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Determination of ampicillin in biological fluids by coupled-column liquid chromatography and post-column derivatization

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ABSTRACT

A rapid and sensitive high-performance liquid chromatographic method has been developed for the determination of ampicillin in plasma and urine. The method involves a simple deproteinization step and separation on a coupled-column chromatographic system followed by post-column derivatization and fluorescence detection. The method has been used for the determination of ampicillin in various clinical studies. The high sensitivity makes it especially useful for small sample volumes, *e.g.* samples from pediatric patients.

INTRODUCTION

Analyses of penicillins have traditionally been performed by microbiological methods. However, these assays are slow and give poor precision and specificity (since active metabolites and other microbiological agents interfere).

The accuracy and selectivity of modern liquid chromatography (LC) has made this technique one of the most widely used for the determination of penicillins in biological fluids [1,2]. It also has the advantage of being easily automated and is, therefore, appropriate for routine analysis of a large number of samples. Several methods to determine ampicillin in biological fluids have been developed. Due to the low UV absorbance of the drug, most procedures involve a pre-column [3,4] or a post-column [5–9] derivatization step.

The aim of this work was to develop a method appropriate for analysis of a large number of samples from clinical studies. The method should involve a simple sample pretreatment and a chromatographic procedure which could be automated. Further, it should be sufficiently sensitive to analyse ampicillin in small sample volumes (*e.g.* from pediatric patients).

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EXPERIMENTAL

Chemicals

Sodium hexylsulphate was obtained from Research Plus (Bayonne and Denville, NJ, U.S.A.). Fluram® (fluorescamine) was purchased from Fluka (Buchs, Switzerland). Sodium ampicillin was obtained from Astra Pharmaceutical Production (Södertälje, Sweden). All other chemicals used were of analytical or HPLC grade.

Citrate–phosphate buffer pH 5.4 was made by dissolving 19.9 g of disodium hydrogenphosphate and 8.4 g of citric acid monohydrate in 250 ml of deionized water. Citrate–phosphate buffer pH 4.85 was made from 10.0 ml of 0.5 *M* disodium hydrogenphosphate and 350 ml of deionized water; the pH was adjusted to 4.85 by the addition of 1 *M* citric acid. Deionized water was then added to a final volume of 500 ml.

The solution for protein precipitation consisted of trichloroacetic acid (70%, w/v) and citrate–phosphate buffer pH 5.4 (5 + 15).

Apparatus

The LC system contained two pumps, LDC ConstaMetric III (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and LKB 2150 (LKB-Produkter, Bromma, Sweden), a WISP 710B autosampler (Waters Assoc., Milford, MA, U.S.A.) and a Shimadzu SF530 fluorescence detector (Shimadzu, Düsseldorf, Germany). The coupled-column system was operated by three pneumatic, automated switching valves from Waters, controlled by an SP 4100 computing integrator (Spectra Physics, Santa Clara, CA, U.S.A.). A Nelson 6000 (Nelson Analytical, Cupertino, CA, U.S.A.) was used for computation of the results. The start of the integrators was controlled by the autosampler. An LDC SpectroMonitor III UV detector was used to check the retention time of ampicillin after the first analytical column.

The post-column reaction system contained an Eldex A60-S2 (Eldex Labs., San Carlos, CA, U.S.A.) to deliver the fluorescamine solution into the effluent via a Valco zero-volume tee (Valco, Houston, TX, U.S.A.) followed by a Teflon tube, 5 m × 0.4 mm, knitted according to Engelhardt and Neue [10].

A Microlab® M (Hamilton, Bonaduz, Switzerland) was used for diluting and dispensing.

Sample handling

After blood sampling, plasma was quickly separated by centrifugation. Both plasma and urine samples were frozen in a carbon dioxide–ethanol bath within 20 min after collection [11] and stored at −70°C or below [5] until analysis. The samples were stable for at least one year.

Sample pretreatment

Plasma samples. To 0.5 ml of plasma in a 4-ml plastic tube, 200 μl of the solution for protein precipitation were added. The samples were mixed on a Buchler Vortex evaporator for 1 min and centrifuged at *ca.* 2400 *g* for 10 min. A 400-μl volume of the supernatant was transferred to a new tube containing 85 μl of 1 *M* sodium hydroxide to adjust pH to *ca.* 5 for optimal stability of ampicillin. After a short mixing, the sample was transferred to a vial and placed in the autosampler. A 100-μl aliquot of the sample was injected.

Pediatric plasma samples. To 100 μl of plasma and 200 μl of deionized water in a 4-ml plastic tube, 100 μl of the solution for protein precipitation were added. After vortex-mixing for 1 min and centrifugation at *ca.* 2400 *g* for 10 min, 300 μl of the supernatant were transferred to a new tube containing 75 μl of 1 *M* sodium hydroxide for adjustment of the pH. After a short mixing, the sample was transferred to a vial and placed in the autosampler. A 100-μl aliquot of the sample was injected.

Urine samples. To 80 μl of urine (diluted for levels higher than 500 μmol/l), 720 μl of citrate–phosphate buffer pH 4.85 were added in an autosampler vial. After vortex-mixing, the vial was placed in the autosampler, and 20 μl were injected.

Chromatography

The chromatographic system (Fig. 1) contained a guard column, New Guard RP18 (15 mm × 3.2 mm I.D.), with 7-μm particles (Brownlee Labs., Santa Clara, CA, U.S.A.). Column 1 was a Perkin-Elmer 3 × 3™ (Norwalk, CT, U.S.A.) with 3-μm particles, and column 2 (100 mm × 4.6 mm I.D.) was a Microspher C_{18} (Chrompack, Middelburg, The Netherlands) with 3-μm particles. For column 1, the mobile phases consisted of 17% methanol in phosphate buffer (pH 7.4, ionic strength 0.05) containing 1 m*M* sodium hexylsulphate; for column 2, the mobile phase was 35% methanol in phosphate buffer (pH 7.4, ionic strength 0.05) for plasma samples and 30% methanol for urine samples. Flow-rates were 1.0 ml/min. The retention time for ampicillin after column 1 was approximately 2.5 min. On column 2 alone, the approximate retention times were 5 and 6 min for plasma and urine samples, respectively.

A UV detector was connected to the waste channel after column 1 to check the retention time on this column; the time for switching valve 2 was adjusted, if necessary.

The scheme for the switching procedures is shown in Table I. The loop in valve 2 had a volume of 1 ml and an I.D. of 0.5 mm.

After column 2, the eluate was mixed with a solution of fluorescamine in acetonitrile (0.16 mg/ml) pumped at a flow-rate of 0.2 ml/min. After a reaction time of 0.5 min in the knitted Teflon tube reactor (ambient temperature), the fluorescence was monitored at 470 nm. The excitation wavelength was 372 nm. During quantification of samples, injections could generally be made in 10-min intervals.

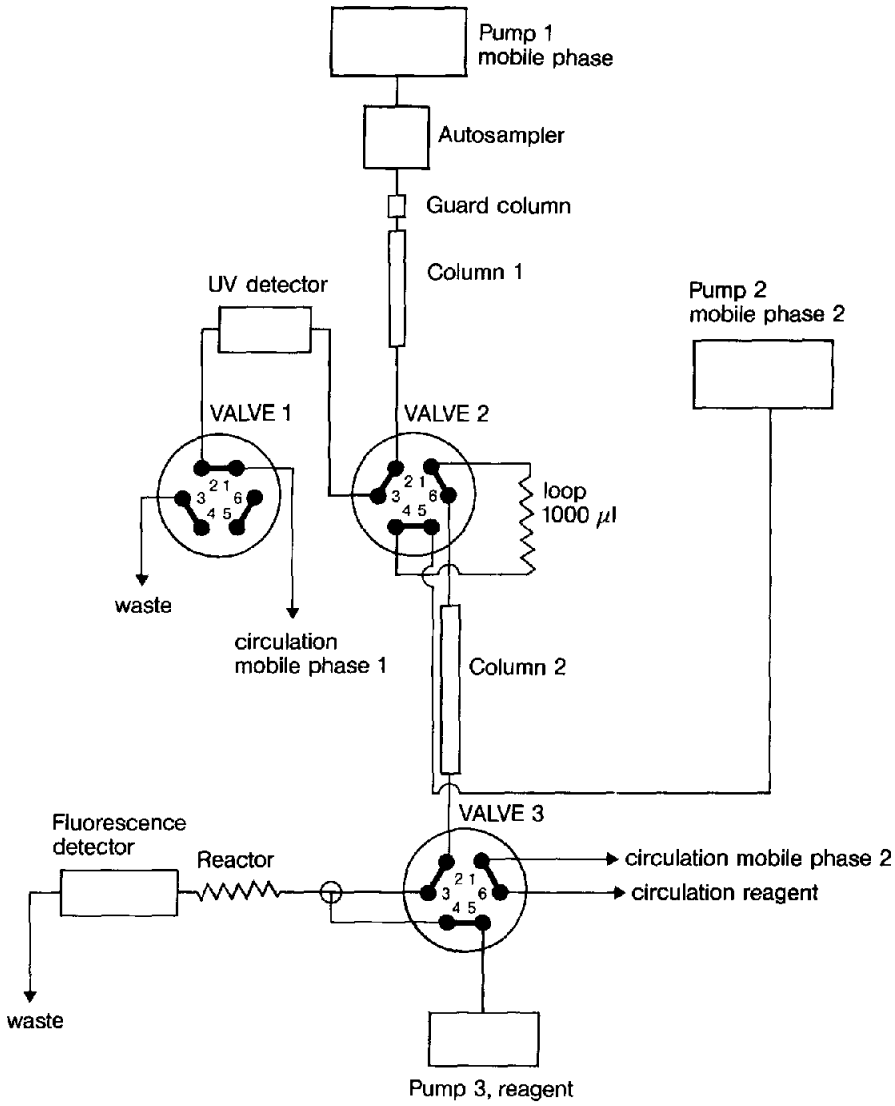


Fig. 1. Scheme of the chromatographic system used.

Calibration and control samples

For the preparation of standard and control samples, aqueous stock solutions of ampicillin were made. From these solutions, known volumes were diluted with plasma or urine to prepare the actual standard and control samples which were treated according to the sample work-up procedures. The concentration of ampicillin in the standard and control samples was chosen depending on the expected

TABLE I

SCHEME OF COLUMN-SWITCHING PROCEDURES

Time after injection (min)	Switch of valve No.	Event
0		Sample is injected on column 1
0.3	1 reset	Mobile phase 1 to waste
2	2	The eluate from column 1 is trapped in the loop
3	2 reset	Trapping completed. The heart cut from column 1 is injected on column 2 for enrichment and separation
5.5	1	Circulation of mobile phase 1
10		Separation completed
11		The next sample is injected
20	3	When injection of sample is completed, circulation of mobile phase 2 and reagent

concentration in unknown samples, which normally means 3–300 μmol/l for urine and 0.1–20 μmol/l for plasma.

Four to six standard samples, evenly spread among the unknowns and containing the same concentration of ampicillin, were used to construct a standard curve through the origin. The origin was confirmed on each occasion by analysing a blank plasma sample. In addition, to check the performance of the analytical system, control samples were run at two to three different concentration levels, chosen according to the levels expected. One control sample was injected in every set of ten injections.

RESULTS AND DISCUSSION

Chromatography and detection

Ampicillin has UV absorbance at low wavelengths only and, therefore, a post-column derivatization was used to obtain higher sensitivity and selectivity. Previously, a post-column reaction based on the formation of the mercuric mercaptide of penicillenic acid and UV detection was used [5]. This reaction is very selective, but the sensitivity is, however, not adequate to analyse small plasma samples (*e.g.* samples from pediatric patients) where, for practical reasons, a dilution prior to the deproteinization step is necessary. Instead, a derivatization of the primary amine group with fluorescamine and fluorescence detection was used. This post-column reaction has previously been used to determine, for example, amoxycillin in biological fluids [11]. The reaction was performed in a knitted Teflon tube to minimize band-broadening [10]. The method involves a simple sample pretreatment (deproteinization and/or dilution) in order to make it suitable for routine analysis. The same kind of pretreatment was used in ref. 5 where it was possible to

use a single-column system because of the high selectivity of the post-column derivatization procedure. In this work the reaction with fluorescamine was used for the post-column derivatization. This reaction, however, is less selective since fluorescamine reacts with most primary amines. Subsequently the selectivity of the chromatographic system had to be increased. This was achieved by use of a coupled-column system. A number of combinations of mobile phases (acetonitrile instead of methanol, addition of quaternary ammonium ions to mobile phase 2) and brands of column 2 (Novapak C_{18} and Microspher C_{18}) were tested. The best selectivity and efficiency were obtained with Microspher C_{18} and methanol as the only modifier in mobile phase 2.

To avoid band broadening due to the large volume injected, it is necessary to enrich the sample on the top of the last column. Therefore, the difference in methanol content between mobile phases 1 and 2 has to be maximal and the methanol content should be lowest on column 1. Methanol contents that are too low on column 1, however, lead to long retention times and subsequently, volumes that are too large to be transferred to column 2. For this reason, hexyl sulphate, which decreases the retention of ampicillin, was added to mobile phase 1. This addition also increased the differences in selectivity between columns 1 and 2.

Evaluation of analytical procedure

During the development of the method, linear standard curves were obtained up to 14 nmol of ampicillin injected.

The accuracy and the intra- and inter-assay precision were determined from

TABLE II

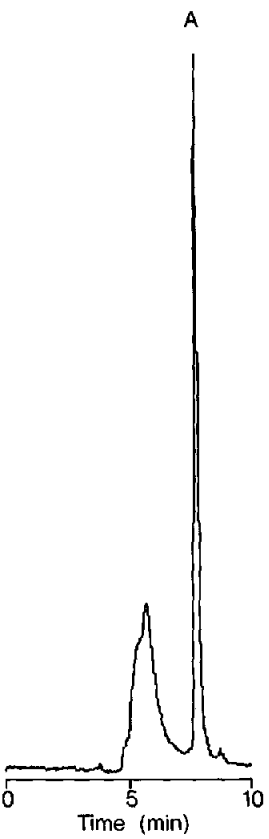
INTRA-ASSAY PRECISION ($n = 10$)

Concentration added (μmol/l)	Found (%)	R.S.D. (%)
Urine (five standards at 185 μmol/l)		
4.4	98.1	1.17
44	100.8	0.41
440	99.3	0.26
Plasma, 0.5 ml (five standards at 6.2 μmol/l)		
0.14	100.6	1.46
1.4	98.6	0.70
14	98.3	0.69
Plasma, 0.1 ml (five standards at 6.2 μmol/l)		
0.14	102.7	3.58
1.4	98.3	1.73
14	100.2	1.31

TABLE III

INTER-ASSAY PRECISION

Concentration added (μmol/l)	Found (%)	R.S.D. (%)	*n*	Interval (days)
Urine (five standards at 232 μmol/l)				
4.8	106.63	4.52	11	6
48	102.94	1.69	11	6
480	98.63	2.97	11	6
Plasma, 0.5 ml (five standards at 10.6 μmol/l)				
1.2	97.51	2.50	28	14
30	96.71	2.00	28	14
Plasma, 0.1 ml (five standards at 4.1 μmol/l)				
0.12	107.9	7.72	12	7
2.8	97.8	4.65	14	7
12	98.5	4.86	14	7



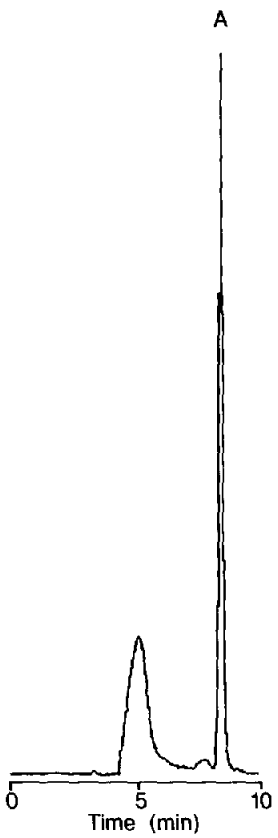


Fig. 2. Typical chromatogram from plasma obtained by analysis according to the described method. The sample, collected 10 min after oral administration of 670 μmol of ampicillin, contained 1.26 μmol/l ampicillin (A).

Fig. 3. Typical chromatogram from urine obtained by analysis according to the described method. The urine was collected between 5 and 7 h after oral administration of 670 μmol of ampicillin with a total volume of 108 ml. The sample contained 52.1 μmol/l ampicillin (A).

spiked plasma and urine samples (Tables II and III). The inter-assay data were obtained from control samples that were kept frozen at −70°C and analysed together with unknowns on different occasions. Typical chromatograms are shown in Figs. 2 and 3.

Detection limits, defined as the concentration that gives a peak height corresponding to three times the noise of the blank baseline, were for urine 570 nmol/l and for plasma 14 nmol/l (0.5-ml sample).

The method has, without problems, routinely been used for more than a year to analyse thousands of samples. The adjustment of pH to *ca.* 5 made the samples sufficiently stable to be analysed overnight using an autosampler.

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