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Note

Determination of 6β-bromopenicillanic acid (brobactam) in human serum and urine by high-performance liquid chromatography

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6β-Bromopenicillanic acid (brobactam, Fig. 1) is a powerful irreversible synthetic β-lactamase inhibitor [1] capable of protecting susceptible β-lactam antibiotics from inactivation by the bacterial enzymes [2]. The antibacterial effect of the combination of 6β-bromopenicillanic acid and ampicillin has been studied in vitro [3–6] and in vivo [2–7].

A microbiological assay has been described for determination of the concentration of 6β-bromopenicillanic acid in rat serum and tissue homogenates [2]. High-performance liquid chromatographic (HPLC) methods with post-column alkaline degradation have been described for the determination of clavulanic acid and sulbactam in plasma/serum and urine [8–11], where the degradation products of clavulanic acid and sulbactam show UV absorption maxima at 267 and 276 nm, respectively. In the present study a similar reaction is used for the determination of 6β-bromopenicillanic acid in serum and urine. The alkaline degradation of 6β-bromopenicillanic acid in methanol results in formation of 2,3-dihydro-2,2-dimethyl-6-methoxycarbonyl-1,4-thiazine-3-carboxylate [12], which exhibits a UV absorption maximum at 315 nm.

EXPERIMENTAL

Reagents and chemicals

6β-Bromopenicillanic acid was synthesized at Leo Pharmaceutical Products. Tetrabutylammonium bromide (puriss) was purchased from Fluka (Buchs, Switzerland). Acetonitrile (HPLC grade) and all the other chemicals (p.a. grade) were purchased from E. Merck (Darmstadt, F.R.G.). Deionized glass-distilled water was used for the preparation of HPLC eluents, post-column reagents and dilutions.

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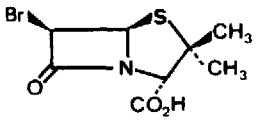

Fig. 1. Structure of 6β-bromopenicillanic acid (brobactam).

HPLC equipment and conditions

A liquid chromatograph consisting of a PU 4010 pump (Pye Unicam, Cambridge, U.K.), a PU 4020 UV detector (Pye Unicam) with an 8-μl flow-through cell and a sample injector (Model 7125 Rheodyne, Berkeley, CA, U.S.A.) with a 100-μl loop was used. A 125 mm × 4 mm I.D. LiChrospher 100 CH-18/II 4-μm column (E. Merck) was used for the serum analysis and a 150 mm × 4.1 mm I.D. Hamilton PRP-1 5-μm column (Hamilton, Bonaduz, Switzerland) was used for the urine analysis. The chromatograms were recorded on a PM 8251 standard compact recorder (Pye Unicam) and an HP 3390 A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The reagent solution for the post-column degradation was delivered from a Milton Roy minipump (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) to the reactor, which consisted of a 250 mm × 4.6 mm I.D. column packed with glass beads (ca. 40 μm), connected to PTFE tubing (2 m × 0.5 mm I.D.). The reactor was inserted between the analytical column and the detector (Fig. 2). The eluent used for serum was 0.05 *M* ammonium hydrogenphosphate–methanol (3:1) at a flow-rate of 0.8 ml/min, and the post-column reagent was 0.75 *M* sodium hydroxide–methanol (3:1) at a flow-rate of 0.8 ml/min. The eluent used for urine was (0.005 *M* sodium dihydrogenphosphate plus 0.005 *M* disodium hydrogenphosphate plus 0.005 *M* tetrabutylammonium bromide)–methanol (1:1) at a flow-rate of 1.0 ml/min, and the post-column reagent was 0.75 *M* sodium hydroxide–methanol (1:1) at a flow-rate of 0.6 ml/min.

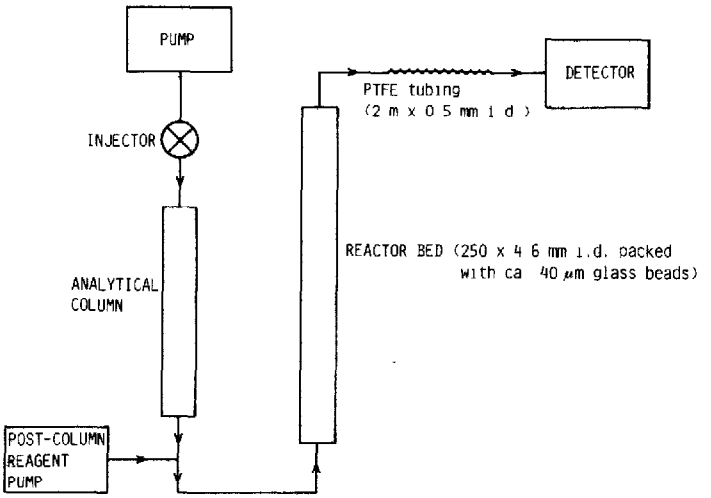

Fig. 2. Schematic diagram of the chromatographic equipment.

Sample preparation

A 1000-μl volume of serum was deproteinized with 2.00 ml of acetonitrile which was mixed on a whirlimixer for ca. 1 min before centrifugation for 5 min at 2300 *g*. Thus 2.00 ml of the supernatant were evaporated to dryness under vacuum at ambient temperature. The dry residue was redissolved in 500 μl of eluent and chromatographed as described above.

A 100-μl volume of urine was diluted ten-fold with water. This dilution was chromatographed as described above, without further preparation.

RESULTS AND DISCUSSION

Fig. 3 shows the chromatograms of blank serum and urine, and of serum and urine spiked with 6β-bromopenicillanic acid. As can be seen, 6β-bromopenicillanic acid is well separated from other compounds in serum or urine. 6β-Bromopenicillanic acid in serum was separated on a LiChrospher 100 CH-18/II column. The eluent was optimized, so there was no interference from serum or from 6α-bromopenicillanic acid or from ampicillin. 6α-Bromopenicillanic acid is the 6-epimer of β-bromopenicillanic acid and could be a possible metabolite, and 6β-bromopenicillanic acid will be coadministered with pivampicillin, an orally active prodrug of ampicillin, which in the organism is hydrolysed to ampicillin.

After optimization of the eluent, the post-column reagent was optimized. The

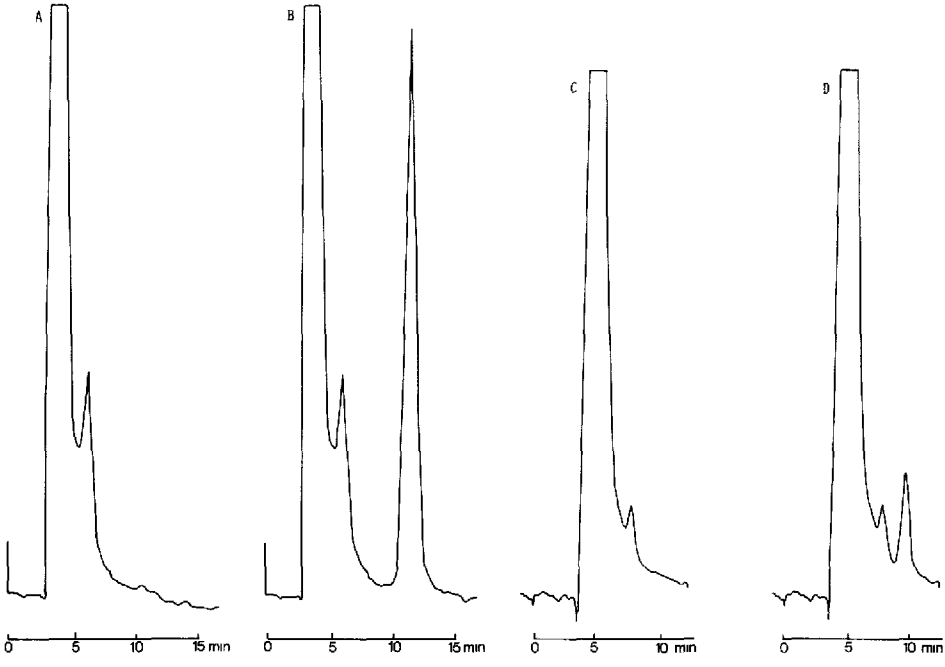

Fig. 3. (A) Chromatogram of blank serum; (B) chromatogram of serum spiked with 5 μg/ml 6β-bromopenicillanic acid; (C) chromatogram of blank urine; (D) chromatogram of urine spiked with 10 μg/ml 6β-bromopenicillanic acid.

concentration of sodium hydroxide has to be high enough to get a fast and reproducible post-column reaction. The concentrations of methanol in the eluent and post-column reagent have to be identical to minimize the baseline noise.

The separation in urine was performed on a Hamilton PRP-1 column, because the LiChrospher 100 CH-18/II column did not give a satisfactory separation of 6β-bromopenicillanic acid and the urine compounds. The eluent and the post-column reagent for the urine assay were optimized as for the serum.

In the post-column system it was necessary, for both assays, to use a column packed with glass beads as well as PTFE tubing to minimize the baseline noise that derives from mixing the eluent and the post-column reagent. For the sample preparation of urine, it was necessary to dilute the urine to suppress interfering compounds.

Linearity

Blank serum was spiked with potassium 6β-bromopenicillanate over the range 0.2–12 μg/ml (calculated as 6β-bromopenicillanic acid). The standard curve was analysed by linear regression analysis to determine the linearity. The correlation coefficient was found to be 0.9998.

Blank urine was spiked with potassium 6β-bromopenicillanate over the range 5–50 μg/ml (calculated as 6β-bromopenicillanic acid). This standard curve was also analysed by linear regression analysis to determine the linearity. The correlation coefficient was found to be 0.9996.

Accuracy

Blank serum was spiked with potassium 6β-bromopenicillanate corresponding to 5.0 μg of 6β-bromopenicillanic acid per ml of serum ($n=5$), as well as urine corresponding to 10.6 μg of 6β-bromopenicillanic acid per ml ($n=6$). The accuracy of the assays was calculated as the percentage deviation of the found concentration from the spiked concentration. The accuracy was found to be 2% for both serum and urine.

Precision

The within- and between-assay standard deviations in blank serum spiked with potassium 6β-bromopenicillanate (ca. 5.0 μg of acid per ml) were found to be 3.4% ($n=5$) and 6.4% ($n=3$), respectively. The blank serum was also spiked with a ten-fold lower concentration of potassium 6β-bromopenicillanate (ca. 0.5 μg of acid per ml) and it gave within- and between-assay standard deviations of 5.6% ($n=5$) and 9.7% ($n=3$), respectively. For urine spiked with potassium 6β-bromopenicillanate (ca. 10.6 μg of acid per ml) the within- and between-assay standard deviations were found to be 2.2% ($n=6$) and 4.3% ($n=3$), respectively.

Sensitivity

The limit of detection is 50 ng/ml 6β-bromopenicillanic acid in serum and 2 μg/ml 6β-bromopenicillanic acid in urine.

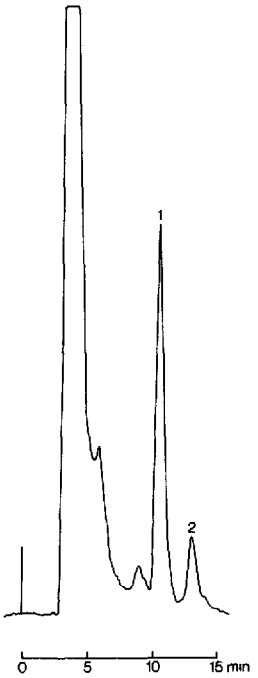

Fig. 4. Chromatogram from serum 1 h after administration of pivampicillin and pivampicillin brobactam. Peaks 1 = brobactam (2.4 μg/ml of serum); 2 = ampicillin (6.7 μg/ml of serum).

Application

The assays have been tested in a pharmacokinetic study in which healthy volunteers were dosed orally with a formulation containing 400 mg of pivampicillin and 100 mg of 6β-bromopenicillanic acid. Fig. 4 shows the chromatogram of a serum sample obtained 1 h after administration. In this study 6β-bromopenicillanic acid was determined in serum samples up to 7 h after dosing, and all concentrations were within the range of linearity and the sensitivity was sufficiently high. In urine 6β-bromopenicillanic acid was determinated up to 24 h after dosing; as for the serum samples the concentrations were in the range of linearity and the sensitivity was sufficiently high.

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