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Note

Determination of benzylpenicillin in plasma and urine by high-performance liquid chromatography

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In spite of the introduction of newer β-lactam penicillins with either a wider spectrum of activity or increased activity against penicillinase-producing bacteria, benzylpenicillin is still frequently considered to be the drug of choice for the treatment of infections due to susceptible organisms [1, 2].

Several high-performance liquid chromatographic (HPLC) methods for benzylpenicillin in dosage forms or as pure drug have been reported [3—5]. In studies examining various aspects of the pharmacokinetics of benzylpenicillin the usual method for assay of the drug has been microbiological [6- 9]. Microbiological assays are slow and also suffer from a lack of selectivity and low precision [10]. HPLC has the advantage of being rapid, highly selective and usually more precise than microbiological assays. HPLC has been developed for a number of penicillins in biological fluids [10—14] but only that of Westerlund et al. [10] assays benzylpenicillin. The method of Westerlund et al. [10] involves post-column derivatisation, a process which requires special equipment and techniques which are often not available.

The present study describes a simple, rapid and selective HPLC assay for the determination of benzylpenicillin in plasma and urine. This method is suitable for clinical and pharmacokinetic studies.

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MATERIALS AND METHODS

Chemicals

Benzylpenicillin was supplied by Commonwealth Serum Labs. (Australia). Methanol and acetonitrile were specially purified for HPLC and supplied by Waters Assoc. (Milford, MA, U.S.A.). All other chemicals were analytical-reagent grade. The water used was deionized, then glass-distilled. Benzylpenicilloic acid, the major metabolite of benzylpenicillin, was prepared according to the method of Cole et al. [8].

HPLC instrumentation and conditions

Reversed-phase HPLC was performed using a Waters M6000A solvent delivery system fitted with a U6K injector. A C_{18} μBondapak column (particle size 10 μm; 300 mm × 3.9 mm I.D.; Waters Assoc.), was used in combination with a guard column (μBondapak C_{18}/Porasil B; Waters Assoc., 23 mm × 3.9 mm I.D.) in all studies. UV absorbance at 214 nm was monitored with a Waters Model 441 UV absorption detector fitted with a zinc lamp. The absorbance was recorded on a dual-channel Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). Injections were made with a 25-μl Hamilton syringe. The mobile phase was a 0.015 *M* phosphate buffer (pH 7.0 ± 0.1)—methanol (72:30) mixture. The flow-rate was 1 ml/min.

Sample preparation

Plasma and urine samples were processed by transferring a 200-μl aliquot of the sample into a glass tube (disposable borosilicate glass culture tubes; 50 × 6 mm; Kimble, IL, U.S.A.) and adding an equal volume of acetonitrile. The sample was vortexed for 1 min, then centrifuged at 1500 *g* for 5 min. An aliquot (20 μl for plasma, 10 μl for urine) of clear supernatant was injected onto the column.

Preparation of standard curves

Plasma standards were prepared at the start of each day by spiking drug-free plasma with known amounts of a freshly prepared aqueous solution of benzylpenicillin to produce concentrations of 0.5—50 mg/l. The standards were then analyzed in the described manner. Urine standards were prepared in a similar manner in drug-free urine to produce concentrations of 20—4000 mg/l. Standard curves were prepared by plotting the peak height of benzylpenicillin versus concentration.

RESULTS AND DISCUSSION

Typical chromatograms of blank plasma and plasma from a subject following intravenous administration of benzylpenicillin are shown in Fig. 1. The retention time for benzylpenicillin is 12.4 min. Fig. 2 shows chromatograms of blank urine and urine following administration of benzylpenicillin from the same subject.

Several HPLC methods for benzylpenicillin in non-biological fluids used UV detection at 254 nm [3, 5, 15]. It was found using the present system that a

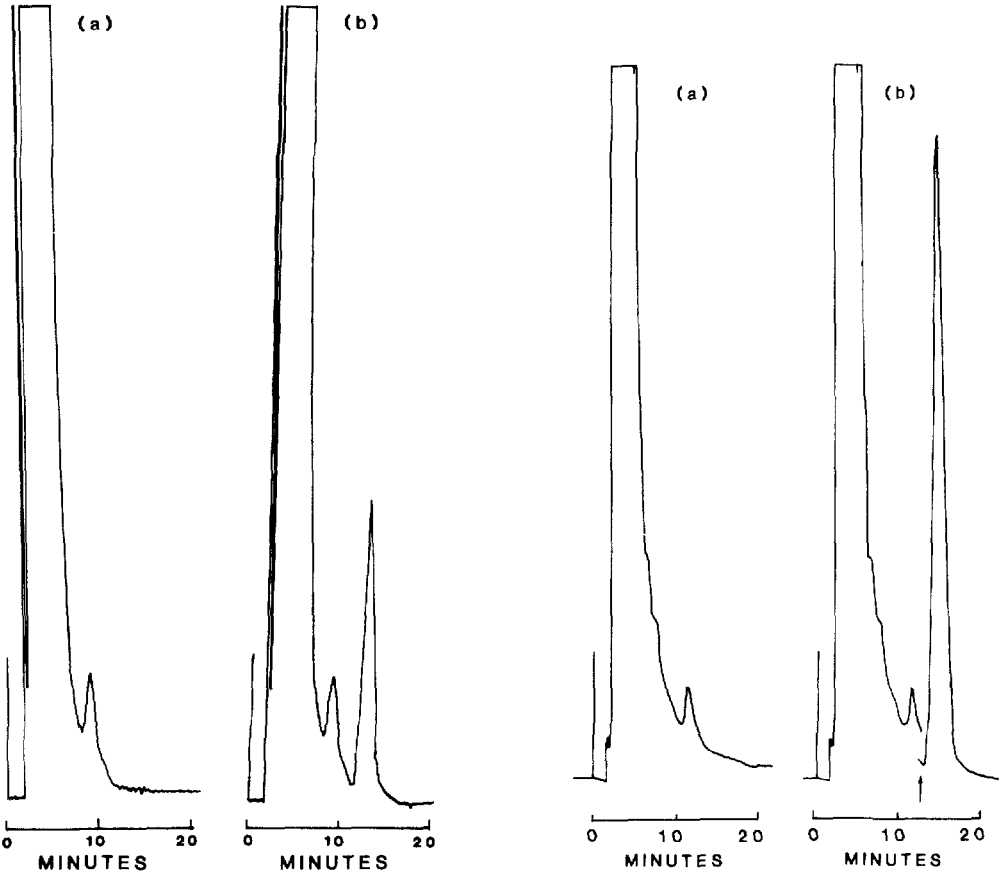

Fig. 1. Chromatograms of (a) blank plasma and (b) plasma from the same subject 30 min after intravenous administration of 600 mg benzylpenicillin. The plasma concentration of benzylpenicillin is estimated to be 7.3 mg/l.

Fig. 2. Chromatograms of (a) blank urine and (b) urine from the same subject collected from 0—8 h after intravenous administration of 600 mg benzylpenicillin. The urine concentation of benzylpenicillin is estimated to be 1310 mg/l. The arrow indicates change in absorbance scale.

greater than twenty-fold increase in sensitivity could be achieved by monitoring the effluent at 214 nm rather than at 254 nm without increased interference or baseline noise. UV detection in the range 210—230 nm has been used by other workers [11—14] to quantitate various other penicillins in biological fluids.

A number of agents may be used to precipitate plasma protein. In HPLC methods for other penicillins, perchloric acid [11] trichloroacetic acid [10] and acetonitrile [13] have been used. In the present study acetonitrile was found to completely precipitate plasma protein and provided the cleanest chromatogram. Acetonitrile was also added to urine samples to reduce the number of endogenous peaks.

Over the concentration ranges studied (0—50 mg/l for plasma and 0—4000 mg/l for urine), linearity of response was found to be good ($r > 0.99$) and consistently reproducible for standard curves based on peak height following injection of a known volume of supernatant.

Under the conditions of this assay, the detection limit for benzylpenicillin in plasma was 0.2 mg/l; 0.5 mg/l could be determined quite readily and reproducibly. In urine the detection limit was about 10 mg/l.

The usual method for the determination of benzylpenicillin in biological fluids is by microbiological means. Such assays have a low precision with an experimental error of ± 15% and their selectivity is reduced if active metabolites or other compounds with antibacterial action are present [10]. The intra- and inter-day reproducibility of the present assay for plasma are shown in Table I. The inter-day coefficient of variation could be reduced to that of the intra-day by the preparation of standard curves each day of assay. The intra-day coefficients of variation for benzylpenicillin in urine at concentrations of 2000, 200 and 20 mg/l were 2.74, 1.69 and 8.5% respectively (n = 6). The selectivity of this method was studied by measuring the retention times of other common penicillins. The results are shown in Table II. Modification of the polarity of the mobile phase could make this system suitable for the assay of these compounds.

The present method was used to follow the disposition of benzylpenicillin administered intravenously and intramuscularly to volunteers. Fig. 3 shows the plasma concentration—time profiles for benzylpenicillin in one subject following the administration of 600 mg of the drug intravenously and intramuscularly on separate occasions. The plasma benzylpenicillin concentration—time profiles for volunteers following intravenous administration showed levels similar to those obtained by Kates et al. [9] following intravenous administration of the same dose but using a microbiological assay.

TABLE I

INTRA- AND INTER-DAY VARIATION OF BENZYLPENICILLIN IN PLASMA

Spiked concentration (mg/l)	Intra-day coefficient of variation* (%)	Inter-day coefficient of variation** (%)
20	2.53	3.59
10	2.26	4.89
4	1.69	5.81
1	7.30	9.16

*n = 6
**n = 5 over two weeks.

TABLE II

RETENTION TIMES OF VARIOUS PENICILLINS

Pencillin	Retention time (min)
Ampicillin	6.0
Methicillin	9.2
Benzylpenicillin	12.4
Amoxycillin	14.4
Oxacillin	26.0
Cloxacillin	37.2
Flucloxacillin	40.0

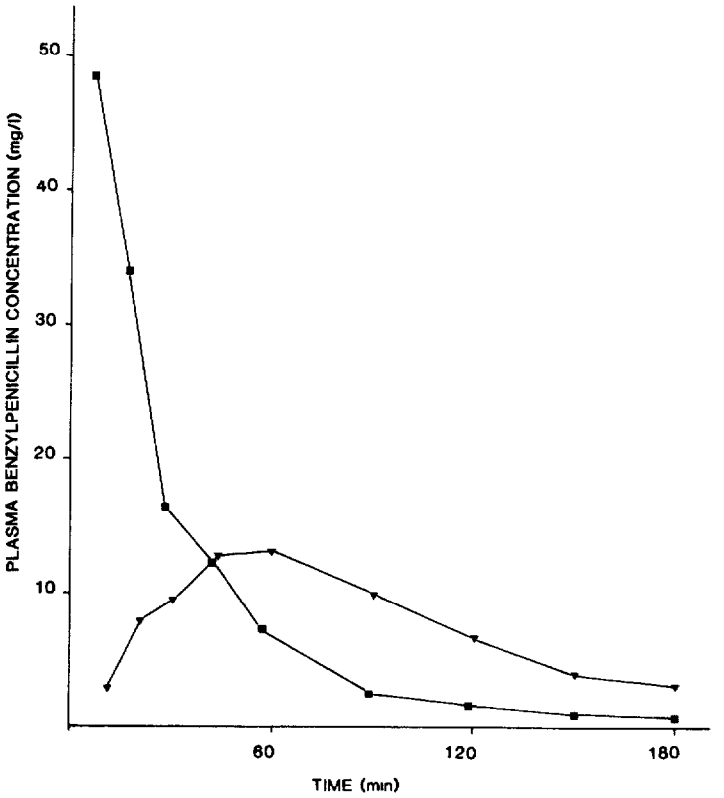

Fig. 3. Time course of plasma benzylpenicillin concentrations found in a subject following (■) intravenous and (▼) intramuscular administration of 600 mg benzylpenicillin.

In summary, the HPLC method presented here provides a selective, reliable and reproducible method for the rapid determination of benzylpenicillin in plasma and urine. The method does not require time-consuming or complex extraction or derivatisation techniques. We have found the method suitable for pharmacokinetic studies of benzylpenicillin.

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