Journal of Chromatography, 617 (1993) 334–338
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6908

Short Communication

Determination of amoxicillin in human plasma by high-performance liquid chromatography and solid phase extraction

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(First received February 4th, 1993; revised manuscript received May 6th, 1993)

ABSTRACT

A high-performance liquid chromatographic method using solid phase extraction has been developed for the determination of amoxicillin in human plasma. After concentration of amoxicillin on a C_8 cartridge, the cartridge was eluted on-line to a reversed-phase column packed with 5 μm Chromspher C_{18}. The mobile phase was methanol–0.08 *M* phosphate buffer (pH 7.6) (20:80), which contained 0.01 *M* tetrabutylammonium dihydrogenphosphate. The column effluent was monitored by UV spectrophotometry at 234 nm. The method has proved to be reliable and is used in bioavailability studies for the development of new amoxicillin formulations.

INTRODUCTION

Amoxicillin (Fig. 1A) is a widely used broad spectrum antibiotic. It belongs to the group of penicillins characterized by a β-lactam group. Several HPLC methods for the quantitation of amoxicillin in plasma have been reported. Most of them use UV detection at low wavelengths [1–4]. To obtain lower limits of detection, fluorescence detection after pre- or postcolumn derivatization has been used [5–7]. A very selective and sensitive method was described by Mascher and Kikuta [6]. They used fluorescence detection after on-line oxidation of amoxicillin.

Owing to its high polarity, the extraction of amoxicillin is problematic. A method for direct injection of plasma samples was proposed by Adamovics [8], but unfortunately this method was suitable for plasma concentrations only be-

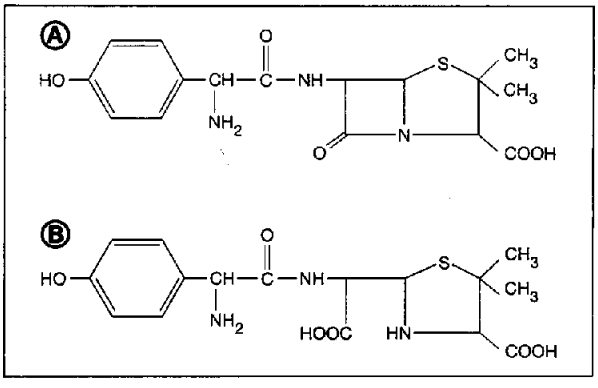

Fig. 1. Molecular structures of (A) amoxicillin and (B) amoxicilloic acid.

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0378-4347/93/$06.00

tween 20 and 100 μg/ml, which is too high for most applications. Tyczkowska *et al.* [9] injected plasma samples after filteration through a 30 000 molecular mass cut-off filter to prevent deterioration of the column. Protein precipitation by adding perchloric acid to the plasma samples was described by several authors [4,6,10]. However, amoxicillin is unstable under acidic conditions, and therefore the sample has to be injected immediately after preparation.

Although the use of solid phase extraction has been described [2,3], none of the authors reported the use of it followed by on-line elution of the solid phase extraction cartridge contents to the HPLC column. The method presented here makes use of a Varian AASP (Advanced Automated Sample Processor) as a fast and simple means of sample preparation.

The method was developed to be used in bioavailability studies of new amoxicillin formulations and has proved to be very reliable.

EXPERIMENTAL

Column

The column used was a 250 × 4.6 mm I.D. stainless steel pre-packed column containing 5 μm Chromspher C_{18} (Chrompack, Middelburg, Netherlands). A μBondapak C_{18} Guard-pak was used as a pre-column (10 μm particle size, irregular, 4 mm × 6 mm I.D.) (Waters Millipore, Milford, USA). The pre-column was replaced every four days (*i.e.* after *ca.* 160 injections). Between the pump and the AASP, a 100 × 3 mm I.D. reversed-phase saturation column (Chrompack) was mounted to saturate the eluent with stationary phase.

Instruments

The following HPLC system was used: a Spectra Physics SP8800 HPLC pump (Spectra Physics Analytical, San Jose, USA); a Varian AASP to prepare and inject the samples (Varian, Walnut Creek, USA). The saturation column, the pre-column and the analytical column were placed in a Chrompack SpH 99 column thermostat. The temperature was maintained at 30°C. A Spectra Physics Spectra 200 variable-wavelength UV detector was used to monitor the effluent at 234 nm. Integration was performed by a Perkin-Elmer LCI-100 integrator (Perkin-Elmer, Norwalk, USA). The samples were extracted using C_8 AASP cartridges (Analytichem International, Harbor City, USA).

Chemicals and solvents

The HPLC eluent was composed of 80.0% phosphate buffer and 20.0% methanol (LABSCAN, Dublin, Eire). The buffer was prepared by dissolving 14.2 g of disodium hydrogenphosphate dihydrate p.a. (E. Merck, Darmstadt, Germany) in 900 ml of deionized water. After addition of 12.5 ml of 1.0 *M* tetrabutylammonium dihydrogenphosphate (TBA) (Aldrich, Milwaukee, USA) the pH was adjusted to 7.60 with diluted sodium hydroxide (EKA Kemi, Surte, Sweden). Water was added to make a total volume of 1000 ml. The buffer and methanol were filtered through a 0.2-μm regenerated cellulose filter (Sartorius, Göttingen, Germany) before use. Both the buffer and methanol were degassed by continuous helium sparging. The solvents were mixed by the pump. The eluent flow-rate was 1.00 ml/min.

The plasma samples were diluted with a 0.067 *M* phosphate buffer (pH 7.0). The AASP purge solvent was prepared by adding 100 mg of sodium azide (extra pure, E. Merck) to 500 ml of 0.067 *M* phosphate buffer (pH 7.6).

Amoxicillin · $3H_2O$ was provided by Gist-brocades (Delft, Netherlands). A stock solution was prepared by dissolving 65 mg of amoxicillin · $3H_2O$ in 45 ml of water. After dissolution, water was added to make a total volume of 50.0 ml. The stock solution was stored at −20°C until use.

Sample collection and preparation

The blood samples were collected in heparinized tubes (Venoject, Terumo Europ, Leuven, Belgium) and immediately centrifuged at 1500 *g*. The plasma was isolated and stored at −70°C until analysis. On the day of analysis the samples were thawed, and 150 μl of each was mixed with 1.5 ml of phosphate buffer and 20 μl of water.

Calibration samples were prepared by adding 1500 μl of buffer and 20 μl of a calibration standard to 150 μl of blank human plasma. After conditioning of the AASP C_8 cartridges with two 1-ml volumes of acetonitrile and two 1-ml volumes of phosphate buffer, 1000 μl of a diluted sample was passed through a cartridge. The cartridges were washed with 200 μl of buffer. Just before elution, the AASP cartridge was purged three times.

Calibration lines were constructed with amoxicillin · $3H_2O$ concentrations between 0.35 and 21 μg/ml plasma. Weighted least-squares regression was used for the calculation of the calibration lines, with the square of the reciprocal of the concentration as a weighting factor.

RESULTS AND DISCUSSION

HPLC separation

The chromatograms (Fig. 2A and B) obtained after dosing of 750 mg of amoxicillin · $3H_2O$ to human volunteers show that the amoxicillin signal (11.8 min) is free of interferences. The main metabolites of amoxicillin, (5*R*,6*R*)- and (5*S*,6*R*)-amoxicillin penicilloic acid (Fig. 1B) are only partially separated from amoxicillin, but are not retained on the C_8 AASP cartridge during isolation and therefore do not interfere.

Recovery of amoxicillin

Owing to its highly polar nature, amoxicillin is difficult to extract. A nearly quantitative recovery was obtained by solid phase extraction on C_8 cartridges after diluting the plasma with a buffer containing TBA as a counter-ion. However, this method has the disadvantage of retaining the penicilloic acids, which are not separated from amoxicillin on the analytical column. By omitting the counter-ion, the recovery of amoxicillin decreased, but it was reproducible and constant over the entire range of the calibration line. The recovery of amoxicillin was determined at three concentrations (Table I) by injecting each level six times and comparing the peak area with the mean peak area obtained from six injections of a pure standard.

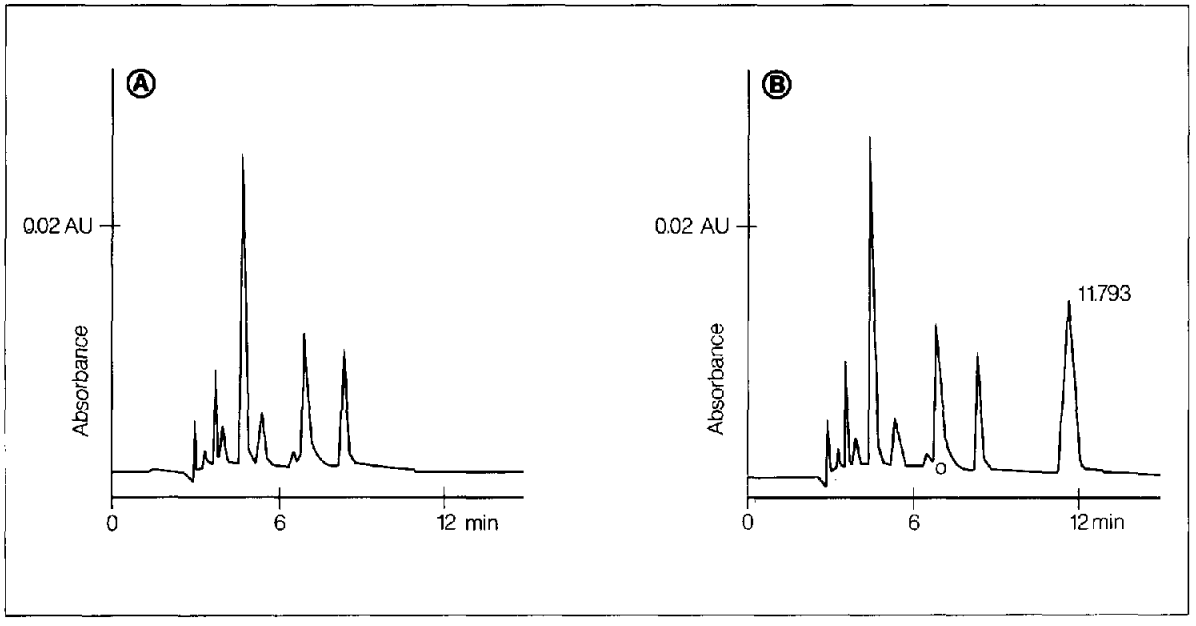

Fig. 2. Chromatograms of (A) blank human plasma and (B) human plasma containing 8.2 μg/ml amoxicillin · $3H_2O$.

TABLE I

RECOVERY OF AMOXICILLIN FROM PLASMA

Concentration (μg/ml)	Recovery (mean ± S.D., n = 6) (%)
0.52	63.5 ± 7.4
5.2	63.5 ± 2.9
17.4	64.0 ± 2.0

Linearity and limit of detection

The linearity of the calibration graph was tested using a lack-of-fit test [11]. Each level was injected twice. The graph proved to be linear between 0.35 and 21 μg/ml plasma and passed through the origin. The correlation coefficient was at least 0.99. The limit of detection was calculated to be 25 ng/ml plasma at three times the baseline noise.

Accuracy and precision

The accuracy and precision of the assay were determined at three plasma levels of amoxicillin (Table II and III) on two days. As can be expected, the precision of the assay improves as the plasma levels increase.

TABLE II

ACCURACY OF AMOXICILLIN ASSAY

Concentration (μg/ml)	Accuracy (mean ± S.D.) (%)	
	Day 1	Day 2
0.52	99.9 ± 3.2	96.3 ± 15.5
5.2	94.9 ± 3.3	104.1 ± 4.2
17.4	98.2 ± 2.2	106.0 ± 4.7

Stability of amoxicillin

The stability of amoxicillin before and after sample pre-treatment was determined. After 4 h at room temperature, 97% of amoxicillin was

TABLE III

PRECISION OF AMOXICILLIN ASSAY

Concentration (μg/ml)	S.D. (n = 6) (μg/ml)	
	Day 1	Day 2
0.52	0.02	0.08
5.2	0.17	0.22
17.4	0.38	0.82

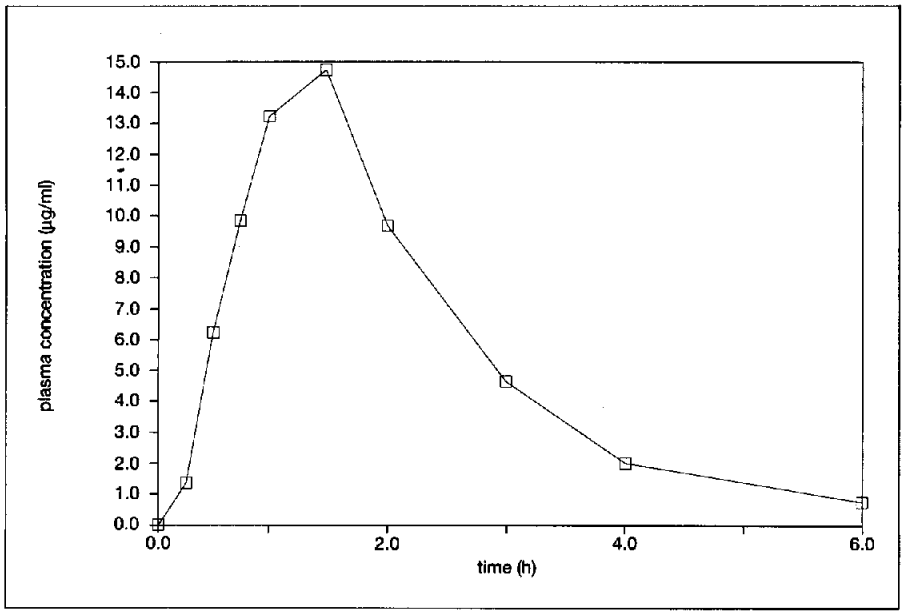

Fig. 3. Plasma concentration–time curve of amoxicillin after oral dosing of 750 mg to a human volunteer.

still present in plasma. Amoxicillin showed peculiar behaviour after sample extraction. After 10 h, the peak area had decreased by only 2.2% but the peak height had decreased by 21.5%. An explanation would be that amoxicillin is not chemically converted but is slowly redistributed over the extraction column and is eluted as a broad band. Therefore, the samples were eluted within 5 h after extraction. During this time the peak area had decreased by only 1.4% and the peak height by 10%.

The method was sensitive and precise enough to be used in pharmacokinetic studies. The simple and rapid clean-up means that *ca.* 40 to 50 samples per day can be processed, including calibration and control samples. The applicability of the method is shown by a typical curve of the plasma concentration against time (Fig. 3) after oral dosing of 750 mg of amoxicillin · $3H_2O$.

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