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Sensitive assay for measuring amoxicillin in human plasma and middle ear fluid using solid-phase extraction and reversed-phase high-performance liquid chromatography

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Abstract

We developed a sensitive assay to measure amoxicillin in human plasma and middle ear fluid (MEF) using solid-phase extraction and reversed-phase HPLC. Amoxicillin and cefadroxil, the internal standard, were extracted from 50–200 μl of sample with Bond Elut C_{18} cartridges. The extract was analyzed on a 15 cm × 2 mm, 5 μm Keystone MOS Hypersil-1 (C_8) column with UV detection at 210 nm. The mobile phase was 6% acetonitrile in 5 m*M* phosphate buffer (pH = 6.5) and 5 m*M* tetrabutylammonium. The average absolute recovery of amoxicillin and cefadroxil were 91.2 ± 16.6% and 91.0 ± 6.8%, respectively. The limit of quantitation was 0.125 μg/ml with 200 μl sample size. The linear range was from 0.125 to 35.0 μg/ml with correlation coefficients greater than 0.999. These analytic conditions produced a highly sensitive amoxicillin assay in human body fluids without derivatization.

1. Introduction

Bacteria like *S. pneumoniae* and *H. influenzae* are still the most common causes of acute otitis media (AOM). Various antimicrobials are used for treatment of children with AOM but amoxicillin remains the most frequent first choice. Amoxicillin is a semi-synthetic β-lactam that inhibits bacterial cell wall synthesis.

To understand the penetration and pharmacokinetic behavior of amoxicillin in human middle ear fluid (MEF) and the correlation of plasma and MEF concentrations, a sensitive and reliable quantitation method of amoxicillin is needed, since sample volume obtained for analysis of plasma and MEF is limited. Currently available methods that measure amoxicillin in biological fluids are mostly based on protein precipitation by acid or organic solvents, since amoxicillin is insoluble in most organic solvents. Also, these assays tend to utilize UV detection [1], though post-column reactions or derivatization enhance assay sensitivity [2,3].

Solid-phase extraction (SPE) has gained popularity over the years in sample preparation for a wide range of analytes in complex matrices due to its better selectivity, easy operation, and reduced solvent consumption. However, only a few reports described SPE methods of extracting amoxicillin by C_{18} cartridges from human plasma

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[4–6]. Systems using solid-phase extraction for other β-lactam antibiotics were few, too [7,8].

After evaluating several liquid-phase extraction methods, we developed a reversed-phase HPLC method using C_{18} cartridge extraction of amoxicillin and cefadroxil, the internal standard, and UV detection at 210 nm. This combination of extraction and detection results in a method that is highly sensitive, reliable, and easy to perform. This method is being used in this laboratory to investigate the clinical pharmacokinetics of amoxicillin in human plasma and middle ear fluid and should be applicable for the quantitation of other related antibiotics in biological matrices as well.

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile was from Fisher (Fairlawn, NJ, USA). HPLC grade water was obtained from a Milli-Q apparatus in this laboratory. Amoxicillin sodium and cefadroxil sodium were purchased from Sigma (St. Louis, MO, USA). The solid-phase extraction cartridges used were Bond Elut C_{18} cartridges (Varian sample preparation products, 500 mg/2.8 ml). All other chemicals were of analytical grade unless indicated otherwise.

All plasma and MEF samples were stored at −70°C after collection and necessary processing.

2.2. Instrumentation

A Hewlett-Packard 1090 L liquid chromatograph and a SpectraSeries UV100 detector (Thermo Separation Product) with a 5-μl flow cell were used throughout the study. The analytical column, maintained at 40°C, was a 15 cm × 2 mm, 5 μm Keystone Scientific (Bellefonte, PA, USA) MOS Hypersil-1 (C_8). A matching 10 × 2 mm guard column was used. The detector was set at 210 nm with 0.0005 AUFS and a rise time of 0.3 s. The mobile phase consisted of 6% acetonitrile in 0.005 *M* phosphate and 0.005 *M* tetrabutylammonium adjusted to pH 6.5 after mixing with acetonitrile. After the elution of the antibiotics, the mobile phase was changed to 25% acetonitrile in the same buffer for 14 to 16 min to elute strongly adsorbed materials and the column was re-equilibrated for 4 min before injecting the next sample. The flow-rate was 0.35 ml/min. Chromatographic data were collected and analyzed by an IBM PC AMD 386SX25 computer using Chrom-Perfect software (Justice Innovations, Palo Alto, CA, USA).

2.3. Stock solutions and standards

Stock solutions of amoxicillin (1.0 mg/ml) and working standard solutions of cefadroxil (60.0 μg/ml) in 5% methanol in water were prepared daily. Stock solutions of amoxicillin were diluted to make working standard solutions of 1.0, 5.0, 10.0, 50.0, and 250 μg/ml. The calibration standards were made by spiking the blank plasma with appropriate amounts of working standards. The quality control (QC) samples were prepared in a similar way. For MEF samples, MEF matrix-based standards and QC samples were used as much as possible. Otherwise, blank plasma was used to prepare the standards. Experience in this laboratory indicated that using plasma does not affect the quantitation of amoxicillin and cefadroxil in MEF.

2.4. Sample preparation

A 35-μl aliquot of the internal standard (60.0 μg/ml of cefadroxil) and 1.0 ml of 0.05 *M* phosphate buffer, pH = 6.8 was added to 200 μl of the plasma samples and to the standards (50 μl MEF sample and the standards). This solution was mixed well by vortex-mixing. The C_{18} cartridge was conditioned with 4.0 ml of methanol and 1.0 ml of 0.05 *M* phosphate buffer, pH = 6.8. The sample was applied to the cartridge and passed slowly under low vacuum (5 kPa). The column was washed with 1.0 ml of the same

buffer and then 1.0 ml of water. High vacuum (50 kPa) was subsequently applied to dry the column completely. Substances retained on the column were eluted with 1.0 ml of methanol–water (40:60) under low vacuum (5 kPa) to yield plasma or MEF extract. The extract was then evaporated to dryness at 40°C under a nitrogen stream and reconstituted in 35 μl of 5% methanol in water. A 25-μl aliquot of the extract was then injected onto the chromatograph. Low vacuum (5 kPa) during sample application or elution and complete drying of the sorbent at high vacuum (50 kPa) are critical to ensure high recoveries of the drugs of interest.

2.5. Calculations

The peak-height ratio of amoxicillin to cefadroxil was used for quantitation, although an internal standard was not absolutely required to achieve acceptable accuracy and precision for human plasma samples (data not shown). A standard curve of amoxicillin at 0.125, 0.25, 0.5, 1.0, 5.0, 20.0, and 35.0 μg/ml ($n = 1$) and quality control samples at 0.4, 10.0, and 30.0 μg/ml ($n = 2$), prepared with blank plasma, were run together with unknowns. The standard curve was analyzed by weighted linear regression (weighting factor: $1/x^2$) and used to calculate the amoxicillin concentration of unknown samples. The run was accepted when the results of at least four out of the six quality control samples were within 15% of the target value and the two failed QC samples were not at the same concentration.

For MEF samples, a standard curve of amoxicillin at 0.5, 1.0, 2.0, 5.0, and 10.0 μg/ml ($n = 1$) and quality control samples at 1.5, 4.0, and 8.0 μg/ml ($n = 2$) were run together with unknowns. The standard curve was analyzed by weighted linear regression (weighting factor: $1/x^2$).

3. Results

Fig. 1a shows a chromatogram of a human plasma sample containing amoxicillin after solid-phase extraction. The retention times were: amoxicillin 6.45 min; cefadroxil 12.30 min. The amoxicillin concentration was 3.48 μg/ml. Fig.

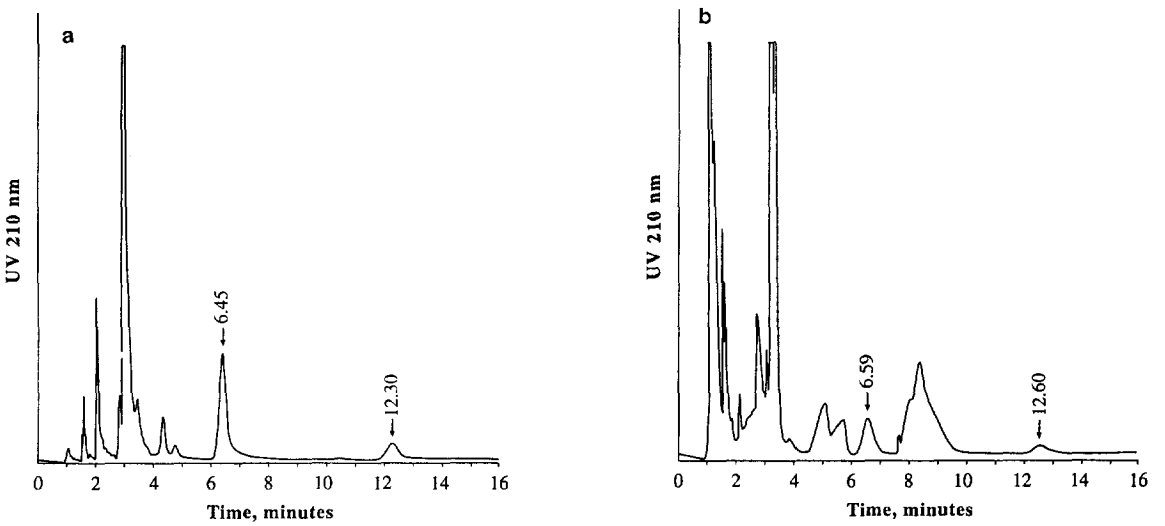

Fig. 1. (a) Chromatogram of a human plasma sample after solid-phase extraction. Retention times: amoxicillin 6.45 min; cefadroxil 12.30 min. Amoxicillin concentration: 3.48 μg/ml. (b) Chromatogram of the same sample after liquid-phase extraction as follows: 200 μl of sample was deproteinized by 2.5 ml of acetonitrile; the dried supernatant was reconstituted in 0.75 ml of 70% methanol in water and washed with 0.75 ml of hexane; the dried extract was reconstituted in 5% methanol in water. Retention times: amoxicillin 6.59 min; cefadroxil 12.60 min. The chromatographic conditions are described in the text.

1b shows a chromatogram of the same sample after liquid-phase extraction (see figure caption for details). The retention times were: amoxicillin 6.59 min; cefadroxil 12.60 min.

Fig. 2 shows the plasma amoxicillin concentration–time profile from a human volunteer who took a 1-g oral dose at time zero.

Fig. 3 shows a chromatogram of a blank human plasma sample after solid-phase extraction. Similar chromatograms were obtained from six different blank plasma samples with no evidence of interfering substances.

Fig. 4 shows a chromatogram of a blank human MEF sample after solid-phase extraction. Similar chromatograms were obtained from fifteen different MEF samples with no evidence of interfering substances.

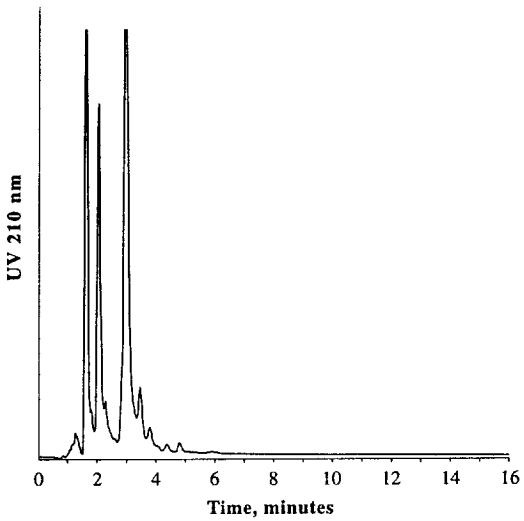

Fig. 3. Chromatogram of a blank human plasma sample after solid-phase extraction. The chromatographic conditions are described in the text.

3.1. Recovery

The absolute recovery of cefadroxil, the internal standard, at 10.5 μg/ml was determined by comparing the peak height of the extracted samples with the average peak height of unextracted cefadroxil samples ($n = 6$) and was found to be 91.0 ± 6.8% (82.1%–99.9%, $n = 11$). The absolute recovery of amoxicillin was de-

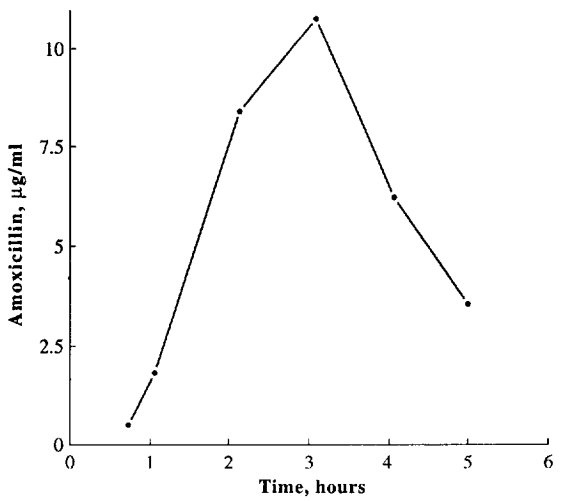

Fig. 2. Plasma amoxicillin concentration–time profile of a human volunteer. The amoxicillin dose taken orally at time zero was 1 g.

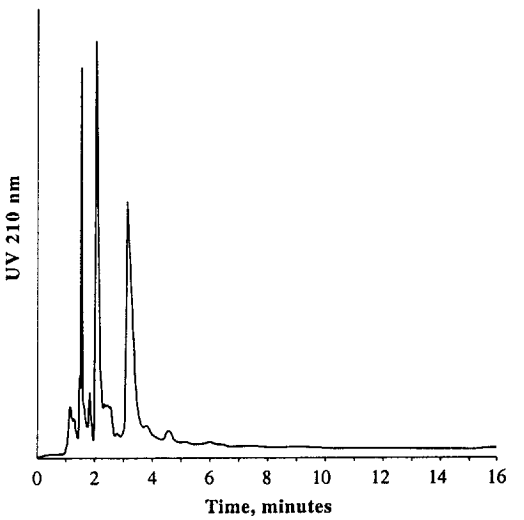

Fig. 4. Chromatogram of a blank human MEF sample after solid-phase extraction. The chromatographic conditions are described in the text.

termined likewise and was found to be 91.2 ± 16.6% (74.2–119.4%, $n = 2$ each at 0.25, 4.0, and 15.0 μg/ml).

3.2. Limit of quantitation and linearity

We defined the limit of quantitation (LOQ) as the lowest concentration at which both within-day and between-day C.V.s were less than or equal to 20% and at which the determined concentration was also within 20% of the target value. The LOQ for amoxicillin is 0.125 μg/ml of amoxicillin for 200 μl of plasma sample and 0.5 μg/ml for 50 μl of MEF sample. Sample volume difference for plasma and MEF accounts for the different LOQ. SPE is equally effective in removing interferences in both plasma and MEF. The standard curves were linear from 0.125 μg/ml to 35.0 μg/ml for plasma and from 0.5 to 10.0 μg/ml for MEF. The correlation coefficients were all greater than 0.999.

Table 1
Within-day precision of amoxicillin determination in human plasma

Concentration spiked (μg/ml)	Concentration determined (mean ± S.D., μg/ml, $n = 3$)	C.V. (%)	Accuracy (%)
Day 1			
0.125	0.126 ± 0.002	1.7	8.0
0.250	0.266 ± 0.006	2.3	6.4
0.500	0.501 ± 0.014	2.7	0.2
1.00	0.981 ± 0.093	9.4	−1.9
5.00	5.31 ± 0.10	9.3	6.2
20.0	19.5 ± 0.6	3.1	−2.7
35.0	35.0 ± 1.4	4.1	−0.1
Day 2			
0.125	0.121 ± 0.007	5.7	−2.7
0.250	0.255 ± 0.009	3.6	2.0
0.500	0.549 ± 0.049	9.0	1.0
1.00	0.945 ± 0.040	5.2	−5.5
5.00	5.16 ± 0.10	1.9	3.1
20.0	19.0 ± 0.2	1.1	−5.1
35.0	38.0 ± 2.1	5.5	8.4

Table 2
Within-day precision of amoxicillin determination in human middle ear fluid

Concentration spiked (μg/ml)	Concentration determined (mean ± S.D., μg/ml, $n = 3$)	C.V. (%)	Accuracy (%)
Day 1			
0.500	0.453 ± 0.037	8.2	−9.0
1.00	0.986 ± 0.052	5.3	−1.4
2.00	2.02 ± 0.05	2.4	1.0
5.00	5.07 ± 0.33	6.6	0.1
10.0	10.2 ± 0.1	0.7	1.2
Day 2			
0.500	0.450 ± 0.015	3.4	−10.0
1.00	1.02 ± 0.03	3.2	1.5
2.00	2.21 ± 0.07	3.1	10.4
5.00	5.20 ± 0.18	3.4	3.9
10.0	9.73 ± 0.42	4.4	−2.7

3.3. Precision

The within-day precisions were determined by analyzing triplicate calibration standards on two different days. The between-day precisions were determined by analyzing the data of the calibration curves at three different amoxicillin concentrations collected on several different days. The within-day and between-day coefficients of variation (C.V.) are listed in Tables 1–4. The within-day and between-day C.V.s for human plasma samples were between 1.1 and 9.4% and

Table 3
Between-day precision of amoxicillin determination in human plasma

Concentration spiked (μg/ml)	Concentration determined (mean ± S.D., μg/ml, $n = 6$)	C.V. (%)	Accuracy (%)
0.500	0.507 ± 0.039	3.6	1.4
5.00	4.94 ± 0.26	5.3	−1.2
20.0	20.1 ± 0.9	4.4	0.7

Table 4
Between-day precision of amoxicillin determination in human middle ear fluid

Concentration spiked (μg/ml)	Concentration determined (mean ± S.D., μg/ml, n = 7)	C.V. (%)	Accuracy (%)
0.500	0.505 ± 0.018	3.6	1.0
2.00	2.03 ± 0.12	5.9	1.4
10.0	10.1 ± 0.5	4.9	5.7

between 3.6 and 5.3%, respectively. For human MEF samples, the within-day and between-day C.V.s were between 0.71 and 8.2% and between 3.6 and 5.9%, respectively.

4. Discussion

The failure rate of antimicrobial treatment for acute otitis media is high (5–10%) [9]. At least 30% of patients will develop a recurrent infection from either poor middle ear drug penetration or resistant organisms [9]. We are studying reasons that explain these treatment failures with the goal of preventing recurrent infections. To do this we are conducting experimental and human studies on the effect of antibiotic penetration and antimicrobial treatment response in acute otitis media [10,11]. Studying antimicrobial penetration into and out of the middle ear requires the development of analytical techniques, such as the one described in this paper, that allow the precise measurement of antimicrobials at low concentrations in very small volumes of middle ear fluid or plasma [12–14].

We initially examined several ways of extracting amoxicillin and cefadroxil from human plasma and MEF by protein precipitation with either perchloric acid or acetonitrile, followed by washing with an organic solvent (such as chloroform or methylene chloride) to remove interfering substances [1]. We found that none of these methods yielded the clean extracts necessary to achieve the sensitivity required in our research. The application of solid-phase extraction to the analysis of β-lactam antibiotics in human body fluids is rare. Many β-lactam antibiotics contain both amine and carboxylic acid groups, and are charged at any pH except at the isoelectric point. This apparently would make it difficult for them to be extracted efficiently by nonpolar C_{18} cartridges.

The present application of a solid-phase extraction for antibiotics from both the penicillin and cephalosporin classes demonstrates the potential usefulness of this approach in the extraction of other amphoteric β-lactam antibiotics from human body fluids by solid-phase cartridges. The present assay improved the sensitivity significantly (20-fold), compared with a previous report using a C_{18} cartridge extraction, where 0.5 μg/ml for 1.0 ml sample size was the limit of quantitation [4]. This is one of the most sensitive methods (limit of quantitation 0.125 μg/ml for 200 μl sample size) that does not require any form of analyte derivatization. The most important factor that makes this assay successful is the clean background and high recovery yielded by SPE. Another contributing factor to the high degree of sensitivity is the use of 210 nm UV light to monitor these antibiotics. This yields a 40% higher absorbance compared with that at 230 nm and even higher absorbance compared with that at 254 nm [1].

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