Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 648–652
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

A sensitive assay of amoxicillin in mouse serum and broncho-alveolar lavage fluid by liquid–liquid extraction and reversed-phase HPLC

Xiaoli Du, Chonghua Li, Heather K. Sun, Charles H. Nightingale, David P. Nicolau *

Center for Anti-Infective Research and Development, Hartford Hospital, 80 Seymour Street, Hartford, CT 06102, USA

Received 23 December 2004; received in revised form 18 April 2005; accepted 19 April 2005
Available online 1 June 2005

Abstract

A sensitive and simple high-performance liquid chromatography (HPLC) method was developed and validated for the analysis of amoxicillin in mouse serum and broncho-alveolar lavage (BAL) fluid. One hundred microlitres of sample were needed for the assay. Sample processing was carried out with liquid–liquid extraction. Cefadroxil was used as an internal standard. The chromatographic separation was achieved on a C_{18} reversed-phase column with a mobile phase consisting of phosphate buffer, 1-octanesulphonic acid sodium salt and acetonitrile. The detection was conducted at 210 nm. The ranges of the standard curves were 0.2–20 and 0.05–5 μg/ml for serum and BAL samples, respectively. The recoveries of amoxicillin from serum and normal saline were 87 and 88%, respectively. The coefficients of variation were 1.78–6.13% for intra-day and 0.82–6.42% for inter-day analyses. The accuracy was within 100 ± 6%. This method was successfully applied to analyze amoxicillin in mouse serum and BAL samples from a pharmacokinetic study.

Keywords: Amoxicillin; HPLC

1. Introduction

Amoxicillin is a β-lactam antibiotic that is widely used clinically for respiratory system infections caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* [1–3]. Recently, the relationship between antibiotic efficacy and drug concentration at the site of infection has been shown to be highly important [4]. In order to evaluate the penetration of amoxicillin into lungs and the correlation between serum and epithelial lining fluid (ELF) concentrations, a pharmacokinetic (PK) study was carried out in mice in our laboratory.

No method for amoxicillin in mouse serum and broncho-alveolar lavage (BAL) fluid has been reported in the literature, although there are a number of high-performance liquid chromatographic (HPLC) methods reported [5–14]. Among the methods used for biological matrices other than mouse serum or BAL fluid, some have low sensitivities with quantification limits higher than 1 μg/ml [12–14], while others apply precolumn or postcolumn fluorescent derivatization, solid-phase extraction (SPE), ultrafiltration, column switching, or couple-column techniques to improve the sensitivity [5–11]. These techniques impose a high requirement for instruments or materials, and also increase the analysis cost and time. Another limitation of the methods mentioned above is that all of them need at least 180 μl of sample, making them unsuitable for our PK study since sample volume obtained from mice is limited, especially for BAL samples.

In this experiment, a reversed-phase HPLC method with liquid–liquid extraction was developed for the assay of amoxicillin in mouse serum and BAL fluid for the first time. This method achieved high sensitivity with only 100 μl of sample size. It proved to be accurate and reproducible and has already been applied to analyze hundreds of mouse serum and BAL samples.

* Corresponding author. Tel.: +1 860 545 3941; fax: +1 860 545 3992.
E-mail address: dnicola@harthosp.org (D.P. Nicolau).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.04.021

2. Experimental

2.1. Chemicals

Amoxicillin and cefadroxil (internal standard, I.S.) were purchased from Sigma Laboratories (St. Louis, MO). The ion-pair reagent, 1-octanesulphonic acid sodium salt, sodium dihydrate phosphate and phosphoric acid were AR grade (Sigma). Dichloromethane and acetonitrile were HPLC grade (Mallinckrodt Baker Inc., Phillipsburg, NJ). Deionized water was obtained from a Milli-Q Plus analytical deionization system (Millipore Corporation, Bedford, MA).

2.2. Instrumentation and chromatography

A HPLC system consisting of a Waters 717 Plus Autosampler (Milford, MA), a Waters 515 Pump and a LDC Analytical Programmable Wavelength Detector (LDC/Milton Roy, Riviera Beach, FL) was used for this experiment. The autosampler was set at 10 °C. The detection wavelength was 210 nm with 0.005 aufs for the serum samples and 0.001 aufs for the BAL samples. A Phenomenex C_{18} column (4.6 mm × 250 mm, 10 μm) was used as the analysis column with a μBondpak C_{18} Guard-pak precolumn (Waters). The column was maintained at room temperature. The chromatographic data were collected by EZCHROM ELITE chromatography system (Scientific Software, San Ramon, CA).

The mobile phase consisted of phosphate buffer (pH 3.0; 0.023 M) containing 4 mM 1-octanesulphonic acid sodium and acetonitrile (87:13, v/v). The flow rate was 1 ml/min.

2.3. Standard solutions and quality controls

The stock solution of amoxicillin (1.0 mg/ml) was prepared in deionized water. For the calibration curves in serum, pooled normal ICR mouse serum (Harlan Bioproducts for Science, Indianapolis, IN) was spiked with amoxicillin stock solution to give a final concentration of 0.2, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml. For BAL samples, due to the difficulty of obtaining a large amount of blank BAL fluid from mice, normal saline (Abbott Laboratories, North Chicago, IL) was used as the proxy matrix to make the standard solution. The final concentrations were 0.05, 0.2, 0.5, 1.0, 2.0 and 5.0 μg/ml, respectively. Aliquots of these standard solutions were stored at −80 °C until analysis.

Three quality control (QC) samples were prepared using the same procedure at concentrations of 0.5, 4.0 and 15.0 μg/ml in mouse serum and 0.1, 0.8 and 4.0 μg/ml in saline, respectively. These QC samples were used for the evaluation of recovery, precision and accuracy of this method and the stability of amoxicillin.

Another three QC samples (0.1, 0.8 and 4.0 μg/ml) were prepared in blank BAL fluid collected from ICR mice (the same species as used in the pharmacokinetic study). These samples were assayed with the standard curve, and their concentrations were calculated using the same method as the saline QC samples. The precision and accuracy of the BAL QC samples were used to confirm the suitability of using saline as the proxy matrix for BAL.

The stock solution of the internal standard, cefadroxil, was prepared in deionized water at a concentration of 1.0 mg/ml. Aliquots of this solution were stored at −80 °C until use. The stock solution was stable for 1 month. The working I.S. solution (20 μg/ml) was freshly prepared by diluting the stock solution with phosphate buffer (pH 3.0; 0.2 M).

When determining unknown samples, the QC samples were randomly distributed among the unknown samples to be extracted and injected. BAL QC samples were also processed with each run when analyzing BAL samples. The accuracies of all of the QC samples were examined to confirm the validity of the assay.

2.4. Extraction procedure [15]

Thirty microlitres of I.S. solution was pipetted into a tube containing 100 μl of standard solution or unknown sample. Then, 500 μl of acetonitrile was added to precipitate the proteins in the sample. The mixture was shaken vigorously for 5 min and centrifuged at 2250 × *g* for 5 min (IEC Centra-HN 2485, Needham Height, MA). The supernatant was transferred to a clean tube, and 2 ml of dichloromethane was added. After shaking slowly for 5 min and centrifuging at 2250 × *g* for 5 min, about 100 μl of the top layer was taken out and 20 μl was injected.

2.5. Assay validation

The peak height ratio (PHR) of amoxicillin to cefadroxil was used for calculation. Calibration curves with six concentration points were constructed by plotting PHR versus spiked concentrations. Weighted least square linear regression (weighting factor: 1/concentration) was used because the calibration curves spanned a range of 100. The calibration curves were used to calculate the amoxicillin concentration of the QC samples and unknown samples.

The recoveries of amoxicillin and the I.S. from mouse serum and saline were determined by comparing the peak heights of amoxicillin and I.S. of the extracted QC samples with those of aqueous solution at the same concentrations.

The precision of the method was determined as the intra-day and inter-day variabilities of the low, medium and high concentration QC samples. The accuracy was evaluated by the relative bias of the calculated concentrations of the QC samples compared with their theoretical values.

To investigate the stability of amoxicillin in the matrices at room temperature, three aliquots of each QC sample were analyzed after being thawed at room temperature and kept at room temperature for 6 h. Freeze–thaw stability was determined after three freeze–thaw cycles. Each cycle consisted of freezing at −80 °C for 24 h and then thawing completely at room temperature. The stability of extracted samples at

10 °C in the autosampler was evaluated up to 24 h after extraction.

Five aliquots of standard solution with the lowest concentrations of the calibration curves were analyzed for the evaluation of the accuracy and precision of the lower limit of quantification (LLOQ). The acceptance criteria for the LLOQ were that the precision, determined by the relative standard deviation (R.S.D.), was under 20%, and the accuracy was within 100 ± 20%.

2.6. Pharmacokinetic study

Immunocompromised ICR mice (25 g, Harlan Sprague Dawley Inc., Indianapolis, IN) were administered a single oral dose of amoxicillin at 12.5, 25 and 100 mg/kg. 0.5 ml blood samples were collected at 8–10 time points for each dose from 0.25 h up to 4 h following drug administration. Six to twelve mice were used for each time point. The blood was centrifuged and the serum was stored at −80 °C until analyzed. At 0.5 and 1.5 h, the mice were simultaneously lavaged four times with 0.4 ml of saline to obtain BAL fluid to determine the amoxicillin concentrations in ELF. The BAL samples were stored at −80 °C until analyzed.

3. Results

3.1. Retention time and specificity

Fig. 1A and B shows the chromatograms of blank mouse serum and serum sample from the amoxicillin PK study. Fig. 2A and B shows the chromatograms of blank mouse BAL fluid and BAL sample from the PK study. There were no endogenous interfering peaks with amoxicillin and the I.S. in the mouse serum and BAL fluid. The retention times

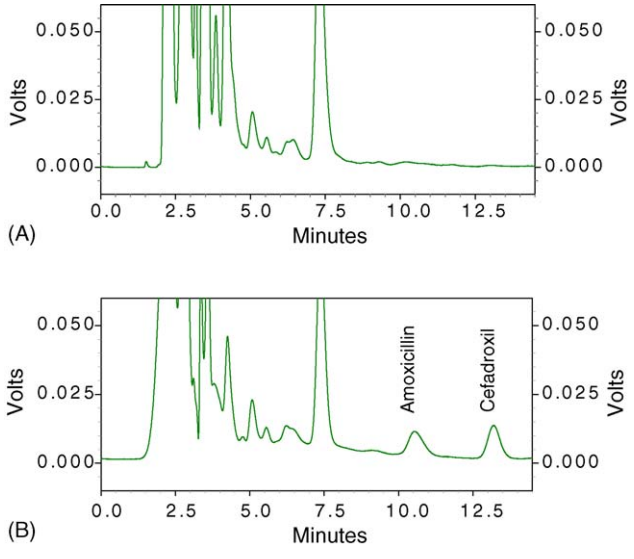

Fig. 1. Chromatograms of (A) blank mouse serum and (B) unknown mouse serum sample (4.41 μg/ml).

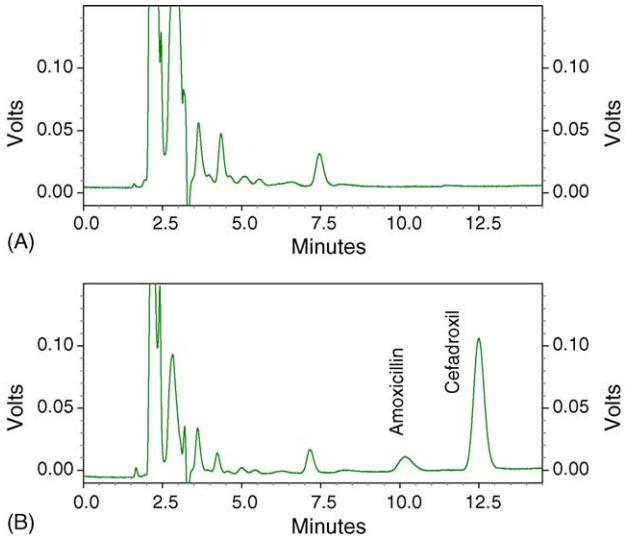

Fig. 2. Chromatograms of (A) blank BAL fluid and (B) unknown mouse BAL sample (0.94 μg/ml).

of amoxicillin and the I.S. were 10.6 and 13.2 min, respectively.

3.2. Linearity

The calibration curve of amoxicillin in mouse serum was linear in the range of 0.2–20 μg/ml. The regression equation was $y = 0.1652x + 0.0035$ ($r = 0.9996$, $n = 11$). The calibration curve of amoxicillin in saline was linear in the range of 0.05–5 μg/ml. The regression equation was $y = 0.1244x - 0.0003$ ($r = 0.9998$, $n = 9$).

3.3. Recoveries

The mean recoveries of amoxicillin from mouse serum and saline at three various concentrations were 87 ± 7% and 88 ± 3% ($n = 3$ for each concentration), respectively. The mean recoveries of the I.S. from mouse serum and saline were 81 ± 2% and 124 ± 3% ($n = 9$), respectively.

3.4. Precision and accuracy

The precision and accuracy of this method were evaluated by assaying each low, medium and high concentration QC samples. As shown in Table 1, the intra-day R.S.D. for mouse serum and saline QC samples were 1.78–3.48% and 3.06–6.13%, respectively; the inter-day R.S.D. for mouse serum and saline QC samples were 2.34–5.11% and 2.07–4.42%, respectively. All the relative biases of the QC samples were less than 6%.

The validity of using saline as the proxy matrix for the BAL calibration curve was proved by the precision and accuracy of the QC samples made in blank BAL fluid (Table 1). The intra-day and inter-day R.S.D. were 2.58–3.22% and 0.82–6.42%, respectively. The relative biases of the three

Table 1
Precision and accuracy of the assay for amoxicillin in mouse serum, saline and BAL

Spiked concentration (μg/ml)	Calculated concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)	Relative bias (%)	Calculated concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)	Relative bias (%)
Serum	Intra-day (n = 10)			Inter-day (n = 11)		
0.5	0.499 ± 0.017	3.48	−0.12	0.503 ± 0.026	5.11	0.30
4	3.865 ± 0.112	2.91	−3.38	3.871 ± 0.141	3.65	−3.07
15	14.758 ± 0.263	1.78	−1.62	14.937 ± 0.349	2.34	0.18
Saline	Intra-day (n = 10)			Inter-day (n = 9)		
0.1	0.100 ± 0.003	3.06	−0.08	0.105 ± 0.003	2.88	4.74
0.8	0.781 ± 0.048	6.13	−2.33	0.821 ± 0.036	4.42	3.65
4	3.768 ± 0.135	3.59	−5.79	3.926 ± 0.081	2.07	−1.88
BAL	Intra-day (n = 6)			Inter-day (n = 5)		
0.1	0.106 ± 0.003	3.21	5.97	0.104 ± 0.004	4.12	3.99
0.8	0.831 ± 0.027	3.22	3.88	0.819 ± 0.053	6.42	2.37
4	3.991 ± 0.103	2.58	−0.23	4.037 ± 0.033	0.82	0.94

BAL QC samples were less than 6%. These data indicated that using saline as the proxy matrix for BAL was acceptable from the HPLC perspective.

3.5. Lower limit of quantification

The LLOQ of the assay for amoxicillin in mouse serum was 0.2 μg/ml with relative bias of 3.17% and R.S.D. of 3.41%. The LLOQ of the assay for amoxicillin in saline was 0.05 μg/ml with relative bias of 1.66% and R.S.D. of 2.04%.

3.6. Stability

Amoxicillin was stable in the matrices at room temperature for 6 h without significant degradation (<10%). After three freeze–thaw cycles, the changes of amoxicillin concentrations were less than 10%. The run-time stability study showed that extracted amoxicillin was stable at 10 °C for up to 24 h (95.03–104.16% compared with 0 h).

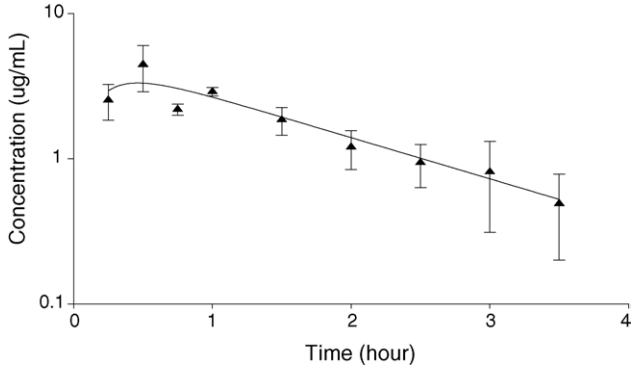

Fig. 3. Serum concentration–time profile of amoxicillin in mice following a single oral dose at 25 mg/kg (mean ± S.D., n = 6–12 for each time point).

3.7. Application

This method was successfully applied to analyze the serum and BAL samples from the PK study conducted in mice. For the purpose of demonstrating, the serum concentration–time profile of the single oral dose of amoxicillin at 25 mg/kg is shown in Fig. 3. The amoxicillin concentrations in BAL samples at 0.5 and 1.5 h following a single oral dose of 25 mg/kg were 0.145 ± 0.023 and 0.097 ± 0.032 μg/ml (n = 6), respectively.

4. Discussion

HPLC analysis for amphoteric, polar substances of low wavelength ultraviolet absorption (such as amoxicillin) contained in biological fluids is always associated with some difficulties, especially when high sensitivity is required. Thus, some employed special analysis columns [5], SPE [6] or ultrafiltration [7] to decrease or remove the endogenous interfering substances to a maximal extent. Others used fluorescent derivatization [9,10] to increase the sensitivity and reduce the interferences. The highest sensitivity reported to date (LLOQ of 0.05 μg/ml with 180 μl sample size) was achieved by coupling a restricted access medium bovine serum albumin octadecyl column to an octadecyl Hypersil column [5]. However, practical and robust analysis methods should be as simple as possible. In this study, a classic liquid–liquid extraction, direct UV detection method successfully achieved the goals of sensitivity, specificity and accuracy.

This assay was sensitive (LLOQ of 0.2 and 0.05 μg/ml for 100 μl of serum and BAL sample, respectively) and did not require any forms of analyte derivatization or special columns or instruments. The achievement of high sensitivity of this method can be attributed to several factors. The first and the most important factor was the clean background

on the chromatogram. In the acidic mobile phase (pH 4), the ion-pair reagent, 1-octanesulphonic acid sodium salt, showed strong retention effect for amoxicillin and cefadroxil [16]. This effect, combined with carefully adjusted ratio of phosphate buffer to acetonitrile, resulted in a clean baseline period for the peaks of interest. Thanks to this period of baseline, a high signal/noise ratio was achieved, which constituted the base for the high sensitivity. The second factor was the use of 210 nm as the monitoring wavelength. This yielded a 56% higher absorbance compared with that at 230 nm. The third and final factor was the high recovery of amoxicillin from the matrices during the extraction process. The use of dichloromethane after acetonitrile deproteination not only removed some organic substances from the sample, but also absorbed the acetonitrile, thereby leaving only the aqueous mixture for assay. Compared with direct assay after deproteination, this procedure efficiently concentrated the sample thus yielding the high recovery of amoxicillin (87 and 88% for serum and saline, respectively).

During the process of method development, it was discovered that the peak shape of amoxicillin was significantly affected by the pH of the sample. When the mouse serum sample, at a pH around 8, was spiked with aqueous I.S. solution, amoxicillin showed a shoulder peak in the chromatogram. The higher the concentration, the more obvious was this phenomenon. However, extracted amoxicillin aqueous solution, at a pH around 6.5, showed a symmetric single peak. It was speculated that since amoxicillin, is amphoteric, it may either exist in two forms or cannot combine with the ion-pair reagent completely in the weakly basic environment. So phosphate buffer (pH 3.0; 0.2 M) was adopted to prepare the I.S. solution in order to adjust the pH of the sample. The resultant pH of the extracted sample was about 5. The single, symmetric peak of amoxicillin in the chromatograms indicated that pH adjusting was effective in optimizing the peak shape.

With high sensitivity, small sample size requirement and simple sample treatment procedures, this method was successfully applied to the analysis of mouse serum and BAL samples. It may also be expected to be employed for clinical pharmacokinetic studies.

5. Conclusion

A sensitive HPLC method for the assay of amoxcillin in mouse serum and BAL fluid was developed for the first time. This method used only 100 μl of sample with simple liquid–liquid extraction and direct UV detection. It proved to be specific, accurate and reproducible. The successful application of this method in the analysis of hundreds of mouse serum and BAL samples confirmed its robustness and reliability.

Acknowledgement

This work was supported in part by a grant from Advancis Pharmaceuticals, Germantown, MD.

References

[1] T.M. File Jr., M.S. Benninger, M.R. Jacobs, Clin. Lab. Med. 24 (2004) 531–551.
[2] Anon., Prescire Int. 12 (2003) 184–189.
[3] D. Felmingham, M.J. Robbins, Y. Tesfaslasie, J. Antimicrob. Chemother. 41 (1998) 411–415.
[4] I.M. Gould, G. Harvey, D. Golder, Thorax 49 (1994) 999–1001.
[5] Q.B. Cass, R.F. Gomes, S.A. Calafatti, J. Chromatogr. A 987 (2003) 235–241.
[6] Z. Yuan, H.Q. Russlie, D.M. Canafax, J. Chromatogr. B 674 (1995) 93–95.
[7] P. Muth, R. Meta, H. Beck, J. Chromatogr. A 729 (1996) 259–266.
[8] L. Tan, J.H. Zhou, N. Luo, Acta Pharma. Sin. 32 (1997) 558–560.
[9] H.J. Mascher, C. Kikuta, J. Chromatogr. A 812 (1998) 221–226.
[10] J.I. Wibawa, D. Fowkes, P.N. Shaw, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 774 (2002) 141–148.
[11] B. Charles, S. Chulavatnatol, Biomed. Chromatogr. 7 (1993) 204–207.
[12] de A.L.R. Pires, R.M. Ortiz, de S.C. Castro, J. Pharm. Pharm. Sci. 6 (2003) 223–230.
[13] A. Menelaou, A.A. Somogyi, M.L. Barclay, F. Bochner, J. Chromatogr. B. 731 (1999) 261–266.
[14] S. Chulavatnatol, B.G. Charles, J. Chromatogr. 615 (1993) 91–96.
[15] K.H. Yoon, S.Y. Lee, W. Kim, J.S. Park, H.J. Kim, J. Chromatogr B 813 (2004) 121–127.
[16] H.S. Huang, J.R. Wu, M.L. Chen, J. Chromatogr. 564 (1991) 195–203.