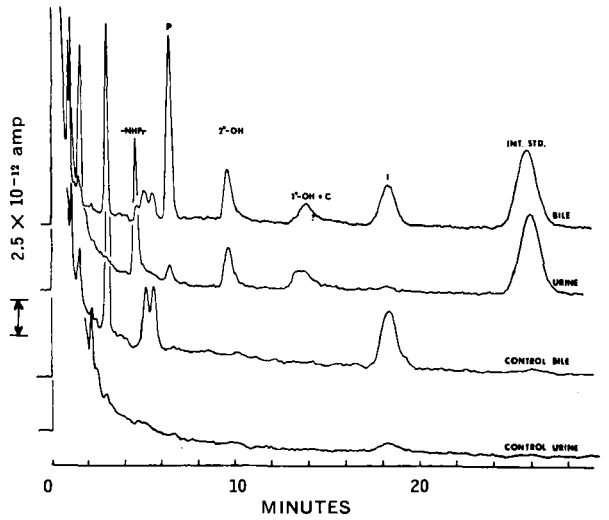


**Figure 1**—*GLC separation of probenecid metabolites in samples of 0–8-hr urine (1 ml) and bile (0.2 ml) from rats given 40 mg/kg iv of probenecid. See text for GLC operating conditions. Key: mono-N-propyl metabolite, —NHPr; probenecid, P; secondary alcohol, 2°-OH; primary alcohol, 1°-OH; terminal carboxy metabolite, C; impurity, I; and internal standard, int. std., N,N-dibenzylbenzenesulfonamide.*

Total metabolites were determined by heating the acidified biological sample prepared initially for 2 hr at 100° before proceeding with the methylene dichloride extraction. Conjugated metabolites were obtained by difference.

The efficiency of the extraction procedure was determined by assaying 1-ml aliquots of urine, to which were added known quantities of a methanolic solution of probenecid, the secondary alcohol, and the mono-*N*-propyl metabolite.

**Standard Curves**—Solutions containing approximately 2 mg/ml of authentic samples of probenecid, *p*-(*N*-propyl-*N*-2-hydroxypropylsulfamoyl)benzoic acid (the secondary alcohol metabolite), and *p*-(*N*-propylsulfamoyl)benzoic acid (the mono-*N*-propyl metabolite) were prepared in methanol. Following esterification of aliquots of these solutions (100–500 μl) as described, the dry residue was taken up in about 2 ml of ether and an aliquot of a methanol solution containing 750 μg of *N,N*-dibenzylbenzenesulfonamide was added as an internal standard. The ether solution was evaporated and the residue was taken up in 2.5 ml of methylene dichloride. Approximately 1-μl aliquots were injected into the gas chromatograph. The retention times (minutes) observed were: probenecid, 6.7; the secondary alcohol, 9.8; mono-*N*-propyl metabolite, 4.7; and internal standard, 26.0. A standard with this retention time was chosen to avoid interference from other metabolites and impurities that emerge over the 10–20-min interval (Fig. 1). The impurity emerging at about 18 min resulted from a contaminant in some batches of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide used to prepare the diazomethane.

Peak areas were determined by multiplying the peak height by the width at half height. The latter was measured using a comparator[2] (No. 30,325) fitted with a reticle (No. 30,323) having a 20-mm scale subdivided to 0.1 mm. A plot of the amount ratio (amount of sample per amount of internal standard) *versus* the area ratio (area of sample peak per area of internal standard peak) was linear over an amount ratio range of at least 0–1. Aliquots of a standard solution containing all three compounds were run daily—before, during, and after the series of biological samples being assayed. Each day the average area ratios for this series of standards was then used to calculate the concentrations in biological samples in the series.

**Biological Studies**—Male, Sprague–Dawley rats, weighing 420–530 g, were anesthetized with urethan (1.3 mg/g body weight ip). The animals were opened with a midline incision and the bile duct was cannulated with PE-10 polyethylene tubing. In some rats the renal pedicles were also ligated. The incision was covered with a gauze sponge which was kept moistened with 0.9% saline solution throughout the experiment. Probenecid was dissolved in a few drops of sodium bicarbonate solution and diluted with distilled water to a concentration of 20 mg/ml. A dose of 40 mg/kg was administered *via* the femoral vein. Bile samples were collected in tared vials at hourly intervals for 6 hr, and a single sample was collected for 6–8 hr. The quantity of bile was determined by weight. In animals in which the renal pedicles were not ligated, the penis was ligated to prevent loss of urine. After 8 hr the urine was withdrawn through the bladder wall with a syringe. The bladder was then rinsed with 1 ml of isotonic saline solution, which was combined with the urine.

To study urinary excretion in unanesthetized animals, male, Sprague-Dawley rats, weighing 300–350 g, were given 40 mg/kg probenecid *via* the femoral vein. The animals were housed individually in metabolism cages, permitting separate collection of urine and feces. Solid food was withheld for the first 8 hr postmedication. Water was given *ad libitum*. A few drops of acetic acid was added to the urine collection vessel, and both urine and feces containers were immersed in ice during collection to minimize hydrolysis of ester glucuronides. Urine was collected at intervals of 0–8, 8–24, 24–48, and 48–72 hr.

**Synthetic Procedures**—Metabolites of probenecid were prepared from *p*-carboxybenzenesulfonyl chloride (I), which was prepared as described previously (1), and the appropriate secondary amine.

p-(*N-Propyl-N-3-hydroxypropylsulfamoyl)benzoic Acid (II)*—A solution of 2.2 g (19 mmoles) of 3-propylaminopropanol (5) and the quantity of I obtained from 5 g (22 mmoles) of sodium *p*-carboxybenzenesulfonate in 30 ml of acetonitrile was allowed to stand for 18 hr at room temperature. Water (50 ml) was added, the acetonitrile was removed by distillation *in vacuo*, and the product was precipitated by acidification with hydrochloric acid. White crystals (II), 1.72 g, 34% yield, were obtained, mp 140–150°. Three recrystallizations from isopropanol–water (1:2, v/v) raised the melting point to 167–168° [lit. (4) mp 168–169°].

*Anal.*—Calc. for $C_{13}H_{19}NO_5S$: C, 51.81; H, 6.35; N, 4.65. Found: C, 51.70; H, 6.19; N, 4.51.

p-(*N-Propyl-N-3-propionitrilosulfamoyl)benzoic Acid (III)*—A solution of 2.8 g (24 mmoles) of propylaminopropionitrile (6), the quantity of I obtained from 6 g (27 mmoles) of sodium *p*-carboxybenzenesulfonate, and 15 ml of triethylamine in 35 ml of acetone plus 5 ml of methanol was allowed to stand for 18 hr at room temperature. The reaction mixture was diluted with 50 ml of water and acidified with hydrochloric acid. White crystals (III), 2.58 g, 41% yield, were obtained, mp 208–214°. Recrystallization from isopropanol raised the melting point to 217–217.5° [lit. (4) mp 213–215°]. The product was obtained in similar yield and purity by reacting 27 mmoles of I with 50 mmoles of propylaminopropionitrile in 10 ml of acetone, followed by the same isolation procedure.

p-(*N-Propyl-N-2-carboxyethylsulfamoyl)benzoic Acid (IV)*—Refluxing 1 g (39 mmoles) of the nitrile (III) for 1 hr in a mixture of 25 ml of acetic acid and 50 ml of concentrated hydrochloric acid followed by cooling, dilution with 50 ml of water, filtration, and drying gave 0.95 g (89% yield) of IV, mp 208–210°. Two recrystallizations from isopropanol–water (1:1, v/v) raised the melting point to 209–210.5° [lit. (4) mp 203–204°].

*Anal.*—Calc. for $C_{13}H_{17}NO_6S$: C, 49.51; H, 5.44; N, 4.44. Found: C, 49.34; H, 5.37; N, 4.33.

## RESULTS AND DISCUSSION

Recoveries of probenecid and the mono-*N*-propyl and secondary alcohol metabolites from urine varied from about 85 to 94% for concentrations of 112–390 μg/ml (Table I). Recoveries from bile samples were not significantly different. The reason for lower recoveries from solutions containing 13 μg/ml was not apparent. Virtually none of the biological samples assayed contained less than 112 μg/ml; therefore, the mean recovery values for the solutions containing 112–390 μg/ml were used to correct the experimental results reported later.

A peak with a retention time of about 14 min, which was observed for extracts of bile from medicated but not from control animals (Fig. 1), was initially presumed to represent the methyl ester

[2] Edmund Scientific Co., Barrington, N.J.

**Table I**—Recoveries of Probenecid and Metabolites from 1 ml of Urine

| Amount Added, μg | Percent Recovered[a] | | |
|---|---|---|---|
| | Probenecid | Mono-*N*-propyl | Secondary Alcohol |
| 336–390 | 87.1 ± 2.7 | 90.7 ± 2.9 | 94.0 ± 3.7 |
| 112–130 | 84.8 ± 3.3 | 87.3 ± 4.1 | 87.6 ± 8.4 |
| 11–13 | 47.3 ± 8.2 | 54.7 ± 6.5 | 75.7 ± 14.5 |

[a] Means of three trials ± standard deviation.

of either the primary alcohol or terminal carboxy metabolite which were reported to be resolved by GLC (2). When authentic samples of these metabolites were later prepared, using the procedures outlined under *Experimental*, their methyl esters were not resolved on the equipment using 10% OV-1 as the stationary phase. On the other hand, resolution of the mono-*N*-propyl metabolite from probenecid was better on 10% OV-1 than on 3% OV-1, 2.5% SE-30, or 3% OV-17. Therefore, composite samples were prepared of the biles from each group of rats and were assayed after conversion of the metabolites to the propyl esters, which were fully resolved by GLC on 10% OV-1. These results are presented in the last two columns of Table II. Details of this procedure using the propyl esters will be given in a later publication.

The excretion of probenecid and four of its metabolites in bile and urine is shown in Table II. Comparison of the results for Groups 1 and 2 illustrates that renal ligation has no significant effect on the qualitative composition of the metabolites excreted in bile. The total amount of each compound excreted in bile, particularly the primary alcohol, is moderately increased by ligation of the renal pedicles. The total of 86.6% of the dose recovered in the bile of rats with the renal pedicles ligated is in reasonable agreement with the 96% recovery obtained, using $^{14}C$-labeled probenecid (2). Only probenecid is found in large amount in the unconjugated state in bile. The free terminal carboxy metabolite is also excreted but in much smaller amounts. It could not be ascertained whether the free probenecid was secreted into the bile by the liver in the free state or whether it arose by hydrolysis of some glucuronide conjugate during passage through the biliary network. However, the composition of the collected bile was stable over several weeks when stored at −15°.

The results for biliary excretion of probenecid in the free and conjugated forms are somewhat higher than the amounts of 10.6 and 7.2%, respectively, calculated from the data of Sabih *et al.* (3). Their failure to detect the remaining metabolites may have resulted from the insensitivity of their assay, which was not validated with authentic samples of these compounds.

The present results support the conclusion that, at a dose of 40 mg/kg, the major metabolites excreted in rat bile are the glucuronic acid conjugates of the alcohols formed by hydroxylation of an *N*-propyl group along with somewhat lesser amounts of both free and conjugated probenecid.

Biliary excretion appears to be the major pathway for excretion of probenecid and its metabolites in rats. Small amounts of the unconjugated alcohols, but very little conjugated material, are excreted in urine. Some metabolites excreted in urine may arise as a result of an enterohepatic circulation. This is in agreement with the finding of less than 5% of the administered dose excreted in the urine of the animals of Group 1, although the low excretion in this case might also be due to impairment of renal function as a result of anesthesia.

The unconjugated mono-*N*-propyl metabolite is excreted in significant amounts in urine, while only traces in either free or conjugated form are found in bile. Therefore, while it is advantageous to use renal-ligated rats to enhance biliary excretion and to provide a concentrated sample for isolation and identification of metabolites (2), it is important that urine also be collected to determine the complete disposition of the drug, since there may be large quantitative differences in the composition of the two fluids resulting from limited excretion of certain metabolites by one of the pathways.

This study was done with the relatively high dose of 40 mg/kg of probenecid to permit direct comparison with previously published studies (2, 3). However, the sensitive GLC assay presented should

**Table II**—Excretion of Probenecid and Its Metabolites in Urine and Bile of Rats after Intravenous Administration of a 40-mg/kg Dose

| Animal Preparation | | | | Amount Excreted in 8 hr, % of Dose[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Probenecid | | | Secondary Alcohol | | | Mono-*N*-propyl[b] | Primary Alcohol[c] | | Terminal Carboxy[b,c] | All Metabolites |
| Group | Anesthesia | Renal Ligation | Excretion in | F[d] | G[d] | T[d] | F | G | T | F | F | G | T | F | T |
| 1 | Yes | No | Bile | 10.0 ± 0.5 | 15.7 ± 2.2 | 25.7 | — | 20.3 ± 0.6 | 20.3 | —[e] | — | 14.2 | 14.2 | 3.6 | 63.8 |
| 2 | Yes | Yes | Bile | 12.8 ± 2.8 | 17.0 ± 3.5 | 29.8 | — | 23.1 ± 1.8 | 23.1 | —[e] | — | 27.9 | 27.9 | 5.8 | 86.6 |
| 3 | No | No | Urine | Trace | Trace | Trace | 5.4 ± 0.7 | —[e] | 5.4 | 11.2 ± 1.3 | 4.5 | — | 4.5 | 3.5 | 24.6 |

[a] Mean ± standard error ($s/\sqrt{n}$) for three animals based on assay of the methyl esters except as noted in c. [b] None excreted as the glucuronide conjugate. [c] Based on a single assay of a composite sample of all three animals as the propyl esters. [d] F = free compound; G = conjugate with glucuronic acid; T = F + G. [e] Not detectable.

also be applicable to urine and plasma samples obtained after administration of conventional doses of the drug.

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# Quantitative GLC Determination of Cyclophosphamide and Isophosphamide in Biological Specimens


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**Abstract** □ A method for measuring the antitumor agent cyclophosphamide was developed and applied to the determination of the drug in biological specimens. After extraction with ether, cyclophosphamide and the internal standard isophosphamide are converted to their trifluoroacetyl derivatives and assayed by GLC, using either an electron-capture or a flame-ionization detector. The minimum detectable amount is 25 pg/injection using the electron-capture detector. Linearity was found up to microgram amounts of substance, without any interference of endogenous substrates. Values from serum, urine, and liver in mice treated with a single dose of cyclophosphamide (85 mg/kg) are also reported.



**Keyphrases** □ Cyclophosphamide—quantitative GLC determination in biological specimens □ Isophosphamide—quantitative GLC determination in biological specimens □ GLC—cyclophosphamide and isophosphamide, quantitative determination in biological specimens


Cyclophosphamide {2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide} (I) is a well-known anticancer drug utilized both in animal studies and in clinical practice (1–3).

Colorimetric, titrimetric, and IR methods have been used for estimating I (4–8), although they suffer from a lack of sensitivity and specificity, particularly when applied to biological specimens. Labeled I has been also utilized, but the results require validation for specificity because I undergoes a complicated metabolic pathway (9–12).

More recently, a GLC method for measuring I was described (13), but its usefulness in quantitative determination was limited by the presence of decomposition products. Compound I was also measured by direct injection into the ion source of a mass spectrometer, but the specificity of the determination was not accompanied by sufficient sensitivity (14).

The method described here does not suffer from some of these disadvantages. It is based on the formation of a stable trifluoroacetyl derivative of I suitable for GLC analysis of biological specimens (Scheme I). Isophosphamide [3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazaphosphorine-2-oxide] (II), an analog of I with similar antitumor activity (15–17), also forms a stable trifluoroacetyl derivative (Scheme I) and was chosen as the internal standard for quantitation of I.

## EXPERIMENTAL

**Standards and Reagents**—The following reagents were used: trifluoroacetic anhydride[1], sodium hydroxide, ether, and ethyl acetate[2].

Cyclophosphamide[3] and isophosphamide[3] were used as the hydrate salts, with all concentrations expressed in terms of the free base. Drugs were dissolved in double-distilled water; methylaminochlorobenzophenone[4] (internal marker) was dissolved in ethyl acetate.

**GLC**—GLC was carried out on a gas chromatograph[5] equipped with a flame-ionization detector[6] or a $^{63}Ni$ electron-capture detec-

[1] Fluka AG, Buchs, Switzerland.
[2] Carlo Erba, Milan, Italy.
[3] Supplied by Asta Werke AG, Brackwede, West Germany.
[4] Supplied by Ravizza, Milan, Italy.
[5] Carlo Erba Fractovap model Gl.
[6] Carlo Erba model 20.