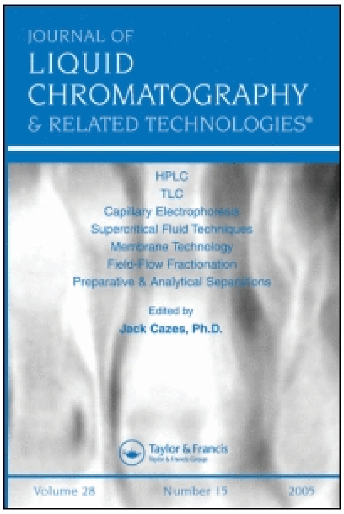

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF AMOXICILLIN IN MICROLITER VOLUMES OF CHINCHILLA MIDDLE EAR EFFUSION AND PLASMA

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ABSTRACT

A reversed-phase high-performance liquid chromatographic procedure was developed to analyze 75 μl volumes of chinchilla middle ear effusion and plasma for amoxicillin. The small sample volumes were dictated by the chinchilla model we use to study otitis media and our need to collect multiple samples over an 8-h dosing interval. Amoxicillin was separated on an octylsilane column using methanol-10 mM sodium dihydrogen phosphate-acetonitrile, (88:10:2, v/v), pH 3. Amoxicillin and the internal standard were detected at 230 nm. Middle ear effusion and plasma samples were precipitated with perchloric acid and neutralized prior to injecting 6 μl onto the column. The limit of quantitation in plasma and middle ear effusion was 0.5 μg/ml (coefficient of variation 14.8% and 18.2%, respectively), and 99% of amoxicillin was recovered.

INTRODUCTION

In an effort to explain the failure of antibiotic therapy in treating acute otitis media in children, we have been studying the penetration of antibiotics into the middle ear of chinchillas with experimentally induced otitis media (1-3). The chinchilla has provided a useful model for studying acute otitis media since middle ear effusion (MEE) can be easily induced for sampling.

We felt that developing an analytic method to measure small concentrations of amoxicillin (AMX) in very small volumes of experimentally induced otitis media, would allow the study of AMX penetration into the middle ear. These studies could then be used to explain otits media treatment failures.

AMX is a hydrophilic β-lactam antibiotic which possesses antibacterial activity against several gram-positive and gram-negative bacteria. High-performance liquid chromatographic (HPLC) analysis of AMX in plasma and urine has been reported using several different modes of detection and sample preparation. There are several good reviews which discuss AMX analyses in plasma and urine (4). Foulstone and Reading (5) described an HPLC method to analyzed AMX and clavulanic acid in plasma and urine using ultraviolet (UV) detection. The procedure required ultrafiltration of the samples, a special blanking procedure to eliminate interfering substances, and did not utilize an internal standard (IS). A post-column derivatization procedure using imidazole and mercuric chloride was reported by Carlqvist and Westerlund (6) which showed sensitivity of 25 ng/ml in plasma and 200 ng/ml in urine, but the column front had to be repacked every 15 injections due to column deterioration and the assay did not utilize an IS. Other methods include post-column fluorescamine derivatization (7), amperometry (8), and bonded-phase extraction with UV detection (9). Sample size requirements generally range from 0.5-1 ml and sample injection volumes up to 200 µl.

We report herein a sensitive reversed-phase HPLC method to quantify AMX in 75 µl volumes of plasma and MEE obtained from chinchillas with experimental otitis media.

MATERIALS AND METHODS

Instrumentation

The chromatographic analysis was performed with a Hewlett Packard 1090 L liquid chromatograph (Hewlett-Packard, Palo Also, CA) with a Beckman model 163 variable wavelength ultraviolet detector (Beckman Instruments, San Ramon, CA). The column was an octylsilane, 150 x 4.6 mm packed with 5 µm spherical material (Zorbax C8, DuPont, Biomedical Products, Wilmington, DE). A 20 x 3.2 mm C8 precolumn (Brownlee Labs, Inc., Santa Clara, CA) was also used. The effluent was monitored at 230 nm for the detection of AMX and the internal standard. The column compartment was maintained at 40°C.

Reagents

The perchloric acid (70-72%), potassium chloride, and disodium hydrogen phosphate were reagent grade and were obtained from Mallinckrodt Inc. The acetonitrile, water, and methanol were HPLC grade and were obtained from Fischer Scientific (Fair Lawn, NJ). Sodium amoxicillin was provided by Bencard (Brentford, Middlesex) and hydroflumethiazide was obtained from Sigma (St. Louis, MO).

Drug Solutions

A 1 mg/ml stock solution of sodium AMX was prepared in water. Two working dilutions, 100 µg/ml and 10 µg/ml, were prepared in water from the stock. A 50 μg/ml solution of hydroflumethiazide, the internal standard (IS), was prepared by first dissolving the drug in a few drops of methanol then diluting to volume with water. All solutions were prepared fresh each day.

Mobile Phase

The mobile phase for analyzing MEE and plasma consisted of 10 mM sodium dihydrogen phosphate, pH 3, methanol and acetonitrile (88:10:2, v/v) and was pumped through the column at 1.4 ml/min.

Standard Curves

Calibration curves (n = 3 for each concentration point) were prepared on three separate days in blank plasma and MEE for computing accuracy, precision, and linearity. All standard curves were constructed by adding AMX to the blank medium to achieve concentration ranges of 0.5-10 μg/ml for MEE, and 0.5-20 μg/ml for plasma.

Quantification

Calibration curves were plotted using measured peak-height ratios of drug to IS versus known concentrations of AMX. The concentrations in unknown samples were subsequently determined from the calibration curves.

Sample Preparation

Plasma and MEE samples were obtained according to previously published methods (3, 10, 11). The plasma samples were collected at 0.5, 1, 2, 3, and 5 hours, while MEE samples were collected at 2, 4, 6, and 8 hours after the administration of AMX.

The samples were prepared by placing 75 μl into a clean 10 mm x 75 mm glass tube containing 50 μl of IS. After mixing, 25 μl of

10% perchloric acid was added, the sample was vortexed and 25 μl of KCL was added to precipitate the unreacted perchlorate. After mixing and centrifugation, the supernatant was transferred to a clean tube. Twenty-five microliters of 800 mM disodium hydrogen phosphate, pH 10.4, was added to neutralize the liquid. A 6 μl volume was injected onto the column.

Recovery

Samples containing known concentrations of AMX (1, 5, and 10 μg/ml) in plasma and MEE were compared with corresponding concentrations of drug and IS added to water to simulate 100% recovery. The two standard curves were analyzed together. The recovery was determined by calculating the drug concentrations found in plasma and MEE using the regression equation obtained from the 100% recovery curve, dividing the plasma or MEE concentration by the recovery curve concentration and recording the difference as a percentage.

Statistics

Linearity was calculated by least squares linear regression analysis and reported as R^2. The intercept and slope determinations included standard deviation estimates. All standard deviation measurements used in calculating between-run and within-run precision were sample standard deviations (n-1). The coefficient of variation (CV) was determined by dividing sample standard deviation by the sample mean and expressing the quotient as a percentage.

TABLE 1.
Linearity and Within-day Precision of AMX in Plasma and MEE

Matrix	Theoretical conc (μg/ml)	Observed conc (μg/ml)*	CV (%)
Plasma	0.5	0.5 ± 0.05	10.0
	1.0	0.9 ± 0.12	12.5
	2.5	2.5 ± 0.13	5.3
	5.0	4.9 ± 0.32	6.4
	10.0	10.2 ± 0.05	0.5
	15.0	15.2 ± 0.25	1.7
	20.0	19.8 ± 0.18	0.9
	R^2 = 0.9992 Intercept = 0.0113 Slope = 0.0558		
MEE	0.5	0.6 ± 0.01	2.5
	1.0	1.0 ± 0.03	3.1
	2.5	2.2 ± 0.07	3.2
	5.0	5.2 ± 0.20	3.9
	10.0	9.9 ± 0.10	1.0
	R^2 = 0.9978 Intercept = -0.0034 Slope = 0.0353		

n = 3 replicates at each concentration
* mean ± standard deviation

RESULTS AND DISCUSSION

Table 1 describes the linearity and within-day precision for AMX in plasma and MEE. The sample precipitation technique used in this study could quantify 0.5 μg/ml with a CV <20%. The limit of detection, however, was 0.25 μg/ml, which gave a CV of approximately 30% in both matrices.

The inter-day precision of AMX in plasma and MEE is shown in Table 2. The precision of drug measurements in plasma and MEE did

TABLE 2.
Inter-day Precision of AMX in Plasma and MEE

Matrix	Theoretical conc (μg/ml)	Observed conc (μg/ml)*	CV (%)
Plasma	0.5	0.4 ± 0.06	14.8
	5.0	5.1 ± 0.26	5.1
	20.0	19.9 ± 0.35	1.8
MEE	0.5	0.6 ± 0.10	18.2
	5.0	5.2 ± 0.13	2.4
	10.0	9.9 ± 0.09	0.9

n = 9 replicates at each concentration
* mean ± standard deviation

TABLE 3.
Recovery of AMX in Plasma and MEE

Matrix	Conc (μg/ml)	Recovery curve conc (μg/ml)	Amount recovered conc (μg/ml)*	Recovery (%)
Plasma	1.0	0.9 ± 0.14	1.0 ± 0.12	104
	5.0	5.0 ± 0.11	4.9 ± 0.02	97
	10.0	10.1 ± 0.06	9.8 ± 0.01	98
			Mean recovery:	99 %
MEE	1.0	0.9 ± 0.13	0.9 ± 0.01	100
	5.0	5.1 ± 0.10	5.2 ± 0.11	104
	10.0	10.1 ± 0.06	9.9 ± 0.03	98
			Mean recovery:	100 %

not differ greatly, and maintained good reproducibility through the concentration ranges studied.

Data describing the recovery of AMX in plasma and MEE are shown in Table 3. The mean recovery of AMX in plasma and MEE was 99% and 100%, respectively. Examination of the lower and higher concentrations showed no appreciable differences in recovery.

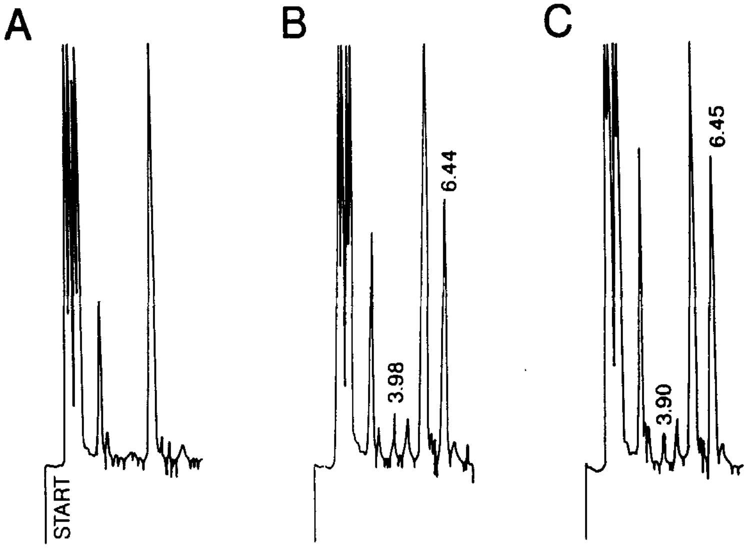

Figure 1. Chromatograms of chinchilla middle ear effusion. (A) Blank MEE; (B) 5 μg AMX per ml MEE standard; (C) chinchilla sample; retention times: AMX = 3.9 min; IS = 6.4 min.

<u>Chinchilla MEE and Plasma</u>.

Chromatograms showing the resolution of AMX and IS in MEE and plasma are shown in Figures 1 and 2, respectively. AMX eluted at 3.98 min while the IS eluted at 6.45 min. Blank MEE (Fig. 1A) and plasma (Fig. 2A) showed no interfering peaks.

Chinchilla MEE and plasma samples were analyzed over an 8-hr period for AMX after administration of 50 mg per kg intramuscularly (Fig. 3). In plasma, AMX reached a concentration of 71 μg/ml at 0.5 hrs with loss of detection by 6 hrs. The concentration of AMX in MEE was 23 μg/ml at 2 hrs and declined steadily to 2 μg/ml by 8 hrs.

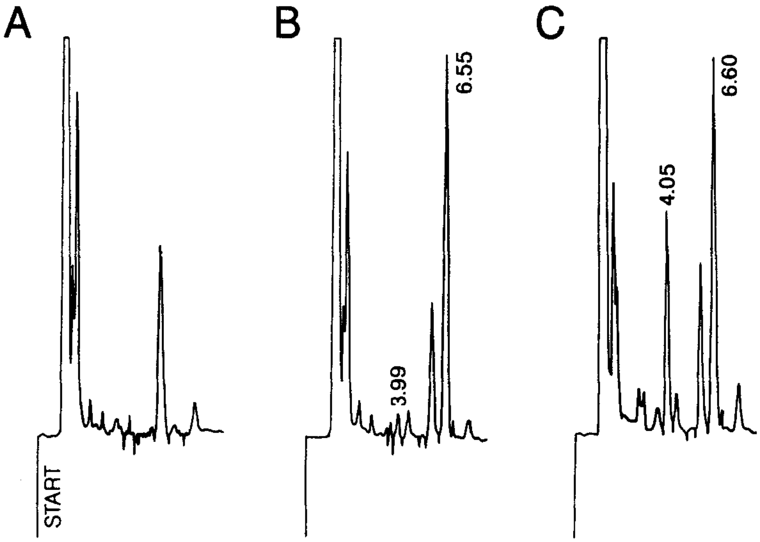

Figure 2. Chromatograms of chinchilla plasma. (A) Blank plasma; (B) 10 μg AMX per ml plasma; (C) chinchilla sample; retention times: AMX = 4.0 min; IS = 6.6 min.

Acute otitis media is a common cause of morbidity in children and can lead to the loss of hearing and delays in the development of speech. Since bacteria are one of the etiologic factors that cause otitis media, AMX is frequently given to treat this disease. Unfortunately, 20-30% of children fail to respond to this first line drug. It is our hypothesis that subtherapeutic middle ear AMX concentrations contribute to this failure.

We developed an HPLC method, described in this paper, that is sensitive at low concentrations and can be performed rapidly, and requires only a small (75 μl) sample volume for AMX quantitation.

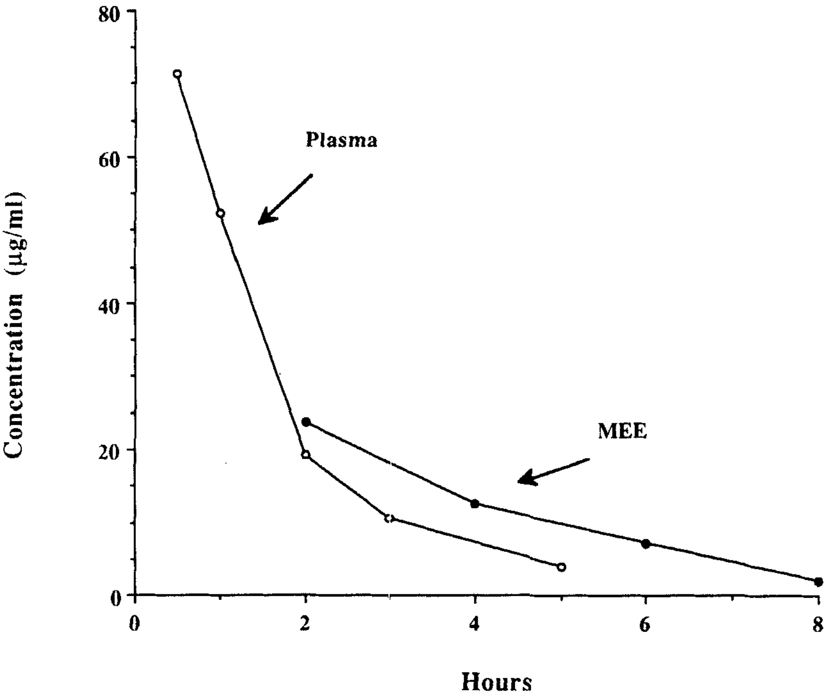

Figure 3. Concentration-time profile of AMX levels in chinchilla middle ear effusion and plasma.

This analytic procedure was found to be linear and precise over a concentration range which covers AMX concentrations commonly achieved during routine therapy (12).

The hydrophilic nature of AMX precluded the use of organic extraction, therefore, precipitation with perchloric acid allowed for good recovery in a small sample volume. The β-lactam moiety could not tolerate extended exposure to acidic and oxidative environment produced by perchloric acid, therefore, neutralizing the sample prior to HPLC injection was essential to enhanced the stability of the AMX. Although neutralization helped when a large number of samples were being analyzed, re-injection of the samples the next day was not reliable after this treatment.

Treatment of compartmentalized infections such as otitis media present a therapeutic problem since most antibiotic doses are chosen empirically on the basis of plasma concentration studies. The difficulty seen in successfully treating these patients may be due to poor antibiotic penetration into the infected area. Studying the kinetics of antibiotic penetration into the middle ear is paramount in determining why treatment failure exists. Developing the methodology to study AMX in small sample volumes allows for better dosage determinations based on the compartmental concentration, and not only the plasma concentration. In the further pursuit of improving otitis media treatment, we are currently using the HPLC and other methodologies to quantify middle ear concentrations of several antibiotics and determine their relationship to plasma levels and treatment outcome.

CONCLUSION

An HPLC method for the analysis of AMX in microliter volumes of chinchilla plasma and MEE has been described. The procedure is sensitive and rapid, requiring only a 75 μl sample size. The procedure involves protein precipitation with perchloric acid followed by neutralization of the sample and detection at 230 nm. The method has been used to quantify AMX in plasma and MEE and is currently being used to study the penetration characteristics of AMX within the middle ear compartment.

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