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High-performance liquid chromatographic analysis of amoxicillin in human and chinchilla plasma, middle ear fluid and whole blood

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Abstract

We extended the application of a sensitive high-performance liquid chromatography assay of amoxicillin developed in this laboratory for human plasma and middle ear fluid (MEF) to other sample matrices including chinchilla plasma or MEF and human and chinchilla whole blood with minor modification and validated the limit of quantitation at 0.25 μg/ml with a 50-μl sample size for human and chinchilla plasmas or MEFs. Amoxicillin and cefadroxil, the internal standard, were extracted from 50 μl of the samples with Bond Elut C_{18} cartridges. The extract was analyzed on a 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 within-day coefficients of variation were 2.7–9.9 (n=4) and 1.7–7.2% (n=3) for chinchilla plasma and MEF samples, respectively; 2.8–8.1% (n=3) and 2.9–4.7% (n=3) for human and chinchilla whole blood, respectively. An alternative mobile phase composition for chinchilla plasma and MEF samples reduced the analysis time significantly.

Keywords: Amoxicillin

1. Introduction

Amoxicillin, a semi-synthetic β-lactam antibiotic that inhibits bacterial cell wall synthesis, is often used as the first-line drug for treating acute otitis media (AOM) because of its activity against the most common AOM pathogens such as *S. pneumoniae* and *H. influenzae* and its low cost. However, the failure rate of the initial treatment is high (5–10%) and is under active investigation [1,2].

To understand the penetration and pharmacokinetic behavior of amoxicillin in human middle ear fluid (MEF) and the correlation of plasma and MEF concentrations as well as to compare these human data with a corresponding chinchilla model for similarities or differences in the pharmacokinetics, a sensitive and reliable quantitation method for amoxicillin is needed for both human and chinchilla samples because sample volumes obtained for analysis were very limited. We developed and validated a sensitive and specific assay of amoxicillin in human plasma and MEF [3]. We have now extended its application to chinchilla plasma and MEF. Very often, the human and chinchilla MEFs were contaminated with blood. Therefore, it is necessary to evaluate the potential interferences of

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blood contamination on sample preparation and analysis. We approached this problem by evaluating the method for human and chinchilla whole blood samples and showed that the method is also applicable to human and chinchilla whole blood, on addition of a filtration step after extraction and reconstitution. We validated a limit of quantitation of 0.25 μg/ml for a 50-μl sample in plasma and MEF samples. In addition, an alternative mobile phase for chinchilla plasma and MEF significantly reduced the analysis time.

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 (SPE) cartridges used were Bond Elut C_{18} cartridges (Varian sample preparation products, 500 mg/2.8 ml). All other chemicals were of analytical-reagent grade unless indicated otherwise.

All plasma, MEF and whole blood 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 Products) 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) column. 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 flow-rate was 0.35 ml/min. Two mobile phases were compared. Mobile phase A was 6% acetonitrile in 5 m*M* phosphate and 5 m*M* tetrabutylammonium hydroxide (TBA), adjusted to pH 6.5 after mixing with acetonitrile, and mobile phase B was 6% acetonitrile in 30 m*M* phosphate, pH 2.8, and 20 m*M* triethylamine. After the elution of the antibiotics, the mobile phase was changed to 25% acetonitrile in the same buffer for 2 min, to elute strongly absorbed materials, and then the column was re-equilibrated for 4 min before injecting the next sample.

Chromatographic data were collected and analyzed using an IBM PC AMD 386SX25 computer with Chrom-Perfect software (Justice Innovations, Palo Alto, CA, USA).

2.3. Stock solutions and standards

Stock solutions of amoxicillin (1.0 mg/ml) and a working standard solution of cefadroxil (50.0 μg/ml) in 5% (v/v) methanol in water were prepared daily. Stock solutions of amoxicillin were diluted to make working standard solutions of 1.0, 5.0, 10.0 and 50.0 μg/ml. A stock solution of the extraction buffer, 0.05 *M* phosphate, pH 6.8, made by weighing and dissolving an appropriate amount of Na_2HPO_4 in HPLC-grade water, was stable for at least one month. The calibration standards were made by spiking the corresponding blank matrices with appropriate amounts of working standards. The quality control (QC) samples were prepared in a similar way.

2.4. Sample preparation

The internal standard (35 μl) and the extraction buffer (1.0 ml) were added to 50 μl of samples (plasma, MEF or whole blood) and the standards. The C_{18} cartridge was conditioned with 4.0 ml of methanol and 1.0 ml of the extraction buffer. The sample was applied to the cartridge and passed slowly under 5 kPa vacuum. The column was washed with 1.0 ml of the extraction buffer and then with 1.0 ml of water. A 50-kPa vacuum was then applied to dry the column completely. The antibiotics were eluted with 1.0 ml of a 40:60 (v/v) mixture of methanol–water under a 5-kPa vacuum. Low vacuum (5 kPa) during sample application or elution and complete drying of the sorbent at high vacuum (50 kPa) are important for high recoveries of the antibiotics. The extract was dried at 40°C under nitrogen and reconstituted in 35 μl of 5% methanol in water. For whole blood samples, the reconstituted extract (40 μl) was filtered using microcentrifuge

filters (MicroSeparation). The volume of the extract after filtration varied. A 25-μl volume of the extract was injected onto the chromatograph. No interfering peaks for amoxicillin and cefadroxil were found for plasma, MEF and whole blood from six different samples.

2.5. Calculations

The peak-height ratio of amoxicillin to cefadroxil was used for quantitation. A standard curve of amoxicillin at 0.25, 0.5, 1.0, 2.0, 5.0 and 20.0 μg/ml and QC samples at 0.75, 4.0 and 15.0 μg/ml ($n=2$) were run together with unknowns. The standard curve was analyzed by weighted linear regression (weighting factor: $1/X^2$). The run was accepted when the results of at least four out of the six QC samples were within ±15% of the target value and the two failed QC samples were not at the same concentration.

3. Results

Fig. 1 shows chromatograms of human whole blood (200 μl) after SPE using mobile phase A. (a) Blank whole blood; (b) with the internal standard, cefadroxil. $t_R=5.78$ min. Amoxicillin should appear around 10.0 min. No interferences were found with samples from six different sources.

Fig. 2 shows the chromatograms of human and chinchilla whole blood spiked with amoxicillin. (a) Human whole blood (200 μl). t_R values: amoxicillin (5.0 μg/ml): 9.68 min; cefadroxil: 5.27 min. (b) chinchilla whole blood (50 μl). t_R values: amoxicillin (20.0 μg/ml): 9.81 min; cefadroxil: 5.33 min.

Fig. 3 shows the chromatogram of a blank chinchilla plasma sample after SPE, obtained with mobile phase B and a 250×2 mm, 5 μm Keystone Hypersil MOS-1(C_8) column. The retention times of amoxicillin and cefadroxil were 3.9 min and 4.5 min, respectively. There were two large background peaks but no evidence of interfering substances.

3.1. Recovery

The absolute recoveries of amoxicillin in chinchilla plasma at 0.25, 2.0 and 10.0 μg/ml were 110.4,

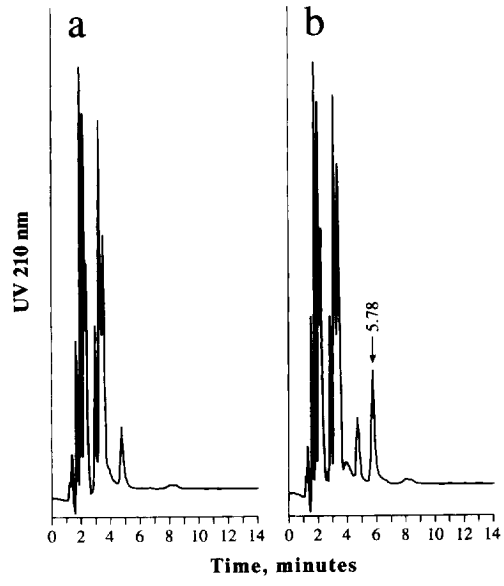

Fig. 1. The chromatograms of human whole blood (200 μl sample size) after solid phase extraction using mobile phase A. (a) Blank whole blood; (b) with the internal standard, cefadroxil. $t_R=5.78$ min. Amoxicillin should appear at around 10.0 min.

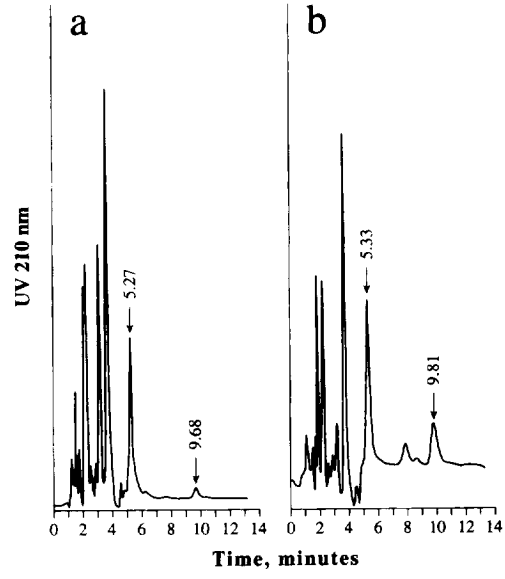

Fig. 2. Chromatograms of human and chinchilla whole blood samples (50 μl) spiked with amoxicillin. (a) Human whole blood. t_R: Amoxicillin (5.0 μg/ml): 9.68 min; cefadroxil: 5.27 min. (b) Chinchilla whole blood. t_R: Amoxicillin (20.0 μg/ml): 9.81 min; cefadroxil: 5.33 min.

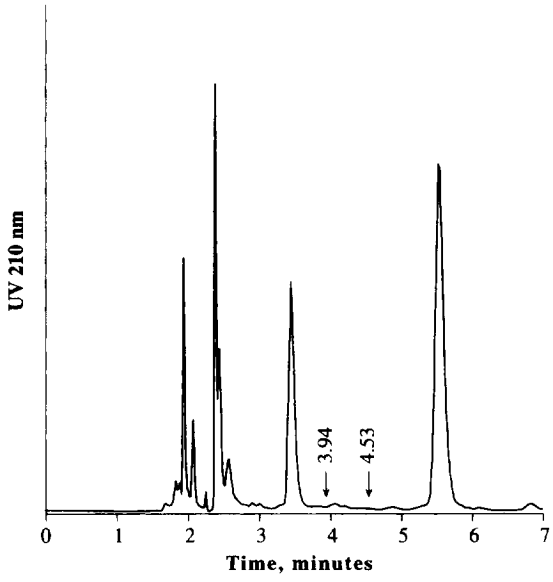

Fig. 3. Chromatogram of a blank chinchilla plasma after solid phase extraction with mobile phase B. Amoxicillin and cefadroxil appear at 3.9 and 4.5 min, respectively.

83.3 and 93.2% (n=3), respectively. The absolute recoveries of amoxicillin in chinchilla MEF at 0.25, 2.0 and 10.0 μg/ml were 89.7, 92.9 and 86.7% (n=4), respectively. Therefore, there was no significant difference in the absolute recoveries between human and chinchilla samples and between plasma and MEF matrices, except at the level of 0.25 μl/ml [3]. The absolute recovery of cefadroxil, the internal standard, for chinchilla plasma and MEF, was not determined, but it was estimated to be comparable with that for human plasma, i.e., ca. 90% [3]. A quantitative determination of recovery for whole blood specimens was not performed due to varying injection volumes after filtration. The recoveries appeared to be slightly less than those for plasma and MEF samples (see Table 2, accuracy data).

3.2. Precision

The within-day precision was determined by analyzing triplicate calibration standards. The within-day coefficients of variation (C.V.) are listed in Table 1 Table 2. For chinchilla plasma and MEF samples, the within-day C.V.s were between 2.7–9.9 (n=4) and 1.7–7.2% (n=3), respectively. For human and chinchilla whole blood samples, the within-day C.V.s were between 2.8–8.1% (n=3) and 2.9–4.7% (n=3), respectively. Additional precision data for human MEF is listed in Table 3. The within-day C.V.s for human MEF at 0.25 and 0.125 μg/ml for a 50-μl sample were 13 and 21.4% (n=5), respectively. The amoxicillin concentrations in whole blood specimens

Table 1
Within-day precision of amoxicillin determination in chinchilla plasma and MEF (50-μl sample)

Concentration spiked (μg/ml)	Concentration determined (mean±S.D., n=3) (μg/ml)	C.V. (%)	Accuracy (%)
Plasma[a]			
0.250	0.302±0.030	9.9	20.8
2.00	2.00±0.054	2.7	0.0
10.0	9.64±0.274	2.8	3.6
MEF			
0.250	0.236±0.017	7.2	−5.6
2.00	2.07±0.035	1.7	3.5
10.0	9.88±0.196	2.0	−1.2

[a] n=4.

Table 2
Within-day precision of amoxicillin determination in human and chinchilla whole blood (50 μl)

Concentration spiked (μg/ml)	Concentration determined (mean±S.D., n=3) (μg/ml)	C.V. (%)	Accuracy (%)
Human			
1.00	0.948±0.077	8.1	−5.2
5.00	4.82±0.135	2.8	−3.6
20.0	18.74±0.626	3.3	−6.3
Chinchilla			
1.00	0.923±0.044	4.7	−7.7
5.00	4.64±0.133	2.9	−7.2
20.0	18.19±0.745	4.1	−9.1

Table 3
Additional within-day precision of amoxicillin determination in human middle ear fluid (50-μl sample size)

Concentration spiked (μg/ml)	Concentration determined (mean±S.D., n=5) (μg/ml)	C.V. (%)	Accuracy (%)
0.125	0.157±0.034	21.4	25.6
0.250	0.297±0.039	13.0	18.8

were calculated using standard curves based on human plasma and were apparently not significantly different ($<\pm 15\%$) from the corresponding values in the plasma matrix. The between-day precision data were not obtained due to operational limitations but they were expected to be close to previously published data (<6%) [3].

3.3. 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 0.25 μg/ml for 50 μl of human and chinchilla plasma or MEF. SPE is effective in removing interferences in plasma, MEF and even in whole blood. The standard curves were linear from 0.25 to 20.0 μg/ml with a 50-μl sample. The correlation coefficients were all greater than 0.99. The LOQ for whole blood specimens is at least 1.0 μg/ml, based on the precision data, although lower concentrations were not evaluated because whole blood is rarely used directly for antibiotic assays.

4. Discussion

The applicability of the amoxicillin assay for human plasma and MEF based on solid phase extraction [3] is demonstrated for different sample matrices, i.e., chinchilla plasma or MEF and human or chinchilla whole blood. The fact that whole blood does not present interferences for amoxicillin and cefadroxil suggests that contaminations of plasma or MEF samples by a small amount of blood do not interfere with the assay.

In order to reduce the run time, mobile phase A and mobile phase B were compared. With mobile phase A, the retention times for cefadroxil and amoxicillin (5.27 and 11.0 min, respectively) and the total run time (20 min) were longer and the background appeared cleaner. All recovery and precision data reported in this article were collected using mobile phase A. With mobile phase B, the retention times of amoxicillin and cefadroxil (3.9 and 4.5 min, respectively) and the total run time (12 min) were significantly shorter. However, mobile phase B cannot be used for human samples due to interfering peaks.

We also observed that the standard curve based on one sample matrix can apparently be used to calculate the amoxicillin concentration in a different matrix, e.g., human plasma based standard curve for calculating the amoxicillin concentration in chinchilla plasma, MEF and whole blood. We thus confirmed our earlier observation that a human plasma-based standard curve can be used to calculate amoxicillin in human MEF (data not shown) [3] and this was also true for chinchilla samples. This suggests that the analyte behaves very similarly with respect to extraction in the different matrices studied by us to date. Furthermore, the C_{18} cartridges used for chinchilla plasma or MEF samples can be reused at least once without there being a noticeable decrease in performance.

This is the most sensitive method (limit of quantitation: 0.25 μg/ml for a 50-μl plasma and MEF sample) compared with previously reported methods [4–8] and does not require any form of analyte derivatization. Other antibiotics, such as ceftriaxone and cefotaxime, can be extracted in a similar way [9]. It is expected that the current method, with or without modifications, will be applicable to other biological matrices as well.

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