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Short Communication

Determination of ampicillin in serum by high-performance liquid chromatography with precolumn derivatization ☆

Jawahar Lal, Jyoti Kumar Paliwal, Pyara Krishen Grover, Ram Chandra Gupta*

Pharmacokinetics and Metabolism Division, Central Drug Research Institute, Lucknow 226 001, India

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Abstract

A high-performance liquid chromatographic assay method using precolumn derivatization and fluorescence detection has been developed and validated for the determination of ampicillin in serum. The presented method is simple and provides improved selectivity and sensitivity over other existing HPLC methods. It is linear over the concentration range of 100 to 10 000 ng/ml (method 1) and 2 to 1000 ng/ml (method 2) and the extraction recovery is more than 75%. The coefficient of variation is found to be less than 10% over the concentration ranges studied.

1. Introduction

The determination of ampicillin (I) in plasma by high-performance liquid chromatography (HPLC) has always been hampered by the problem of low selectivity and sensitivity due to its poor detectability [1] as its molar absorption coefficient (= 3036 M^{-1} cm^{-1} at 257 nm [2]) is too low to afford sufficient sensitivity. UV detection is usually carried out at 220 to 230 nm, which is more sensitive but less selective compared with detection at 257 nm [3–9]. Alternatively, pre- or postcolumn reactions have been reported to convert the compound either to a derivative that displays more useful light-absorbing properties (λ_{max} 300 to 330 nm) [1,10–12] or to a fluorescent breakdown product [13–16]. Although the methods of Miyazaki *et al.* [14] and Lanbeck-Vallen *et al.* [16] have a sensitivity of 0.5 and 5 ng/ml for I, these techniques involve tedious sample preparation.

The purpose of this study, therefore, was to develop a simple and more sensitive HPLC method using a precolumn derivatization technique for the trace level analysis (2 ng/ml) of I in serum.

2. Experimental

2.1. Chemicals and reagents

Pure reference standards of I-trihydrate and I-citric acid were supplied by Ranbaxy Laboratories (New Delhi, India). Analytical-grade potassium hydroxide, potassium dihydrogen orthophosphate, trichloroacetic acid (Glaxo, Bombay, India), formaldehyde solution (37–41%, w/v) and HPLC-grade acetonitrile were obtained from S.D. Fine Chem. (Bombay,

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* Corresponding author.

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India). Diethyl ether (anaesthetic grade IP, Ether India, Bombay, India) was purified before use. Triply-distilled water from an all-quartz apparatus was used in this study. Serum was purchased from a local blood bank to generate a drug-free serum pool.

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Model PU 4015 pump (Pye Unicam, Cambridge, UK), a Model 7125 injector (Rheodyne, Berkeley, CA, USA) with a fixed 50-μl loop, a Model RF-530 variable-wavelength fluorescence detector (Shimadzu, Kyoto, Japan). Separations were achieved on a RP-18 (10 μm) cartridge column (100 × 4.6 mm I.D.) preceded by a precolumn (10 μm, 30 × 4.6 mm I.D.) (Pierce Chemical, Rockford, IL, USA). The column effluent was monitored at an excitation wavelength of 346 nm and an emission wavelength of 422 nm. Chromatograms were recorded by a Model C-R5A Chromatopack recorder (Shimadzu). A Model SVC-200H speedvac concentrator (Savant Instruments, New York, NY, USA), was used to evaporate the organic solvent after extraction.

The mobile phase consisted of acetonitrile–potassium dihydrogen orthophosphate (0.1 *M*, pH 5.6) (23:77, v/v). It was filtered and degassed before use. Chromatography was performed at ambient temperature and a 1.0 ml/min mobile phase flow-rate.

2.3. Stock and standard solutions

A stock solution (1 mg/ml) of I was prepared by dissolving 11.53 mg of I-trihydrate (equivalent to 10 mg of I) in 10 ml triply-distilled water and stored at 4°C. Working standards were prepared in mobile phase in the range of 2 to 10 000 ng/ml.

Serum calibration samples (2 to 10 000 ng/ml) were prepared by adding varying volumes of stock solution of I to 10 ml serum. The standards were vortex-mixed and stored at −80°C until analysis.

2.4. Precolumn derivatization and extraction

To 0.5 ml of standard, calibration and drug-free serum samples was separately added 1 ml trichloroacetic acid (20%, w/v). The mixtures were vortex-mixed for 15 s followed by centrifugation for 20 min at 1000 *g*. To 1 ml supernatant in a 10-ml screw-capped glass tube was added 0.5 ml of 0.4 *M* citric acid solution containing formaldehyde (7%, w/v). The sample was vortex-mixed for 15 s, heated in a water bath at 90°C for 2 h, and cooled to room temperature. The fluorescent product thus formed was either directly injected (100 to 10 000 ng/ml, method 1) or extracted with 2 × 3 ml diethyl ether to increase the sensitivity (2 to 1000 ng/ml, method 2). The combined ether extract was evaporated to dryness under reduced pressure. The residue was reconstituted in 0.1 ml mobile phase and injected onto the HPLC system.

2.5. Extraction efficiency

To determine the extraction efficiency, a standard solution containing I was added to aliquots (0.5 ml) of serum to give concentrations of 5, 50, 200, 500, 1000 and 5000 ng/ml. Samples were processed as outlined above and injected for analysis onto the HPLC system. The recovery of I was calculated by comparing the peak height of I from extracted sample with those obtained from the analysis of equivalent amounts of standard solution after derivatization as outlined above.

2.6. Accuracy and precision

Compound I was added to serum ($n = 6$) at three concentration levels. Aliquots (0.5 ml) of serum spiked with 200 ng/ml, 1 and 5 μg/ml (method 1) and 5, 50 and 500 ng/ml (method 2) of I were processed as described and the concentrations were determined against the corresponding standard curves. The accuracy of the method was calculated based on the difference between the mean calculated and added concentrations, while precision was evaluated by

calculating the within- and inter-day coefficients of variation (C.V.).

Drug-free human serum was routinely processed and analysed as described above. The resultant chromatograms were examined for the presence of endogenous impurities which could possibly interfere with the measurement of I.

3. Results and discussion

In the method described here, precolumn derivatization followed by extraction and reconstitution of the residue with a small volume (100 μl) of mobile phase and the use of a fluorescence detector resulted in a major improvement in the selectivity and excellent sensitivity (2 ng/ml, which is substantially lower than the previous values obtained). Initially, the method of Rogers *et al.* [8] was tried. The method utilizes protein precipitation with perchloric acid and employs the less selective UV detection at 225 nm. However, when those conditions were applied in our chromatographic system, the endogenous peak that eluted interfered with the elution position of I.

Figs. 1 and 2 show representative chromatograms of standard containing 500 ng/ml (method 1) and 10 ng/ml (method 2) (A) of I, drug-free serum (B), serum standard containing 500 ng/ml (method 1) and 10 ng/ml (method 2) (C) of I, and a serum sample from a volunteer taken 45 min (method 1) or 24 h (method 2) after administration of a dose of sultamicillin (D), respectively. No significant endogenous peaks coeluted with I, as shown in the corresponding chromatogram of drug-free serum by either method. Based on a signal-to-noise ratio of 3, the sensitivity of the assay from 0.5 ml of serum was 2 ng/ml. The method provided adequate sensitivity and specificity for quantitative detection of I.

In order to cover a large (2–10 000 ng/ml) concentration range and to increase the sensitivity without sacrificing accuracy or precision, it was advisable to use two standard curves. The high range standard curve was designed for 100

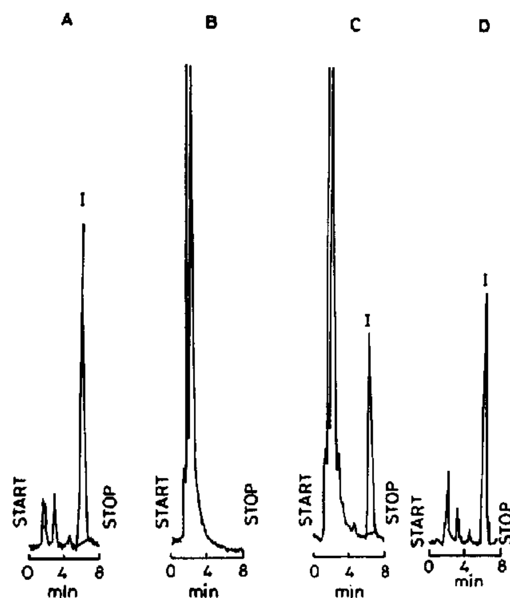

Fig. 1. Chromatograms (method 1) of (A) standard containing 500 ng/ml of I, (B) drug-free serum, (C) serum containing 500 ng/ml of I, and (D) volunteer serum sample taken 45 min postdose of sultamicillin.

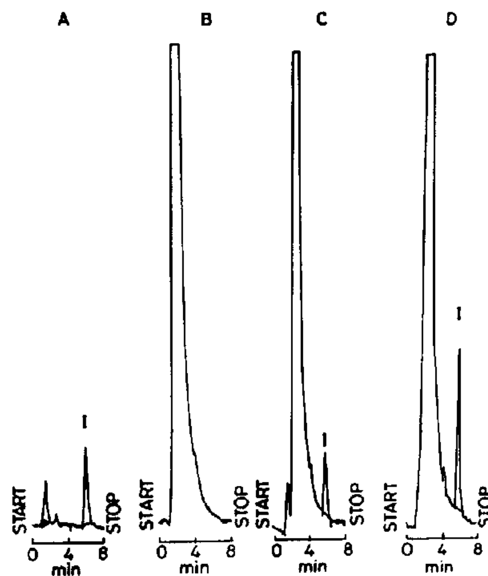

Fig. 2. Chromatograms (method 2) of (A) standard containing 10 ng/ml of I, (B) drug-free serum, (C) serum containing 10 ng/ml of I, and (D) volunteer serum sample taken 24 h postdose of sultamicillin.

Table 1
Precision and accuracy

Nominal serum concentration (ng/ml)	Within-day		Day-to-day	
	Concentration found (Mean ± S.D.) (ng/ml)	C.V. [a] (n = 6) (%)	Concentration found (Mean ± S.D.) (ng/ml)	C.V. [a] (n = 7) (%)
Method 1				
200	215.04 ± 7.70	3.58	217.27 ± 9.17	4.22
1000	1001.19 ± 81.58	8.15	1014.90 ± 44.52	4.39
5000	4924.70 ± 88.52	2.00	4920.65 ± 90.37	1.84
Method 2				
5	4.99 ± 0.31	6.19	5.18 ± 0.26	5.00
50	52.92 ± 0.81	1.53	49.08 ± 2.20	4.47
500	483.83 ± 27.01	5.58	495.94 ± 13.16	2.65

[a] C.V. = (S.D./mean) × 100.

to 10 000 ng/ml (method 1) and the low range standard curve for 2 to 1000 ng/ml (method 2) concentrations. Each standard curve consisted of at least seven points covering the anticipated assay range.

The slopes, intercepts and correlation coefficients of the calibration curves were calculated by linear regression analysis. Linearity was observed for both methods with correlation coefficients (r) that exceeded 0.9998. The extraction recoveries of I were always higher than 75%. The reproducibility and accuracy of the methods were determined by processing six spiked serum samples for each concentration at three concentration levels and calibration curves were run each day. Variability tests showed that the C.V.s were 2.00–8.15, 1.84–4.39 (method 1), 1.53–6.19 and 2.65–5.00 (method 2) for within-day and between-day precision, respectively (Table 1).

The between-day assay variation was also estimated by comparing the linear regression slopes of seven standard curves. Over a period of one month the slopes (mean ± S.D.) were 4.25 ± 0.15 (method 1) and 25.54 ± 1.84 (method 2). These results demonstrate that the method is reproducible and accurate.

3.1. *Application of the method in clinical pharmacokinetics*

The assay described here was applied to bioavailability studies of two formulations of sultamicillin in normal healthy volunteers. The method was sensitive enough to follow ampicillin up to 24 h after a single 750-mg oral dose of sultamicillin. The results of this study will be reported elsewhere. The method will also be applicable for the assay of ampicillin in pharmaceutical formulations.

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