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DETERMINATION OF BENZYLPENICILLIN AND PROBENECID IN HUMAN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the determination of benzylpenicillin and probenecid concentrations in human body fluids using ion-pair reversed-phase chromatography with UV detection has been developed. For plasma samples two extraction techniques were investigated. Precipitation of the plasma proteins with acetonitrile followed by liquid—liquid extraction offered the best results. The limits of detection were 0.5 μg/ml for benzylpenicillin and 0.25 μg/ml for probenecid, which offer sufficient sensitivity for application in pharmacokinetic experiments.

INTRODUCTION

Probenecid, (*p*-dipropylsulphamoyl)benzoic acid, is used as a uricosuric

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agent as well as an adjuvant to therapy with benzylpenicillin and other β-lactam antibiotics, since probenecid inhibits the renal tubular excretion of penicillin and other weak acids [1]. Accordingly, it has been assumed that the increased penicillin plasma levels observed upon co-administration of probenecid are due to competition for active tubular excretion, resulting in a markedly diminished renal clearance of penicillin.

The analysis of penicillins in biological fluids is traditionally performed by microbiological methods, which often offer adequate sensitivity but poor accuracy. Furthermore, the presence of probenecid in samples has been reported to disturb the quantitation of benzylpenicillin [2].

Several reversed-phase high-performance liquid chromatographic (HPLC) methods have been reported for the determination of penicillins [3—10] as well as probenecid [11—15] in body fluids. For pharmacokinetic studies of the interaction of benzylpenicillin with probenecid measurement of both compounds is required. For practical reasons such a method should be simple and rapid; preferably the analysis time for one sample should not exceed 15 min. Unfortunately none of the methods reported so far fulfils these requirements.

This report describes the development of a rapid, sensitive and reproducible reversed-phase HPLC method for the determination of benzylpenicillin and probenecid levels in plasma, urine and cerebrospinal fluid in one single run.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a dual-piston constant-flow pump (Spectroflow 400, Kratos Analytical Instruments, Ramsey, NJ, U.S.A.), a syringe-loaded injection system (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) and a variable-wavelength UV detector (Kratos Spectroflow 773) operating at 231 nm. Chromatograms were registered on a flat-bed recorder (BD 40, Kipp & Zonen, Delft, The Netherlands). The analytical column (stainless-steel precision-bore tubing, 100 × 3.0 mm I.D.) was packed with MOS-Hypersil 5-μm C_8 (Shandon, London, U.K.) according to a method described elsewhere [16].

Chemicals and materials

Probenecid was kindly donated by Merck, Sharp & Dohme (Haarlem, The Netherlands). Benzylpenicillin (potassium salt) was purchased from Gist Brocades (Delft, The Netherlands). Methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was purified by means of a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Sodium acetate was purchased from Merck (Darmstadt, F.R.G.), tetrabutylammonium bromide (TBABr) and tetrabutylammonium hydrogen sulphate ($TBAHSO_4$) were purchased from Sigma (St. Louis, MO, U.S.A.).

Chromatographic procedures

The mobile phase consisted of mixtures of methanol, acetonitrile and a sodium acetate buffer (0.005 *M*); TBABr was used as an ion-pair forming

agent. For sample analysis the mobile phase was 0.005 *M* sodium acetate (pH 5.0)—acetonitrile–methanol (62:12:26) with 0.003 *M* TBABr. Both mobile phase and column were kept at 22°C.

The capacity ratio (k') was calculated from the retention time of the compound and that of an unretained compound (potassium periodate). The selectivity coefficient for compounds j and i is given k'_j/k'_i.

Sample preparation

Plasma. (1) To 0.5 ml of plasma, 250 μl tetrabutylammonium hydrogen sulphate solution (0.15 *M*) were added. After mixing on a whirlmixer for 30 s, 4 ml of diethyl ether were added and mixed. After mixing and centrifugation at 1000 *g* for 30 s, 3 ml of the organic layer were transferred to another tube and evaporated to dryness using a rotary evaporator. The residue was reconstituted in 250 μl of Sörensen buffer. Aliquots of 20 μl were injected onto the HPLC column.

(2) The proteins in 0.5 ml of plasma were precipitated by adding 500 μl of acetonitrile during mixing on a whirlmixer. After mixing for 10 min on a Rotationmischer 3300 (Eppendorf, Hamburg, F.R.G.) and centrifugation for 5 min at 1000 *g*, 700 μl of the supernatant were transferred to another tube and 3.5 ml of dichloromethane were added. After mixing for 30 s and centrifuging at 1000 *g* for 1 min, 20 μl of the aqueous layer were injected onto the column [3].

Urine. Urine samples were diluted with Sörensen buffer and injected onto the column without further sample pretreatment.

Cerebrospinal fluid. Samples of cerebrospinal fluid were injected directly onto the HPLC column.

RESULTS AND DISCUSSION

Chromatographic system

Mobile phases consisting of binary mixtures of sodium acetate buffer (pH 5.0) and either methanol or acetonitrile did not offer the required results; the analysis time was far too long for application in routine analysis. It was found that acetonitrile and methanol affect the retention of benzylpenicillin and probenecid differently. Therefore the possibilities of ternary systems consisting of methanol, acetonitrile and sodium acetate buffer were investigated.

In phase systems without counter-ion the ratio of the acetonitrile and methanol contents was varied while the total percentage of organic modifier remained constant. The influence of the modifier ratio on the capacity ratio for benzylpenicillin and probenecid is shown in Fig. 1. With increasing methanol—acetonitrile ratios the capacity ratios increase owing to the lower elution strength of methanol, which is in agreement with the results of other studies [17].

Although the capacity ratio for probenecid was less strongly influenced than the capacity ratio for benzylpenicillin, the selectivity coefficient was barely influenced in the investigated range.

Although the analysis time was short (ca. 2 min) when a mobile phase consisting of 28% methanol and 9.5% acetonitrile in an acetate buffer (pH 5.0,

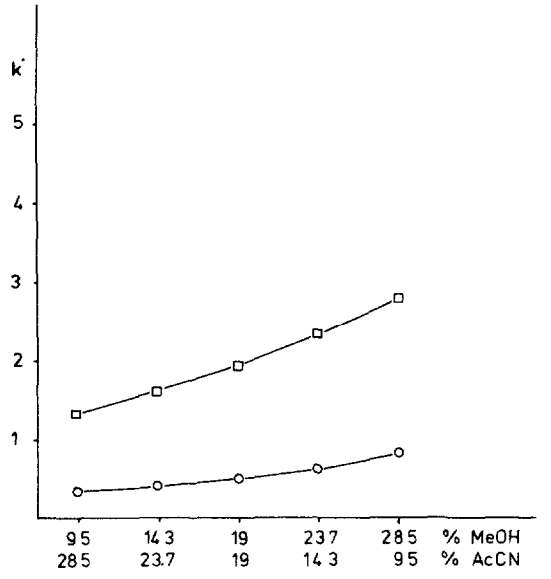

Fig. 1. Plots of the capacity ratio (k') versus the methanol—acetonitrile concentration (v/v) in the mobile phase (0.005 *M* sodium acetate, pH 5.0) without counter-ion for benzylpenicillin (○) and probenecid (□).

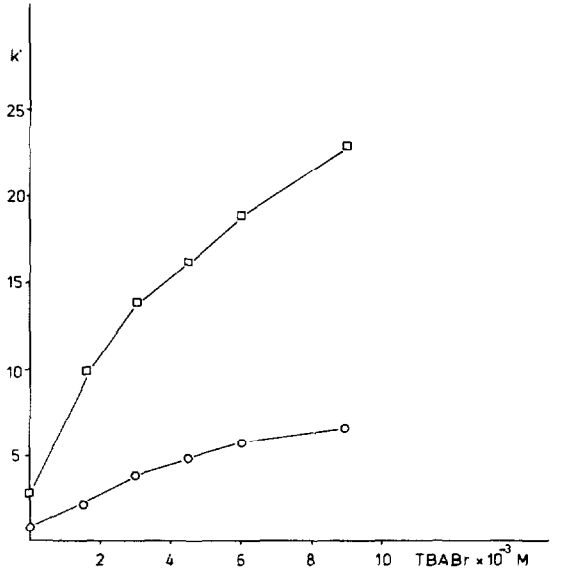

Fig. 2. Plots of the capacity ratio (k') versus the concentration of the counter-ion TBABr in the mobile phase (0.005 *M* sodium acetate, pH 5.0, 26% methanol and 12% acetonitrile) for benzylpenicillin (○) and probenecid (□).

0.005 *M*) was used, this system could not be applied for analysis of plasma samples since both peaks were masked by matrix peaks. Unfortunately a complete separation of benzylpenicillin and the interfering plasma constituents could not be accomplished with any of the mobile phases investigated, premising that probenecid had to be eluted within 15 min.

In order to increase the capacity ratio for benzylpenicillin, the effect of the addition of a counter-ion in the mobile phase was investigated. The effect of various TBABr concentrations in the mobile phase on the capacity ratios for benzylpenicillin and probenecid is shown in Fig. 2.

The capacity ratios increase with increasing concentration of TBABr. For

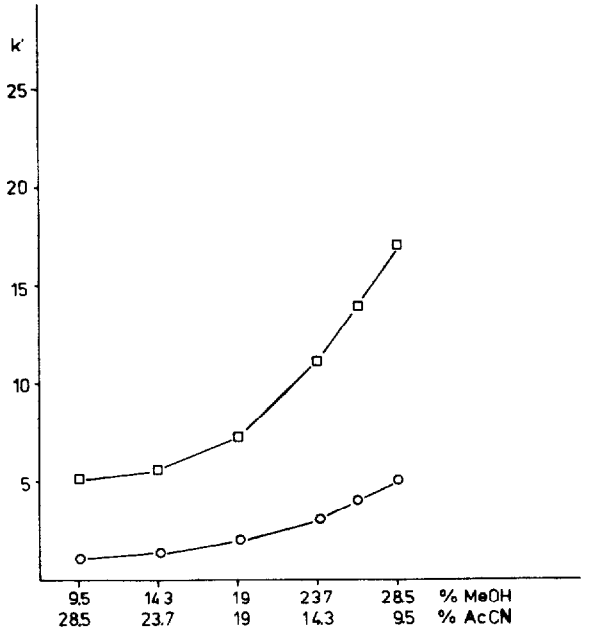

Fig. 3. Plots of the capacity ratio (k') versus the methanol—acetonitrile concentration (v/v) in the mobile phase (0.005 *M* sodium acetate, pH 5.0, 0.003 *M* TBABr) for benzylpenicillin (○) and probenecid (□).

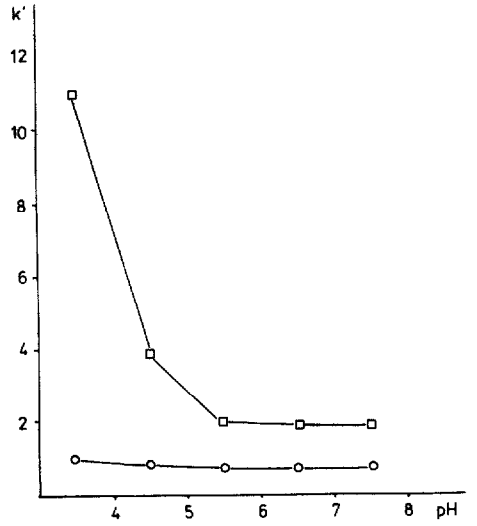

Fig. 4. Plots of the capacity ratio (k') versus the pH of the mobile phase without counter-ion, 26% methanol and 12% acetonitrile (v/v) for benzylpenicillin (○) and probenecid (□).

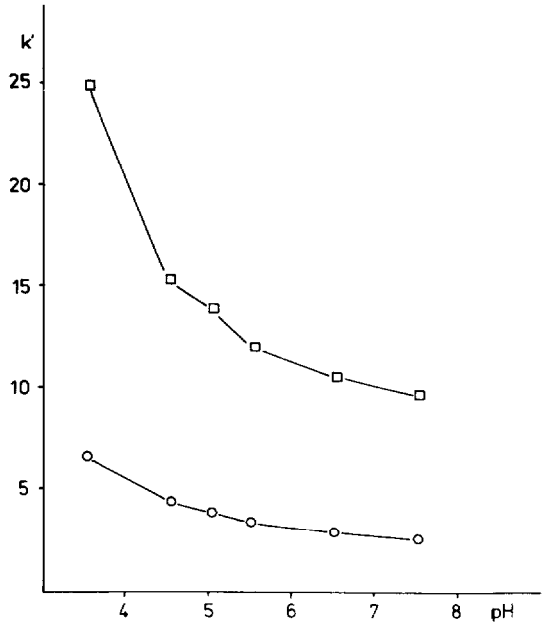

Fig. 5. Plots of the capacity ratio (k') versus the pH of the mobile phase with 26% methanol, 12% acetonitrile (v/v) and 0.003 M counter-ion TBABr for benzylpenicillin (○) and probenecid (□).

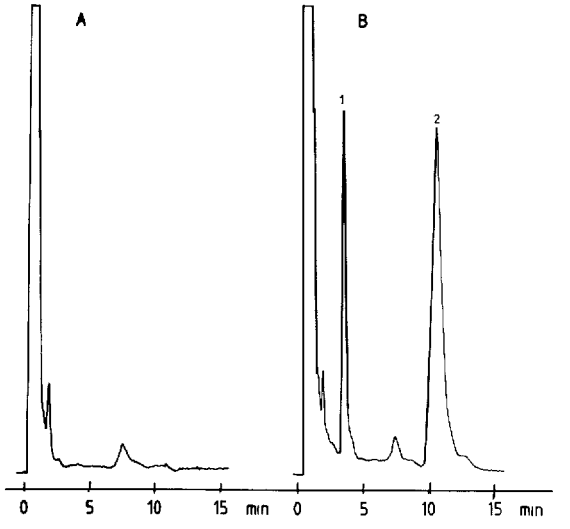

Fig. 6. Chromatograms of (A) blank plasma and (B) a patient plasma sample during continuous intravenous administration of benzylpenicillin and probenecid. The concentrations are for benzylpenicillin (1) and probenecid (2) are 50 and 25 μg/ml, respectively.

benzylpenicllin they leveled off at higher concentrations of TBABr. The addition of TBABr increased the retention of benzylpenicillin and probenecid considerably. At a concentration of 0.003 M TBABr the capacity ratio for benzylpenicillin was high enough for complete separation from the matrix peaks, while the capacity ratio for probenecid was sufficiently high for complete separation from a more retained plasma peak.

The influence of the modifier ratio on the capacity ratio in the presence of 0.003 *M* TBABr at a total modifier content of 38% in 0.005 *M* sodium (pH 5.0) is shown in Fig. 3. Comparing this figure with Fig. 1, the selectivity coefficients for probenecid and benzylpenicillin turn out to be in the same range, but the selectivity with respect to the biological background is markedly improved after addition of 0.003 *M* TBABr.

The relation between the pH and the capacity ratios for benzylpenicillin and probenecid in the absence and in the presence of TBABr is shown in Figs. 4 and 5, respectively. The marked effect of the pH on the retention behaviour of probenecid in the system without TBABr can be seen (pK_a probenecid 3.4, pK_a benzylpenicillin 2.8).

A mobile phase with pH 5.0 was chosen because the selectivity with respect to the sample background appeared to be optimal at this pH.

The mobile phase chosen was 0.005 *M* sodium acetate (pH 5.0)—acetonitrile—methanol (62:12:26) with 0.003 *M* TBABr. Chromatograms for blank and patient plasma extracts are presented in Fig. 6. Benzylpenicillin is completely separated from the plasma matrix ($k' = 4$), while probenecid elutes within 12 min ($k' = 14$) at a flow-rate of 1.0 ml/min.

Sample preparation

The liquid—liquid extraction method (procedure 1) resulted in high recoveries (85—90%) and a good reproducibility (S.D. <5%) for both probenecid and benzylpenicillin in plasma. Unfortunately benzylpenicillin degrades under the acidic conditions that exist after adding $TBAHSO_4$. This entails working on a time schedule and handling only one sample at a time.

As an alternative, the possibility of precipitation was investigated. Although the precipitation method takes more than 30 min several samples can be handled simultaneously; moreover, penicillin does not degrade under the prevailing circumstances.

Method parameters

Correlation coefficients for the calibration curves were at least 0.999 in the range 0.5—200 μg/ml for both compounds. Replicate analyses of spiked serum gave coefficients of variation ranging from 2 to 5% for day-to-day reproducibility.

The limits of detection for benzylpenicillin and probenecid were 0.5 and 0.25 μg/ml, respectively, for the liquid—liquid extraction as well as the precipitation procedure.

Analyses of urine and cerebrospinal fluid samples, even without sample clean-up, yielded a good separation of the compounds from the sample background.

CONCLUSION

A rapid, sensitive and reproducible method for the determination of benzylpenicillin and probenecid levels has been developed. The method can be applied to the determination of these compounds in plasma, urine and cerebrospinal fluid samples. Sample pretreatment is rapid and does not affect the stability of benzylpenicillin.

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