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Micro-method for the determination of piperacillin in plasma by high-performance liquid chromatography

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Abstract: A simple and sensitive high-performance liquid chromatographic method for the determination of piperacillin in plasma is described. A C_8 reversed-phase column was used with a mobile phase consisting of methanol–water–triethylamine (550:450:4, v/v/v) adjusted to pH = 3 with orthophosphoric acid and UV detection at 270 nm. Cephalothin was used as internal standard. The method involves a plasma protein precipitation with acetonitrile followed by extraction of endogenous compounds with chloroform and injection of the upper aqueous phase into the chromatograph.

Within-day and between-day assays give relative standard deviations ≤5.7%. The detection limit is 0.2 μg ml^{-1}. Stability studies show that piperacillin degradation starts at −4°C. Therefore, samples have to be processed promptly and stored at −20°C. The method described is convenient for clinical monitoring and for pharmacokinetic studies.

Keywords: *Reversed-phase HPLC; penicillin; piperacillin.*

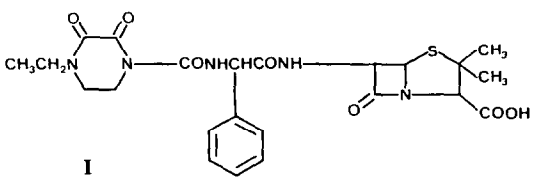
Introduction

Piperacillin (**I**) is a semi-synthetic penicillin with a broad-spectrum activity against gram-positive and gram-negative organisms including *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus* species and *Bacteroides fragilis* [1, 2, 3]. Like other ureidopenicillins, it is poorly absorbed after oral administration and is only intended for parenteral use.

Two types of methods for the determination of piperacillin in human body fluids have been reported, namely microbiological assays [4] and reversed-phase high-performance liquid chromatography (RP-HPLC) [5–10]. Generally, bioassay procedures are less selective than HPLC, especially when the drug is analysed in the presence of active metabolites or other antibiotics.

Among the previously described HPLC assays, some required at least 1 ml of plasma [6, 9, 10] or involved a cumbersome extraction procedure [6, 8]. Others employed an internal standard with molecular structure quite different from that of piperacillin [5, 7] and one used no standard at all [6]. The rapid microscale method described is performed in the presence of an internal standard, cephalothin, that has a structure similar to piperacillin. As described by Aravind *et al.* [5] and Jung and Nahajan [7], sample pretreatment includes protein precipitation (by acetonitrile), but also includes another purification step, namely the removal of interfering endogenous compounds with an organic solvent (chloroform). Thus, under the present HPLC conditions, these substances can no longer interfere with the determination of piperacillin, as they did when the supernatant was introduced after a single protein precipitation.

The stability of piperacillin during storage and analytical treatment has been studied, since the β-lactam ring shows high fragility in aqueous solution.



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Experimental

Chemicals

Piperacillin was supplied by Lederle (Oullins, France) and cephalothin by Glaxo (Paris, France). Acetonitrile was supplied by Rathburn (Walkburn, Scotland, UK) and methanol by Prolabo (Paris, France). Water was deionized and twice distilled from glass.

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All other chemicals (ammonium acetate, acetic acid, orthophosphoric acid, triethylamine and chloroform) were of analytical reagent grade (Merck, Darmstadt, FRG).

HPLC conditions

The Waters Association (Milford, MA, USA) chromatographic system used, consisted of a Model 590 constant-flow pump, a Wisp Model 710 B automatic injector and a Lambda Max Model 480 ultraviolet detector operated at 270 nm and interfaced with an Omniscribe recorder (Houston Instruments, Houston, TX, USA). Separation of compounds was carried on a Spherisorb C_8 column (15 cm × 4.6 mn i.d., particle size 5 μm). The mobile phase was methanol–water–triethylamine (550:450:4, v/v/v), the pH being adjusted to 3.0 by orthophosphoric acid. Before use, the mobile phase was filtered through a Durapore 0.22 μm filter (Millipore, Milford, MA, USA). The flow rate was maintained at 1.2 ml min^{-1} at ambient temperature. The chromatograms were recorded at a chart speed of 0.5 cm min^{-1} and peak-height ratios of piperacillin to cephalothin were measured. After use, the chromatographic system was flushed with methanol–water (550:450, v/v) and the column was kept in methanol while not in use.

Standard solutions

Stock solutions of piperacillin and cephalothin (1 mg ml^{-1}) were prepared in methanol. They could be stored at −20°C for 12 months without degradation. Appropriate dilutions of piperacillin were made in drug-free human plasma to provide concentrations of 10–100 μg ml^{-1} (internal standard at 10 μg ml^{-1}) and of 50–500 μg ml^{-1} (internal standard at 50 μg ml^{-1}). The internal standard dilutions were made in 0.1 M ammonium acetate buffer (pH = 5).

Sample preparation

In a conical centrifuge tube, 100 μl of plasma was added to 100 μl of the internal standard solution (cephalothin) and 500 μl of acetonitrile. The mixture was vortexed for 30 s and centrifuged for 2 min at 8700 ***g*** (Beckman Microfuge Centrifuge). The clear supernatant was transferred to another conical centrifuge tube containing 500 μl of chloroform, vortexed for 30 s and centrifuged for 2 min. A 40 μl aliquot of the upper aqueous phase was introduced to the chromatograph.

Results and Discussion

Representative chromatograms of plasma samples are shown in Fig. 1. The retention times of piperacillin and cephalothin were 3.6 and 2.8 min, respectively. The potential interference of some other compounds was investigated (Table 1). All these drugs were eluted before both cephalothin and piperacillin.

The UV spectral study showed that piperacillin and cephalothin had a maximum absorbance at 234 nm. Under the present chromatographic conditions, interferences occurred at such a wavelength. However the preferred wavelength for determination is 270 nm, where the analytes were detected without interference from indigenous materials. The detection limit (signal-to-noise ratio ≥2) was 0.2 μg ml^{-1}.

The reproducibility (intra-day assay, $n = 10$) and the repeatability (inter-day assay, $n = 10$) of the HPLC procedure were determined on drug-free human plasma samples spiked with piperacillin at different concentrations: 16, 80 and 400 μg ml^{-1} (Table 2). The within-day precision data had a relative standard deviation (RSD) of 0.9% for 16 μg ml^{-1} (accuracy = 104.7%), 2.1% for 80 μg ml^{-1} (accuracy = 104.7%) and 2.2% for 400 μg ml^{-1} (accuracy = 100.1%). The between-day data gave a RSD of 1.6% for 16 μg ml^{-1} (accuracy = 99.6%), 3.7% for 80 μg ml^{-1} (accuracy = 96.2%) and 5.7% for 400 μg ml^{-1} (accuracy = 102.0%).

The HPLC response was found to be linear in the range 10–500 μg ml^{-1} in plasma. A good correlation was obtained between plasma concentration and peak-height ratio (piperacillin/cephalothin): $r = 0.997$ (low concentrations, 10–100 μg ml^{-1}, $n = 8$), $r = 0.993$ (high concentrations, 50–500 μg ml^{-1}, $n = 8$).

The stability of piperacillin was studied using a plasma sample (80 μg ml^{-1}) stored at −20, 4 and 20°C. No degradation of piperacillin was noted after 16 days, on storage at −20°C. After 3 h storage at 4 and 20°C, concentrations began to decrease, and after 30 h, 87% of the initial amount remained at 4°C but only 65% at 20°C (Fig. 2). The degradation of piperacillin at 20°C was shown to be a first-order rate process with a degradation half-life around 50 h (Fig. 3).

This observation is in agreement with that of Yamana *et al.*, who had noted a similar process of degradation [11]. The results, however,

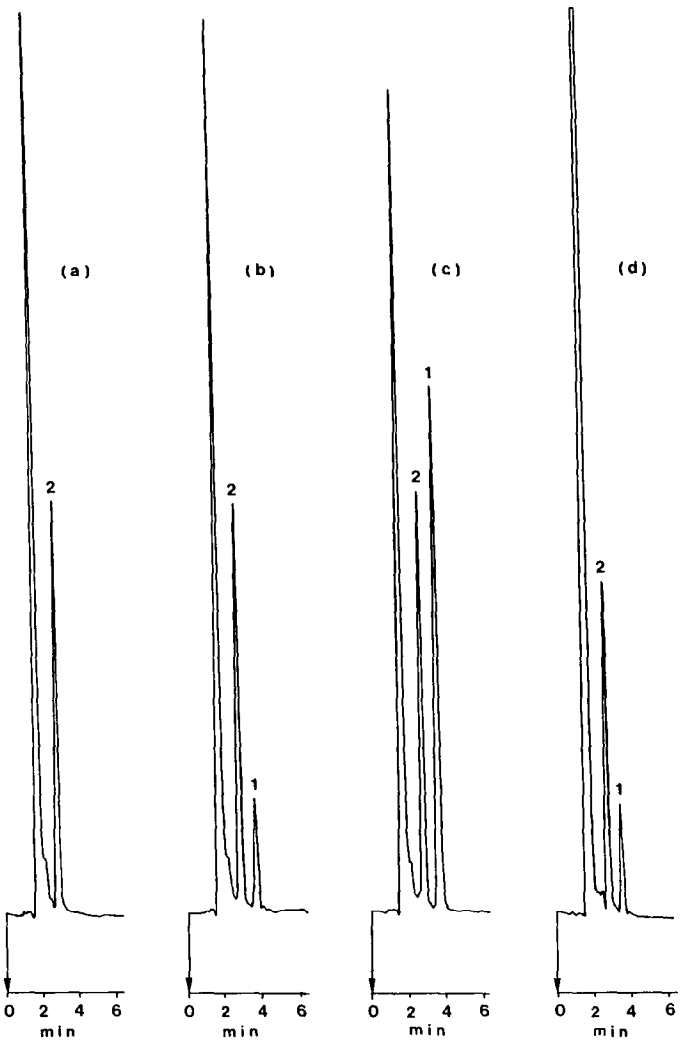

Figure 1
Chromatograms of: (a) human drug-free plasma; (b) plasma spiked with 50 μg ml^{-1}; (c) plasma spiked with 250 μg ml^{-1}; (d) patient plasma (68 μg ml^{-1}). Peaks: 1 = piperacillin; 2 = cephalothin.

Table 1
Retention time of some drugs

Drugs*	Relative retention time†
Cefotiam	0.40
Cefadroxil	0.50
Ceftriaxone	0.50
Cefotaxime	0.50
Desacetylcefotaxime	0.50
Ceftazidime	0.50
Cefotetan	0.50
Cefonicid	0.50
Ceforanide	0.55
Cephaloridine	0.60
Cefazolin	0.60
Cefpiramide	0.60
Cefoperazone	0.60
Cephalothin	0.80

*0.50 μg of each drug was introduced to the column.
†Relative to piperacillin: 3.60 min.

differed from those previously reported by Jung and Nahajan [7]. He had observed a percentage of degradation of 100% after 6 weeks at −20°C and of 25% after 6 h at 20°C. However, Riegel [8] had noted no degradation after 5 weeks at −20°C. Therefore, in the present work, plasma samples were processed as promptly as possible and stored at −20°C.

The sample pretreatment left the drug in an aqueous phase which had to be of suitable pH to prevent the degradation. Previous studies [10] have shown that piperacillin was more stable at neutral pH around 6. Under the described conditions, the pH of the upper aqueous phase was 6.5 [12]. The stability was demonstrated on a human plasma sample spiked with 50 μg ml^{-1} piperacillin. The sample was treated as described above and

Table 2
Accuracy and precision for plasma spiked with piperacillin

Spiked concentration ($\mu g\ ml^{-1}$)	Intra-assay determination (n = 10)			Inter-assay determination (n = 10)		
	Mean ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy* (%)	Mean ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy* (%)
16	16.8	0.9	104.7	16.0	1.6	99.6
80	83.8	2.1	104.7	77.0	3.7	96.2
400	400.2	2.2	100.1	407.8	5.7	102.0

*Accuracy = (amount found/amount added) × 100.

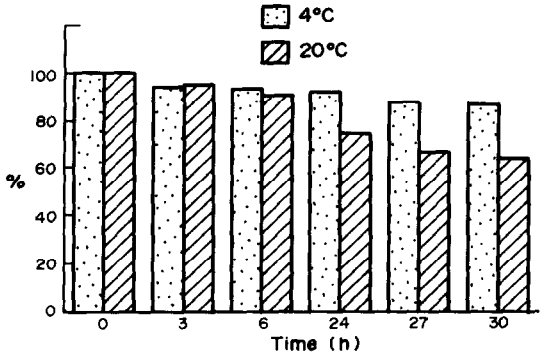

Figure 2
Stability of plasma spiked with 80 $\mu g\ ml^{-1}$ at 4 and 20°C.

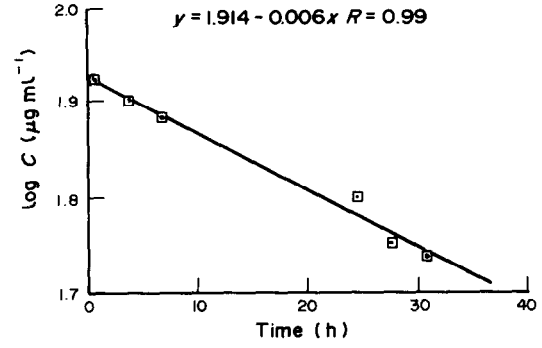

Figure 3
Degradation of piperacillin in plasma at 20°C. C = concentration.

kept at ambient temperature for 5 h. Every hour an aliquot of the aqueous phase was injected and the peak-height ratio of piperacillin to cephalothin was measured. The RSD was 0.62% (n = 6).

Plasma samples spiked to a final concentration of 50 $\mu g\ ml^{-1}$ were processed and recoveries of piperacillin and cephalothin were determined by comparison of known amounts of these drugs. The mean values were, respectively, 77.5 ± 1.6% and 92.5 ± 0.8% (n = 4).

The method was used for the determination of piperacillin in plasma from patients after administration of 4 g of **IV** [chromatogram (d), Fig. 1].

Conclusion

The method described is rapid (for each sample the total time analysis is about 5 min), sensitive (detection limit 0.2 $\mu g\ ml^{-1}$) and has the requisite accuracy and precision for routine use in a laboratory. It is suitable for therapeutic monitoring and pharmacokinetic studies in adults and also in paediatric subjects, where sample size is a major concern. However, it is necessary to take care over the carriage and storage conditions of samples in order to prevent any degradation of piperacillin before its determination.

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