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Note

Rapid high-performance liquid chromatographic method for the determination of probenecid in biological fluids

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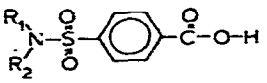
In the course of a study on the effect of probenecid (Fig. 1, I) on the renal excretion of other drugs we felt the need to develop a rapid and sensitive quantitative method for the detection of I in plasma and urine. The various



I $R_1 = R_2 = -CH_2-CH_2-CH_3$
II $R_1 = R_2 = -CH_2-CH_3$
III $R_1 = R_2 = -CH_2-CH(CH_3)-CH_3$

Fig. 1. Structural formulae: I, dipropylsulfamoyl benzoic acid (probenecid); II, diethylsulfamoyl benzoic acid; III, diisobutylsulfamoyl benzoic acid.

older spectrophotometric methods that have been reported [1—3] lack the required sensitivity and specificity, whereas the more recently introduced gas chromatographic procedures [4—7] make derivatization of I necessary, which is a complicating and time-consuming step in the analysis.

In this case high-performance liquid chromatography is the method of choice. The procedure described in this report combines sensitivity and specificity with ease of handling. Chromatography is performed in a soap chromatography mode using a C_8 hydrocarbon stationary phase. As internal standards the diethyl analog (II) and the diisobutyl analog (III) of probenecid are applied.

MATERIALS AND METHODS

Materials

Probenecid was obtained from Sigma (St. Louis, Mo., U.S.A.). Compounds II and III were synthesized from *p*-carboxybenzenesulfonylchloride and the appropriate amines, according to a procedure adopted from Mieler [8]. Stock solutions of I, II and III were prepared in 5% sodium bicarbonate. LiChrosorb RP-8 (5 μm) and sodium dodecyl sulphate were obtained from E. Merck (Darmstadt, G.F.R.). All other reagents were of analytical grade.

Apparatus

The equipment used was a high-pressure liquid chromatograph of Waters Assoc. (Milford, Mass., U.S.A.) consisting of an M6000A pump, a 46K universal injector and an M440 absorbance detector.

Chromatographic conditions

A stainless-steel column (15 cm × 0.46 cm I.D.) was packed with LiChrosorb RP-8 (5 μm). Chromatography was performed in a soap chromatography mode using a mobile phase of methanol—water—acetic acid with 0.005 *M* sodium dodecyl sulphate added as a counter-ion. For plasma samples the mobile phase contained methanol—water—acetic acid in a volume ratio of 49.5:49.5:1; for urine samples the ratio was 64.5:34.5:1. The flow-rate was fixed at 1.5 ml/min. Some typical examples of chromatograms obtained from plasma and urine samples are given in Fig. 2.

Experimental

Extraction procedure: Samples of plasma (1.0 ml) or urine (1 ml) were trans-

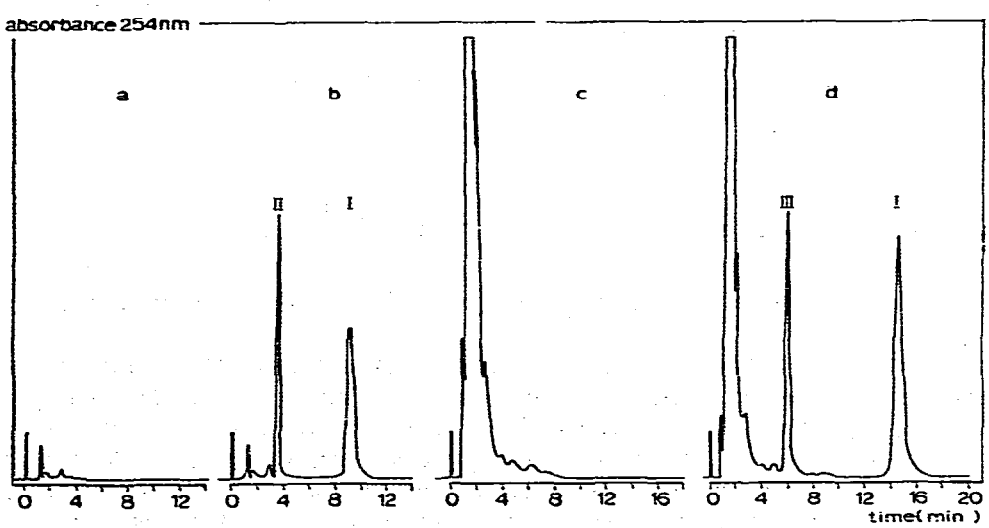


Fig. 2. Chromatograms obtained from plasma and urine samples. (a) Blank plasma; (b) plasma containing compounds I and II; (c) blank urine; (d) urine containing compounds I and III.

ferred to conical test-tubes, spiked with 1 ml of internal standard solution and acidified by the addition of 1 ml of 2 *N* HCl. Extraction was carried out by shaking this mixture mechanically with ethyl acetate (5 ml) for 10 min. After centrifugation the ethyl acetate layer was transferred to another test-tube and evaporated to dryness under a stream of dry, filtered air in a waterbath of 45°. The residue was dissolved in 250 µl of methanol, an aliquot of which (10 µl) was injected into the liquid chromatograph.

Calibration procedure. Samples of blank plasma (1.0 ml) or urine (1 ml) were spiked with various known amounts of probenecid, ranging from 0.5 to 40 µg, and with a fixed amount of internal standard (usually 25 µg). Since interfering peaks necessitated different mobile phases for plasma and urine samples, optimal chromatographic conditions required different internal standards. In the case of plasma, compound II was used as internal standard, in the case of urine compound III. The calibration samples were extracted as outlined above and after injection into the liquid chromatograph peak height ratios of probenecid to internal standard were plotted against the amount of probenecid added.

RESULTS AND DISCUSSION

Both for probenecid and compounds II and III the efficiency of the extraction process was estimated by comparing peak heights obtained after injection of standard solutions to peak heights obtained after injection of extracted standard solutions. The mean recoveries found were 98 ±2% ($n = 6$) for probenecid and for compound III, and 95 ± 2% ($n = 6$) for compound II. This recovery appeared to be constant over the concentration range 0.5—40 µg/ml. The detection limit of probenecid with the method described was below 0.5 µg/ml, but reproducible analytical results were obtained only when the concentration was 0.5 µg/ml or higher. Then the following equations are obtained for the calibration graphs from plasma

$$C_{\text{probenecid}} = 3.64 \times \text{amount compound II} \times \frac{\text{peak height probenecid}}{\text{peak height compound II}}$$

and from urine

$$C_{\text{probenecid}} = 0.563 \times \text{amount compound III} \times \frac{\text{pH probenecid}}{\text{pH compound III}}$$

Over the whole range studied the standard deviation of the plasma and urine determination was 2—5%.

This method of probenecid analysis was successfully applied to pharmacokinetic studies, details of which will be reported elsewhere. As an example of these investigations Fig. 3 shows the plasma curve obtained after oral administration of a 500-mg dose of probenecid to a human volunteer. Although this curve suggests a linear pharmacokinetic profile, experiments with higher doses clearly indicate the occurrence of nonlinear, saturable elimination. Whereas after a single 500-mg dose plasma concentrations are near to the limit of detection at 24 h after dosing, this period may be substantially prolonged

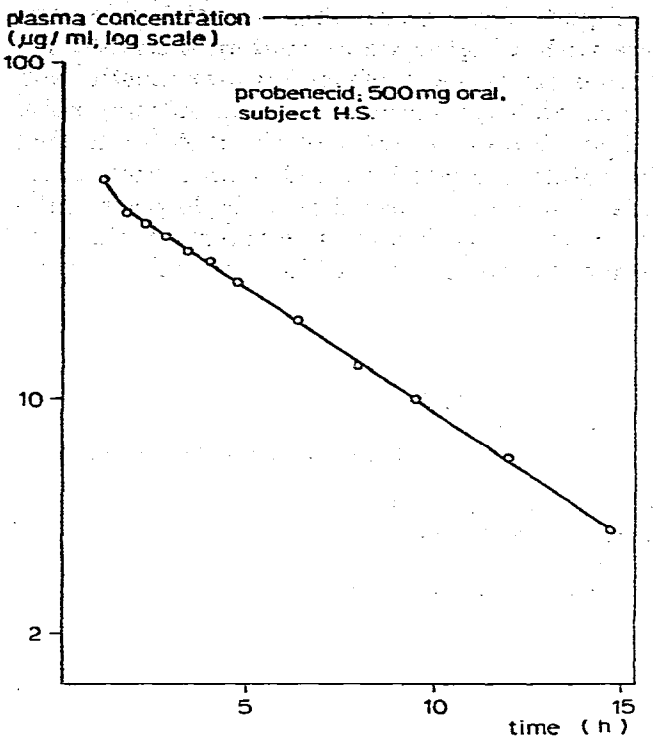


Fig. 3. Plasma concentration curve obtained after oral administration of 500 mg of probenecid to a human volunteer.

after application of higher doses. Less than 5% of a 500-mg dose is recovered unchanged from urine, the rest is metabolised. One of the metabolites is the probenecid acyl glucuronide (IV, Fig. 4), which can be measured in urine after hydrolysis with the aid of β-glucuronidase and which accounts for about 25%

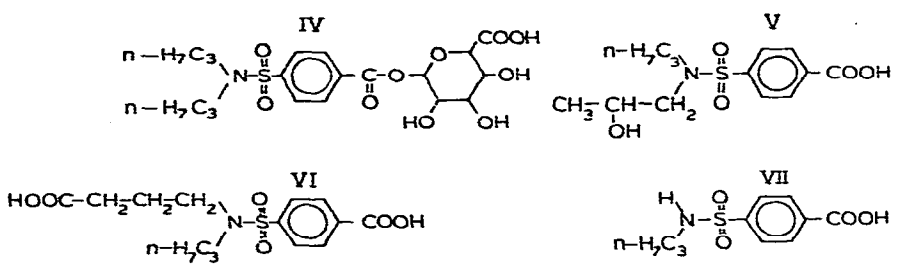


Fig. 4. Structural formulae of the principal metabolites of probenecid: IV, acyl glucuronide of probenecid; V, *sec.*-hydroxylated metabolite; VI, carboxy metabolite; VII, monopropyl metabolite.

of the dose. For the analysis of the other metabolites (Fig. 4) some modifications of the procedure described above are necessary. Extraction of these metabolites from urine is preferably performed with a 5-ml aliquot of a dichloromethane—diethyl ether (1:2) mixture. As internal standard the diethyl

analog (compound II) is used. Liquid chromatographic separation of the metabolites requires a mobile phase with a higher water content than used for probenecid itself. By using a mobile phase of methanol—water—acetic acid (39.5:59.5:1) with 0.005 *M* sodium dodecyl sulphate, the cumulative renal excretion of compounds V and VI was found to represent about 20% and that of compound VII about 5% of the total dose administered. Under these chromatographic conditions compounds V and VI appeared as a single peak in the chromatograms, showing a retention time of 5.9 min. The retention times of compounds VII and II (internal standard) were 4.5 and 7.7 min, respectively.

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