Journal of Chromatography, 615 (1993) 91–96
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6767

High-performance liquid chromatographic determination of amoxicillin in urine using solid-phase, ion-pair extraction and ultraviolet detection

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(First received November 3rd, 1992; revised manuscript received January 26th, 1993)

ABSTRACT

A simple, robust high-performance liquid chromatographic method is described for assaying amoxicillin in urine. Sample clean-up involved solid-phase, ion-pair extraction onto Sep-Pak C_{18} cartridges followed by elution with acetonitrile (3%, v/v) in pH 4.85 buffer. Separations were performed on an Ultrasphere C_{18} column with a mobile phase comprising acetonitrile (32.5 ml) and pH 7.1 phosphate buffer (0.01 *M*, 1000 ml). Peaks were detected at 229 nm. Recovery was greater than 94%. Plots of peak area against urinary amoxicillin concentration were linear ($r > 0.999$) from 5 to 500 mg/l. Between-day and within-day imprecision (coefficient of variation) ranged between 3.0 and 15.5%. Inaccuracy was 7.1%, or less.

INTRODUCTION

Amoxicillin, a broad-spectrum, α-amino-substituted β-lactam antibiotic, is widely used in clinical practice. The measurement of this drug in biological fluids is challenging since its amphoteric state precludes extraction with organic solvents. Previous analytical methods encompass microbiological assays [1–4] which are time-consuming and lack specificity and accuracy, or specialized detection using fluorimetry [5,6], polarography [7] or derivative spectrophotometry [8]. HPLC methods for the determination of amoxicillin in urine have also been developed by diluting the urine sample and submitting to analysis with detection at 230 nm [9,10]. At this wavelength, however, endogenous compounds also have high absorbance and in our experience these approaches have lacked adequate selectivity and sensitivity. Attempts to improve assay performance have included derivatization before or after chromatographic separation [11–15], column switching [15] and ion-pairing [13–16]. These HPLC methods are sensitive and specific, but require lengthy sample work-up and often involve complex instrumentation.

We describe a selective and precise method for assaying amoxicillin in urine which uses a simple extraction procedure and basic HPLC instrumentation. No internal standard is required, and the assay is sensitive enough for application in amoxicillin pharmacokinetic studies.

EXPERIMENTAL

Drugs and reagents

Amoxicillin trihydrate BP (Batch No. MO313) was supplied by Alphapharm (Brisbane, Australia). An amoxicillin metabolite, amoxicillin (2*R*)-

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0378-4347/93/$06.00

piperazine-2′,5′-dione, was kindly donated by Dr. J. Haginaka (Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Hyogo, Japan). The penicilloic acid metabolite of amoxicillin was prepared by alkaline hydrolysis of amoxicillin as described previously [17]. Acetonitrile and methanol (Mallinckrodt, Clayton, Australia) were HPLC grade. Water was freshly distilled, filtered through a 0.45-μm membrane and degassed under vacuum before use. Anhydrous disodium hydrogen orthophosphate, tetrabutylammonium bromide, phosphoric acid, citric acid and sodium hydroxide were A.R. grade. Citrate–phosphate buffer (pH 5.4) [15] was prepared by dissolving 19.9 g of disodium hydrogen orthophosphate anhydrous and 40.0 ml of 1 *M* citric acid in distilled water and made up to 250 ml. pH-Adjusting solution (pH 4.85) was made up from 100 ml of 0.5 *M* disodium hydrogen orthophosphate and 350 ml of distilled water then adjusted with 1 *M* citric acid to pH 4.85 before adding water to 500 ml [15].

Instrumentation

The HPLC system was from Millipore–Waters (Milford, MA, USA) and comprised a Model 501 pump, a Model 712 WISP automatic sampler and a Model 484 tunable absorbance detector. Separations were performed on an Ultrasphere C_{18} column (250 mm × 4.6 mm I.D.) packed with 5-μm particles (Beckman Instruments, San Ramon, CA, USA). Chromatograms were recorded and peak areas were integrated on a Model 745B data module. Solid-phase extractions were performed in conjunction with a ten-place Vac-Elut unit (Analytichem International, Harbor City, CA, USA) connected to a vacuum source.

Standards and controls

A master stock solution of amoxicillin trihydrate was freshly prepared on each day of analysis by dissolving 57.4 mg of amoxicillin trihydrate (equivalent to 50 mg of amoxicillin base) in 50 ml of citrate–phosphate buffer, pH 5.4. Standards were prepared by supplementing drug-free human urine with the master stock to give concentrations of 500, 400, 300, 100, 50, 10 and 5 mg/l amoxicillin base. Seeded controls containing amoxicillin base concentrations of 490, 200 and 7.5 mg/l were prepared in drug-free urine and stored at −70°C.

Sample preparation

A 1-ml volume of sample, standard or control was pipetted into an appropriately labelled tube and 100 μl of 0.5 *M* tetrabutylammonium bromide in water were added. The contents were agitated with a vortex-mixing action for 30 s, then applied to a Sep-Pak C_{18} cartridge (Millipore–Waters), previously conditioned with methanol (5 ml), then water (5 ml). Amoxicillin was eluted with 9 ml of acetonitrile (3%, v/v) in pH-adjusting solution under 26 kPa vacuum pressure without leaving the cartridge to dry. The final volume was adjusted to 10 ml with the eluent and an aliquot injected into the HPLC column. Urine standards, unknown samples and controls were analysed in duplicate.

Chromatography

HPLC analyses were carried out in an air-conditioned laboratory at 20 ± 2°C. The mobile phase comprising acetonitrile (32.5 ml), 0.1 *M* disodium hydrogen phosphate pH 7.1 (100 ml) and distilled water (900 ml) was filtered through a 0.45-μm membrane filter and degassed under vacuum before use. The flow-rate was 1.2 ml/min which generated a back pressure of 77 bar. The injection volume was 30 μl and peaks were detected at 229 nm, at a sensitivity of 0.5 a.u.f.s. The recording integrator was set to an attenuation of 16 and a chart speed of 0.1 cm/min. The total run time was 25 min.

Quantitation

Standard calibration plots were constructed by least-squares linear regression of peak area on amoxicillin concentration, weighted by the reciprocal of the variance [18]. Unknown amoxicillin concentrations were calculated by inverse prediction.

Recovery

Amoxicillin was added to distilled water and to drug-free urine to give two series of concentrations each of 500, 100 and 10 mg/l. The absolute recovery of amoxicillin was assessed by assaying these samples as described and comparing the peak area of amoxicillin with that obtained from direct injection of a control solution of amoxicillin in distilled water.

Imprecision

Within-day imprecision was estimated by analysing the three seeded controls ten times over one day in no fixed order. Between-day imprecision was estimated from the analysis of seeded controls over 36 assay days as part of a clinical study. Assay imprecision (C.V.) in both cases was defined as standard deviation normalised by the mean value, expressed as a percentage.

Stability

Stability was determined in samples containing 500, 100 and 10 mg/l amoxicillin in urine under the following conditions: urine specimens stored at −70°C for five months; processed specimens reinjected periodically during overnight storage in the HPLC autosampler carousel.

Selectivity

Assay selectivity was examined in relation to interferences from endogenous substances in the drug-free urine of six healthy volunteers. Potential interference from two known amoxicillin metabolites, amoxicilloic acid and amoxicillin (2*R*)-piperazine-2′,5′-dione, was assessed by injecting aqueous samples of these agents onto the column and comparing their retention times with amoxicillin.

Application of the method

Six different oral doses of amoxicillin sodium solution (97, 194, 388, 776, 1552 and 3103 mg) were assigned to six healthy volunteers in a randomised cross-over study. Urine samples were collected at half-hourly intervals for 4 h, then hourly until 10 h after administration, and assayed for amoxicillin by the described method.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of (a) drug-free urine, (b) a urine standard containing 300 mg/l amoxicillin, (c) a urine standard containing 10 mg/l amoxicillin and (d) a urine sample from a volunteer who had taken a single oral dose of 97 mg amoxicillin sodium 2 h previously (178 mg/ml amoxicillin). Amoxicillin was eluted after 20 min as a sharp symmetrical peak in an area of the chromatogram free from interfering peaks. The two amoxicillin metabolites did not interfere. Amoxicilloic acid, the product of hydrolysis of the ß-lactam ring of amoxicillin, is more polar than the parent compound and was eluted in the solvent front. A solution of the dione metabolite in mobile phase produced peaks at 39 and 51 min, however, no such peaks were detected in the urine of subjects administered a wide range of oral doses of the drug.

Solid-phase extraction offered a potential solution to the non-extractability of amoxicillin which exists as a zwitterion over the entire pH range. Others [13–16] have used ion-pairing with a cationic or anionic counter-ion to improve the reversed-phase chromatography of the antibiotic. The formation of the amoxicillin–tetrabutylammonium ion-pair and subsequent elution with 9 ml of a 3% (v/v) acetonitrile in pH 4.85 buffer solution gave extracts of the drug from 1-ml urine samples which were free from interference.

The choice of pH in the extractive and chromatographic stages was critical. A pH 4.85 buffer was found to give the minimum number of extraneous peaks while offering a reported maximum stability of amoxicillin in biological fluids [15]. We confirmed that amoxicillin is stable at this pH by storing extracted samples (10, 100 and 500 mg/l) in the autosampler at room temperature up to 24 h, conditions which often prevail when large numbers of specimens are analysed in clinical studies. No degradation was observed in any sample and satisfactory stability was also found for the storage of urinary amoxicillin samples at −70°C up to five months (Table I).

Amoxicillin retention time could be shortened

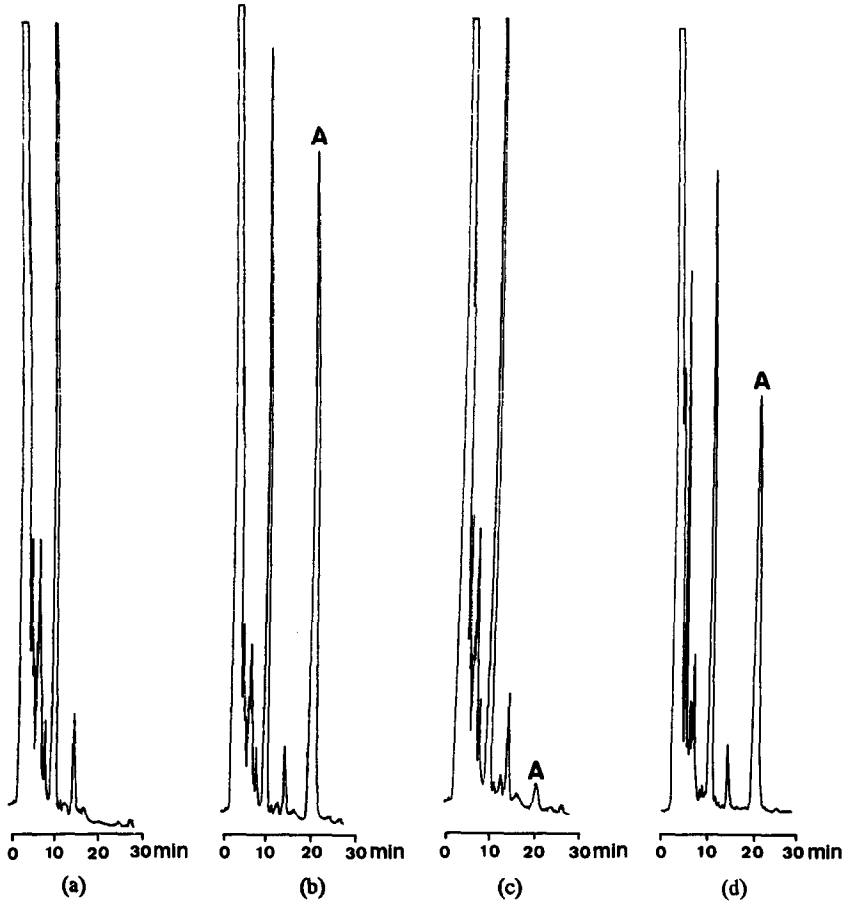

Fig. 1. Chromatograms of (a) drug-free urine, (b) urine containing 300 mg/l amoxicillin, (c) urine containing 10 mg/l amoxicillin and (d) urine from a volunteer 2 h after taking a single oral dose of 97 mg amoxicillin sodium (178 mg/l amoxicillin). Peak A = amoxicillin.

or lengthened by adjustment of the pH of buffer in the mobile phase below or above 7.2 respectively, which corresponds to the reported pK_a of the α-amino group on the side-chain [15]. Minor alterations of this pH (without concomitant change in acetonitrile concentration) were occasionally required to fine tune the chromatography of some specimens. A column life of at least 800 injections was routinely achieved by flushing the column with methanol (30 ml), followed by dimethylsulphoxide (1 ml), then 50% (v/v) methanol in methylene chloride (30 ml), whenever any peak broadening or shifting was evident.

The lack of internal standardisation may appear, at first, to be a potential drawback of the assay. Of more than 60 possible compounds screened none had the required extractive, spectrophotometric or chromatographic properties. Nonetheless, we established that the within-day and between-day imprecision of the assay was acceptable without internal standardisation when used in combination with the WISP autosampler (Table I). An internal standard is not necessary if sample clean-up is relatively simple and volume control can be achieved, and, in fact, the incorporation of internal standards may introduce additional assay variation in some circumstances [19]. The minimum quantifiable concentration was set at 7.5 mg/l, the lowest concentration of amoxicillin in urine at which maximum acceptable im-

TABLE I

WITHIN-DAY AND BETWEEN-DAY IMPRECISION AND INACCURACY

The values in parentheses are coefficients of variation.

Concentration (mg/l)		Inaccuracy[a] (%)
Target	Found (Mean ± S.D.)	
Within-day (n = 10)		
490	485.4 ± 14.7 (3.03)	0.9
200	202.5 ± 16.6 (8.19)	1.3
7.5	7.2 ± 1.12 (15.5)	3.7
Between-day (n = 36)		
490	477.5 ± 17.9 (3.75)	2.5
200	189.5 ± 6.64 (3.50)	5.3
7.5	7.0 ± 1.07 (15.4)	7.1

[a] [Absolute (target − found)/target] × 100.

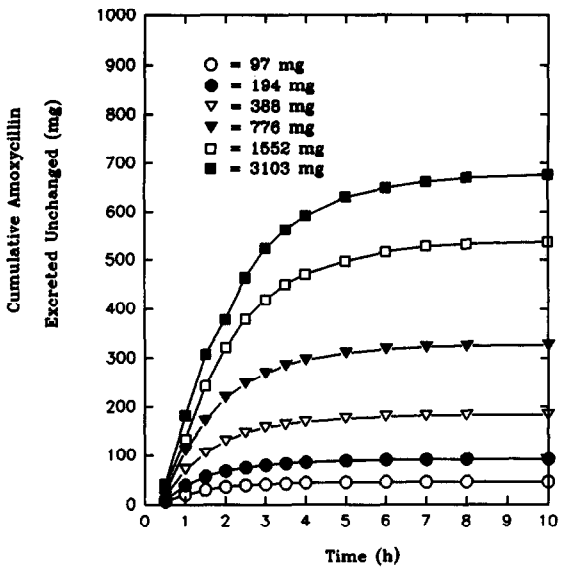

Fig. 2. Mean cumulative urinary excretion of unchanged amoxicillin assayed in samples collected after administration of six different doses of amoxicillin sodium solution to six healthy volunteers.

precision (*ca.* 15%) was obtained. Within-day and between-day inaccuracy was 7.1%, or less, between 7.5 and 490 mg/l (Table I). The absolute recovery from distilled water and urine (Table II) was high (>94%) and independent of amoxicillin concentration. Calibration data pooled from 36 replicate standard curves of peak area *versus* concentration (mean response range: 26 395 area units at 5 mg/l to 2 978 250 area units at 500 mg/l) showed highly significant linearity ($P < 0.0001$; $r = 0.9993$) with no significant non-linear elements in the residual sums of squares, as determined by analysis of variance.

The utility of the assay was demonstrated in a pharmacokinetic study of amoxicillin absorption in six healthy volunteers who took oral doses of amoxicillin sodium solution ranging from 97 mg (equivalent to 0.25 mmol amoxicillin base) to 3103 mg (8 mmol base). Profiles of mean cumulative amounts of amoxicillin excreted unchanged in urine are shown in Fig. 2. This is a convenient and valid application of the method

TABLE II

ABSOLUTE RECOVERY OF AMOXICILLIN

The values in parentheses are coefficients of variation.

Concentration (mg/l)	Experiment No.	Absolute recovery (mean ± S.D.) (%)	
		Water	Urine
500	1	100.9 ± 0.40 (0.39)	98.1 ± 1.37 (1.40)
	2	100.0 ± 1.28 (1.28)	97.7 ± 0.71 (0.73)
100	1	94.8 ± 0.89 (0.94)	94.1 ± 3.12 (3.32)
	2	99.1 ± 1.01 (1.02)	96.7 ± 2.30 (2.38)
10	1	97.8 ± 6.52 (6.67)	113.0 ± 7.53 (6.66)
	2	96.3 ± 5.67 (5.89)	100.0 ± 3.27 (3.27)

since urine collection is a non-invasive method and amoxicillin is largely excreted in the urine unchanged. Useful bioavailability and pharmacokinetic data can be obtained provided that a sufficient number of urine samples are collected [20]. Further, the assay has potential for clinical application since urine concentrations of antibiotics are often useful in treating some acute, uncomplicated urinary tract infections [21].

ACKNOWLEDGEMENT

The authors wish to thank Dr. M. J. Faddy, Department of Mathematics, The University of Queensland, for statistical advice.

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