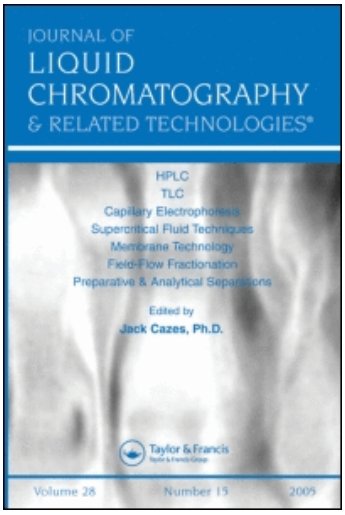


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# Determination of Ampicillin in Plasma by Paired Ion High Performance Liquid Chromatography

A. H. Hikal[a]; A. B. Jones[a]
[a] School of Pharmacy University of Mississippi University, Mississippi

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# DETERMINATION OF AMPICILLIN IN PLASMA BY PAIRED ION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A. H. Hikal and A. B. Jones
*School of Pharmacy*
*University of Mississippi*
*University, Mississippi 38677*



ABSTRACT

A new, accurate, precise, and specific method has been developed for the assay of ampicillin in plasma. No extraction of ampicillin from plasma is called for. Plasma is treated with acetonitrile containing propiophenone as the internal standard, vortexed, centrifuged, and the supernate is injected. A C18 reverse phase column is used. The mobile phase consisted of a mixture of methanol and 0.02M phosphate buffer of pH 6.00, and contained alkyldimethylamine C10 as an ion pair ligand. Detection was by UV at 220 nm. A linear relationship between concentration and peak area ratio was obtained. Recovery, day-to-day, and within-day variation were determined.

## INTRODUCTION

Ampicillin (D(-)-α-aminobenzyl penicillin) is an antibiotic derived from 6-aminopenicillanic acid. It is used therapeutically on a wide scale because of good systemic availability, low toxicity, and broad spectrum of activity against Gram-positive and several Gram-negative pathogens. Official (1) methods for assay-



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ing ampicillin currently consist of: (a) microbiological agar diffusion, (b) iodine titration, (c) hydroxylamine colorimetry, (d) non-aqueous acid titration, and (e) non-aqueous base titration. Other methods which have been reported include fluorometry (2), high performance liquid chromatography (HPLC) (3-7), DC-polarography and spectrophotometry (8), and high performance thin layer chromatography (9).

Microbiological techniques guarantee the determination of microbiologically active principles, including active metabolites. However, the long time required for the analysis, and the lack of specificity are definite disadvantages. Chemical and spectrophotometric methods depend on the availability of functional groups which may occur in other components of the sample. HPLC techniques are convenient, specific, and accurate. Some HPLC methods cited previously (3,5,6) have been applied to the determination of ampicillin in pharmaceutical preparations(3) in urine (5), or in the pure substance (6), while another (4) produced interfering peaks when tried in our laboratory. The present procedure is specific, accurate, precise, and requires no extraction.



## EXPERIMENTAL

### Chemicals And Reagents

Ampicillin sodium (Omnipen-N, Wyeth Laboratories Inc., Pennsylvania), ADMA C10 (Alkyldimethylamine, Ethyl Corporation, Louisiana) was used as the ligand; propiophenone (Pierce Chemical Co., Illinois) was the internal standard; methanol and acetonitrile (Burdick and Jackson Laboratories, Inc., Michigan) were "distilled in glass" quality; and water was also distilled in glass.

### Instrumentation

The instrument used consisted of a solvent delivery system (Model 6000A), an injector (Model U6K, both Waters Associates,

Massachusetts), a variable wavelength detector (Tracor 970, Tracor Inc., Texas) and an integrator (model 3380A, Hewlett Packard, Pennsylvania). The column was 30 cm x 4 mm. id. reverse phase (μ Bondapak C18, Waters Associates). A guard column (50 mm x 4 mm i.d.) packed with Bondapak $C_{18}$ Corasil (Waters Associates) was attached to the inlet of the analytical column. The mobile phase consisted of 200 ml methanol, 250 ml 0.02M phosphate buffer of pH 6.00 ± 0.05, and 0.25 ml ADMA C10.

Conditions For Quantitation

The analysis was conducted at ambient temperature. Solvent flow rate was 1.0 ml/min. Wavelength for detection was set at 220 nm; detector sensitivity was set at 01 with the attenuation of the integrator set at 2, 4, or 8 depending on the concentration; chart speed was 0.5 cm/min. Peak area ratio (ampicillin to internal standard) was used for quantitation.

The mobile phase was degassed under vacuum for a minimum of 30 min. The column was conditioned by passing mobile phase through it at 2 ml/min for 30 min before starting the injections, and was washed with a 50:50 methanol-water mixture for 30 min at 2 ml/min at the end of each day.



Ampicillin Solutions

An accurately weighed amount (30-50 mg) of ampicillin (as the sodium salt) was dissolved in 0.02M phosphate buffer of pH 6.0 to reach a concentration of 10.0 mg/ml. This stock solution was then used to prepare a series of five standard solutions covering the range 50-1000 μg/ml.

Internal Standard Solution

Propiophenone was accurately weighed and dissolved in acetonitrile to a concentration of 100 μg/ml.

Preparation Of The Calibration Curve

Blood was obtained from rabbits and immediately centrifuged to separate the plasma. Five 100-μl aliquots of plasma were

placed in small centrifuge tubes, and 10 µl of a standard ampicillin solution was added to each tube and vortexed for 1 min. Thus, the concentration of ampicillin sodium in the plasma was 5-100 µg/ml. To each tube was added 100 µl of acetonitrile containing the internal standard. The tubes were vortexed for 1 min and centrifuged for 15 min. Fifty microliters of the supernate was injected into the chromatograph. The peak area ratio of ampicillin to internal standard was plotted against the concentration in plasma.

Test For Recovery Rate

The experiments described above were repeated using 100 µl of 0.02M phosphate buffer in lieu of the plasma. Peak area ratios obtained were compared with those obtained using plasma.

Between-Day And Within-Day Variation

In order to determine the day-to-day variation of the calibration curve, the previously-described procedure was repeated on six different days, and the resulting data were compared. Within-day variation was determined at two different concentrations as follows. Twelve 100 µl, aliquots of plasma were placed in small centrifuge tubes. To each of six tubes was added 10 µl of standard ampicillin solution containing 50 µg/ml, and to the remaining tubes, 10 µl of standard ampicillin solution containing 1000 µg/ml was added. All tubes were treated as described previously. The coefficient of variation of the peak area ratios obtained for each set was determined.

## RESULTS AND DISCUSSION

Figure 1 (A,B) shows that ampicillin is well separated from other endogenous constituents of plasma and from the internal standard. Ampicillin has a retention time of about 7.5 min. while the internal standard peak appears at 11.2 min. The relationship between peak area ratio and concentration is linear with a correlation coefficient of better than 0.99, and a Y

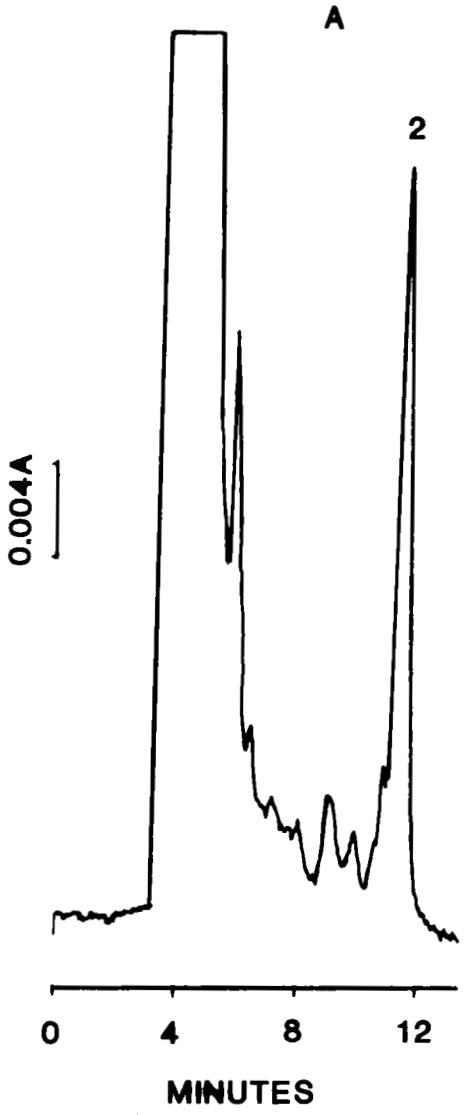


FIGURE 1A

Chromatogram of blank plasma. Peak "2" is the internal standard.

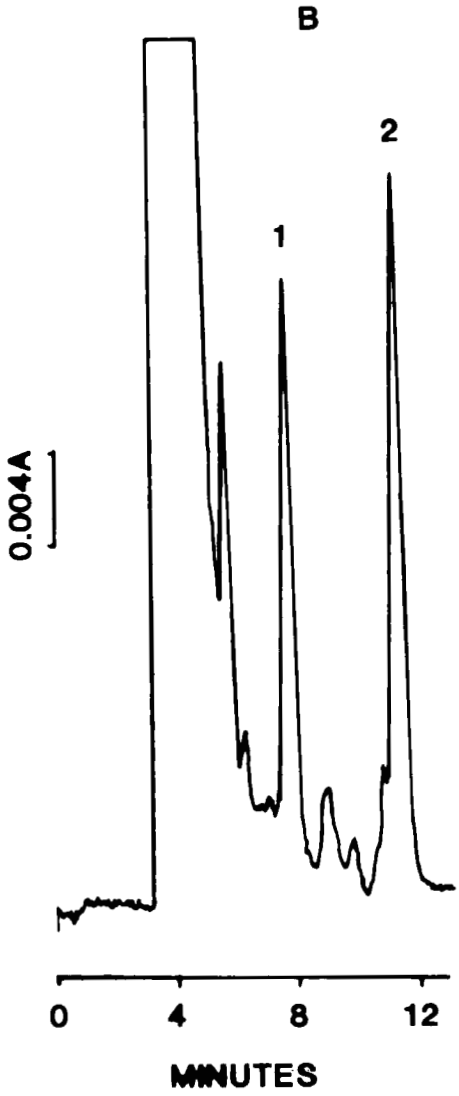


FIGURE 1B

Chromatogram of plasma spiked with 20 µg/ml of ampicillin. Peak "1", ampicillin; peak "2", internal standard.

TABLE 1

Day-to-Day Variation of the Calibration Curve for Ampicillin Assay

| Conc. | Peak Area Ratio | | | | | |
|---|---|---|---|---|---|---|
| µg/ml. | 1* | 2 | 3 | 4 | 5 | 6 |
| 5 | 0.180 | 0.158 | 0.065 | 0.095 | 0.113 | 0.170 |
| 10 | 0.318 | 0.298 | 0.237 | 0.200 | 0.174 | 0.315 |
| 20 | 0.666 | 0.606 | 0.535 | 0.445 | 0.460 | 0.681 |
| 50 | 1.564 | 1.585 | 1.395 | 0.963 | 1.186 | 1.528 |
| 100 | 2.680 | 3.175 | 2.976 | 2.354 | 2.404 | 3.475 |
| Y intercept | 0.103 | -0.016 | -0.084 | -0.056 | -0.035 | -0.043 |
| slope | 0.027 | 0.032 | 0.030 | 0.023 | 0.024 | 0.035 |
| r | 0.996 | 0.999 | 0.999 | 0.996 | 0.999 | 0.998 |

*Day



intercept of almost zero. Table 1 shows that this linear relationship between peak area ratio and concentration could consistently be achieved from day to day.

Since no extraction is involved, recovery should be virtually complete. Indeed, this was found to be the case. When spiked plasma was compared with spiked phosphate buffer, rate of recovery was calculated to be almost 100%.

Precision of the procedure was tested by spiking 6 plasma samples with the equivalent of 5 µg/ml of ampicillin, and 6 other samples with the equivalent of 100 µg/ml of ampicillin. Table 2 shows that the precision of the present assay was 99.8 ± 5.65% (C.V.) at the 5 µg/ml level and 99.8 ± 6.18% (C.V.) at the 100 µg/ml level.

This procedure is presently being used in a pharmacokinetic investigation. Figure 2 shows a chromatogram obtained from a

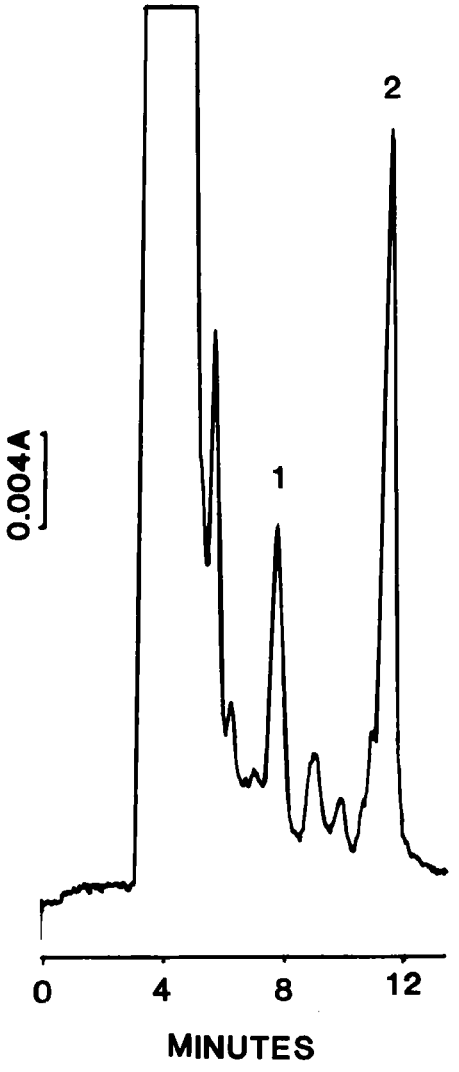


FIGURE 2

Chromatogram of plasma obtained from a rabbit that was given 10 mg/Kg of ampicillin. Peak assignments, same as in previous figure.

TABLE 2

Within-Day Variation of Ampicillin Assay at Two Concentration Levels

| | 5 µg/ml Added | | 100 µg/ml Added | |
|---|---|---|---|---|
| Run No. | Peak Area Ratio | Concentration Found | Peak Area Ratio | Concentration Found |
| 1 | 0.133 | 5.03 | 3.359 | 97.2 |
| 2 | 0.140 | 5.23 | 3.750 | 108.4 |
| 3 | 0.143 | 5.31 | 3.531 | 102.1 |
| 4 | 0.122 | 4.71 | 3.168 | 91.7 |
| 5 | 0.118 | 4.60 | 3.595 | 103.9 |
| 6 | 0.134 | 5.06 | 3.298 | 95.5 |
| Mean | | 4.99 | | 99.8 |
| C.V.% | | ±5.65 | | ± 6.18 |



blood sample withdrawn from a rabbit that was given 10 mg/kg of ampicillin.

The use of paired ion technique enables one to separate ampicillin peak from interference by endogenous constituents of biological fluids. By varying the nature and concentration of the ligand and the ratio of methanol in the mobile phase, relative retention times may be varied to reach optimum separation.

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