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Short communication

Simultaneous analysis of amoxicillin and sulbactam in human plasma by HPLC-DAD for assessment of bioequivalence☆

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ABSTRACT

A simple and accurate high-performance liquid chromatography with diode array detection-based (HPLC-DAD) method has been developed and validated for simultaneous determination of amoxicillin and sulbactam in human plasma. Sample preparation was involved in protein precipitation with acetonitrile followed by one-step extraction procedure. Chromatographic separation was achieved on a C18 column with an isocratic mobile phase consisting of water (containing 30 mM potassium dihydrogen phosphate, pH 2.8) and acetonitrile. The detection wavelengths of a diode array detector were set at 210 nm for amoxicillin and sulbactam, and 263 nm for the internal standard (cefadroxil). The method was validated for linearity, accuracy, precision, and stability. The calibration curve was linear from 0.163 to 14.7 μg/mL with correlation coefficient squared of 0.9991 for amoxicillin and 0.250–15.0 μg/mL with correlation coefficient squared of 0.9988 for sulbactam using 500 μL plasma samples. The lower limit of quantification was 0.163 and 0.250 μg/mL for amoxicillin and sulbactam, respectively. The imprecisions of intra- and inter-day validations for amoxicillin and sulbactam were <11% and their accuracies (%) were within the range of 95.4–105.7%. Mean recoveries were 75.9, 72.8, and 70.0% for amoxicillin, sulbactam, and cefadroxil, respectively. The established method was successfully applied to a bioequivalence study of two combination formulations of amoxicillin and sulbactam pivoxil in healthy male volunteers.

1. Introduction

Amoxicillin is a semi-synthetic penicillin widely used in clinical therapy as a broad-spectrum bactericidal. Like other penicillins, it is also susceptible to various betalactamases produced by many gram-positive and gram-negative microorganisms [1,2]. Sulbactam is a betalactamase inhibitor with a poor absorption in the gastrointestinal tract. In contrast, sulbactam pivoxil [3], a prodrug of sulbactam, has better absorption than its parent drug. Sulbactam pivoxil is hydrolysed by non-specific plasma esterases after absorption and produces high serum levels of sulbactam after oral administration. Amoxicillin sulbactam pivoxil, a penicillin-beta-lactamase inhibitor combination drug, is widely used to improve antibacterial therapy and counteract bacterial resistance [1,4,5].

Recently, a combination formulation containing amoxicillin (125 mg) and sulbactam pivoxil (125 mg sulbactam) has been approved to carry out the bioequivalence study by the State Food and Drug Administration of China. Therefore, a suitable method for simultaneous determination of these drugs is required. Many HPLC methods are available for the measurement of amoxicillin [6–21] or sulbactam [22–27] in biological samples, individually [6–12] or in combination with other drugs or metabolites [13–27]. In past, the plasma concentrations of amoxicillin and sulbactam are mainly measured via HPLC–UV or HPLC–mass spectrometry. Unfortunately, there is no method available for simultaneous determination of amoxicillin and sulbactam in human plasma.

Although the selectivity and sensitivity offered by LC–MS/MS should provide the possibility for the accurate simultaneous determination of amoxicillin and sulbactam in plasma, it is not always available in some laboratories. In this article, we describe a sensitive, rapid and specific HPLC-DAD method for simultaneous determination of amoxicillin and sulbactam in human plasma.

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The method has been successfully applied to a bioequivalence study.

2. Experimental

2.1. Chemicals and reagents

Amoxicillin standard (purity 85.0%) was supplied by Hunan Anbang Pharmaceutical Co. Ltd. (Changsha, China). Sulbactam standard (purity 89.2%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cefadroxil (internal standard, I.S., purity > 99.7%) was supplied by Hunan Zhonghe Pharmaceutical Co. Ltd. (Changsha, China). HPLC grade acetonitrile and dichloromethane were obtained from Tedia Co. Ltd. (Fairfield, OH, USA). Water was purified by double distillation. All other chemicals and solvents were of analytical grade.

Reference formulations (North China Pharmaceutical Group Formulation Co, Ltd., China) and test formulations (Hunan Anbang Pharmaceutical Co. Ltd., Changsha, China) containing amoxicillin (125 mg) and sulbactam pivoxil (125 mg sulbactam) per capsule were used in this study.

2.2. Instrumentation and chromatography

Shimadzu LC-10A chromatographic system consisted of a SCL-10AVP system controller, two LC-10ATVP pumps, a SIL-10ADVP auto-injector, a CTO-10AVP column oven, a SPD-M10AVP diode array detector, and a Class-VP 6.2.1 work station (Shimadzu, Japan). Chromatographic separation was achieved on an Inertsil ODS column (150 mm × 4.6 mm, 5 μm, GL Sciences, Tokyo, Japan) thermostated at 30 °C. The mobile phase was a mixture of 30 mM potassium dihydrogen phosphate buffer (pH 2.8)–acetonitrile (97.5:2.5, v/v) at a flow rate of 1.0 mL/min. The detection wavelengths were set at 210 nm for amoxicillin and sulbactam and 263 nm for I.S.

2.3. Analytical procedure

2.3.1. Preparation of stock solutions, calibration standard, and quality control samples

Standard stock solutions of amoxicillin (1.03 mg/mL), sulbactam (1.05 mg/mL), and I.S. (1.00 mg/mL) were prepared in acetonitrile and stored at 4 °C. Calibration range was selected according to the expected concentrations in real plasma samples. Calibration standard plasma samples (0.163, 0.327, 0.981, 2.45, 4.90, 9.80, and 14.7 μg/mL for amoxicillin; and 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, and 15.0 μg/mL for sulbactam) were prepared by adding known amounts of stock solutions of both drugs to pooled drug-free plasma. Quality control plasma samples (0.327, 2.45, and 12.3 μg/mL for amoxicillin, and 0.500, 2.50, and 12.5 μg/mL for sulbactam) were prepared in the same way. All plasma samples were stored at −20 °C until use.

2.3.2. Samples preparations

Frozen human plasma samples were thawed at room temperature. Then, 50 μL of I.S. solution (66.7 μg/mL) were added to 500 μL of plasma sample. The samples were vortexed and 500 μL of acetonitrile was added to precipitate the protein. After a thorough vortex mixing for 2 min, the mixture was centrifuged at 13,000 rpm for 5 min. Then 700 μL of supernatant was transferred to another tube and 700 μL of dichloromethane were added. After samples were vortexed for 2 min and then centrifuged at 13,000 rpm for 5 min, 50 μL of the clear supernatant was injected into liquid chromatograph.

2.4. Method validation

The evaluation of assay included the determinations of specificity, linearity, quantification limit, precision, accuracy, extraction recovery, and stability.

The specificity and selectivity of the method were evaluated by comparing chromatograms of six sources of blank plasma, blank plasma spiked with standard, and human plasma sample after oral administration of amoxicillin sulbactam pivoxil.

To evaluate the linearity, plasma calibration curves were prepared and determined in triplicate on three different days. Calibration curves were calculated by the peak area ratio *vs.* analyte concentrations using a $1/X$ weighted linear least-squares regression model. Lower limit of quantification (LLOQ) was defined as the lowest drug concentration on the calibration curve.

Intra- and inter-day precisions were determined by repeated analysis of quality control plasma samples five times at low, medium, and high concentrations on the same day and on three different days. Imprecision was expressed as relative standard deviation (RSD, %) and accuracy was evaluated by comparing the calculated concentration with the nominal concentration.

Extraction recoveries of amoxicillin, sulbactam, and I.S. were determined by comparing their peak areas obtained from blank plasmas spiked with standards with those of un-extracted standard solutions at the same nominal concentrations.

The stock solution, short-term room temperature, long-term storage, freeze–thaw, and post-preparative stabilities were tested. The stock solution stabilities of amoxicillin, sulbactam, and I.S. were examined at room temperature for 8 h and at 4 °C for 20 days. The short-term stability was tested at room temperature for 8 h, and the long-term stability was examined at −20 °C for 20 days. The freeze–thaw stability test was performed by three freeze–thaw cycles. The post-preparative stability was tested by injecting extracts immediately after preparation and re-injected 8 h later. The obtained concentrations of stabilities are compared to the nominal concentrations. The deviation should be within ±15%.

2.5. Application to bioequivalence study

The bioequivalence study was approved by the Ethical Committee of Third Xiangya Hospital of Central South University, and all subjects signed the informed consent before beginning of study. The study was performed based on a single dose, randomized, two-treatment, and two-period cross-over design. Twenty male healthy volunteers took 1.0 g oral dose of amoxicillin sulbactam pivoxil (500 mg amoxicillin, 500 mg sulbactam) with 200 mL of water. Blood samples (4 mL) were collected in separate vacutainers containing heparin pre-dose (0 h) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, and 8 h post-dose. The blood samples were immediately centrifuged at 4000 rpm for 5 min and the supernatants were stored frozen at −20 °C until analysis.

The main pharmacokinetic parameters were calculated by non-compartment model using Drug and Statistics Software (DAS, version 2.1, Mathematical Pharmacology Professional Committee of China).

3. Results and discussions

3.1. Method development

3.1.1. Optimization of chromatographic conditions

In order to minimize the undesirable UV absorption, high-purity acetonitrile was selected as the organic phase. Phosphate buffer systems (pH values between 2.5 and 5.5) were tested to optimize the chromatographic behaviors of the analytes. As a result,

the retention time of amoxicillin and sulbactam increased as the pH values of the buffer decreased. As compared with amoxicillin, the chromatographic retention of sulbactam was more sensitive to the change of pH values. Therefore, a mixture of 30 mM potassium dihydrogen phosphate buffer (pH 2.8)–acetonitrile (97.5:2.5, v/v) at a flow rate of 1.0 mL/min on an analytical column (Inertsil ODS: 150 mm × 4.6 mm, 5 μm) was proved to be the best chromatographic conditions.

3.1.2. Selection of internal standard

Allopurinol [16], acetaminophen [28], and cefadroxil [11,12] were used as internal standards in previous studies. Under above chromatographic conditions, the retention time of allopurinol, acetaminophen, and cefadroxil were approximately 4.0 min, 8.3 min, and 8.9 min, respectively. Because of the short retention time, allopurinol was not separable from endogenous interfering substances. Although the retention time of acetaminophen appears appropriate, its extraction recovery was less than 30%. Therefore, in this study cefadroxil was selected as an internal standard due to its favorable retention time and high extraction recovery. Furthermore, cefadroxil is the most appropriate I.S. for our analytes because cefadroxil was rarely prescribed together with our analytes.

3.1.3. Selection of detection wavelength

In order to obtain the LLOQ of amoxicillin and sulbactam determination, the detection wavelength was set at 210 nm for amoxicillin and sulbactam. Unfortunately, the endogenous substance interfered with the detection of I.S. (cefadroxil) at this detection wavelength. The UV absorption of the interfering substance decreased gradually when the wavelength increased from 190 to 370 nm. The interfering substance was neglectable when the wavelength was greater than 250 nm. Therefore, the detection wavelength was set at 263 nm for I.S., which was the maximum detection wavelength for I.S. within the range of 250–370 nm.

3.2. Method validation

3.2.1. Specificity and selectivity

The typical chromatograms including three sources of blank plasma, blank plasma spiked with amoxicillin, sulbactam and I.S., and a plasma sample collected at 2 h after administration of 1.0 g amoxicillin sulbactam pivoxil capsule were shown in Fig. 1. No interfering peak was observed at the retention time of amoxicillin, sulbactam, and I.S., which demonstrated that the determination condition had better specificity and selectivity.

3.2.2. Linearity and lower limit of quantification

The calibration curve was linear over the range of 0.163–14.7 μg/mL for amoxicillin and 0.250–15.0 μg/mL for sulbactam in human plasma. The linear regression equation ($n = 4$) was $Y = (1.290 \pm 0.033)X - (0.0112 \pm 0.0064)$ ($r^2 = 0.9991 \pm 0.0006$) for amoxicillin and $Y = (4.851 \pm 0.178)\ X + (0.0188 \pm 0.0067)$ ($r^2 = 0.9988 \pm 0.0007$) for sulbactam. The LLOQ was 0.163 μg/mL for amoxicillin and 0.250 μg/mL for sulbactam. The accuracy and imprecision were 94.4 and 7.9% for amoxicillin, and 106.7 and 9.5% for sulbactam, respectively. These data showed that this method is sensitive enough for the simultaneous determination of amoxicillin and sulbactam in human plasma after single oral administration of 1.0 g amoxicillin sulbactam pivoxil capsules.

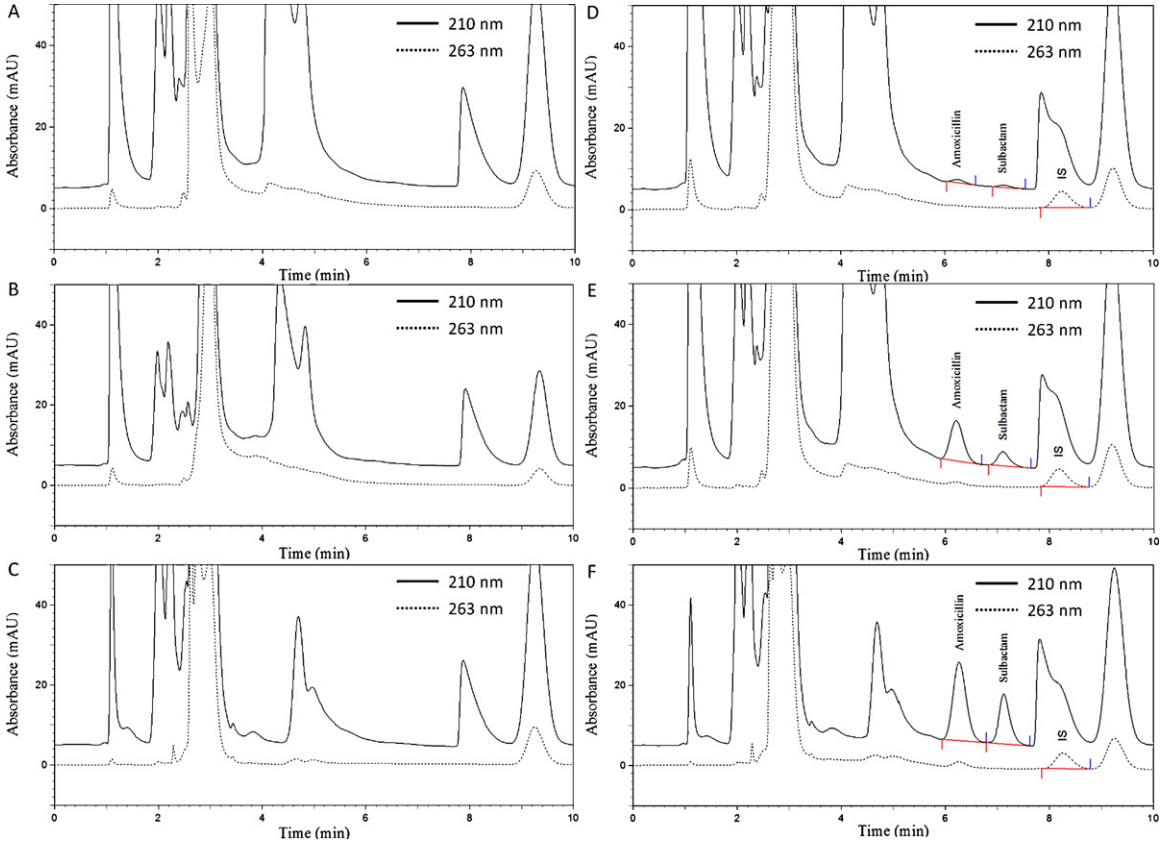

Fig. 1. Typical HPLC chromatograms including three sources of blank plasma (A–C); blank plasma spiked with 0.163 μg/mL amoxicillin (LLOQ), 0.250 μg/mL sulbactam (LLOQ), and I.S. (D); blank plasma spiked with 2.45 μg/mL amoxicillin, 2.50 μg/mL sulbactam and I.S. (E); plasma sample collected at 2 h after single oral administration of 1.0 g amoxicillin sulbactam pivoxil capsule to a volunteer (F).

Table 1
Inter- and intra-day imprecision, accuracy and recovery of amoxicillin and sulbactam in human plasma.

Concentration (μg/mL)	Intra-day (n = 5)		Inter-day (n = 15)		Recovery (n = 5)	
	Imprecision (RSD %)	Accuracy (%)	Imprecision (RSD %)	Accuracy (%)	Mean (%)	CV%
Amoxicillin						
0.327	8.1	103.2	10.1	105.4	71.2	8.4
2.45	7.4	95.4	8.7	97.1	76.9	7.1
12.3	4.7	99.1	8.2	98.6	79.5	6.5
Sulbactam						
0.500	8.8	99.3	9.5	101.5	68.3	8.6
2.50	7.2	104.3	8.7	105.7	75.4	4.8
12.5	5.3	96.8	6.4	97.3	74.6	5.4

Table 2
Stability analysis of amoxicillin and sulbactam.

Concentration (μg/mL)	8 h, room temperature (n = 5)		20 days, −20 °C (n = 5)		Three cylces, freeze/thaw (n = 5)		8 h, post-preparative (n = 5)	
	Bias (%)	RSD (%)	Bias (%)	RSD (%)	Bias (%)	RSD (%)	Bias (%)	RSD (%)
Amoxicillin								
0.327	4.1	9.5	−6.6	10.2	1.9	9.4	4.7	7.2
2.45	−1.5	7.3	1.6	6.7	−3.9	9.8	−2.7	5.0
12.3	2.2	4.5	−4.7	7.3	−5.0	7.9	−1.8	4.3
Sulbactam								
0.500	−2.7	8.2	5.6	11.9	−5.5	9.7	2.6	7.3
2.50	4.8	8.0	−1.6	10.7	−3.2	9.0	5.2	6.4
12.5	2.8	4.9	−4.8	7.0	−0.4	8.6	−4.2	5.3

3.2.3. Precision, accuracy, and extraction recovery

The inter- and intra-day imprecisions, accuracy and extraction recovery of amoxicillin and sulbactam in human plasma at QC concentrations are summarized in Table 1. The imprecisions (RSD, %) for amoxicillin and sulbactam were <11% and the accuracies (%) were within the range of 95.4–105.7%. The average extraction recovery of amoxicillin, sulbactam and I.S. were 75.9 ± 4.2%, 72.8 ± 3.9%, and 70.0 ± 3.3%, respectively.

3.2.4. Stability studies

With regard to the stock solution stability, there was no little loss for amoxicillin, sulbactam, and I.S. after storage at room temperature for 8 h or at 4 °C for 20 days. The amoxicillin and sulbactam were found to be stable in human plasma kept at room temperature for 8 h, −20 °C for 20 days, or three freeze–thaw cycles at −20 °C. The post-preparative samples were stable at room temperature for at least 8 h. All values of bias obtained were within ±15% (Table 2).

3.2.5. Bioequivalence study

This method was successfully applied to measure the real plasma samples collected from volunteers after drug administration in our bioequivalence study. The mean plasma concentration–time profiles of amoxicillin and sulbactam after a single dose of 1.0 g of either formulation were shown in Fig. 2. The pharmacokinetic parameters were summarized in Table 3. The pharmacokinetic parameters of amoxicillin and sulbactam in our

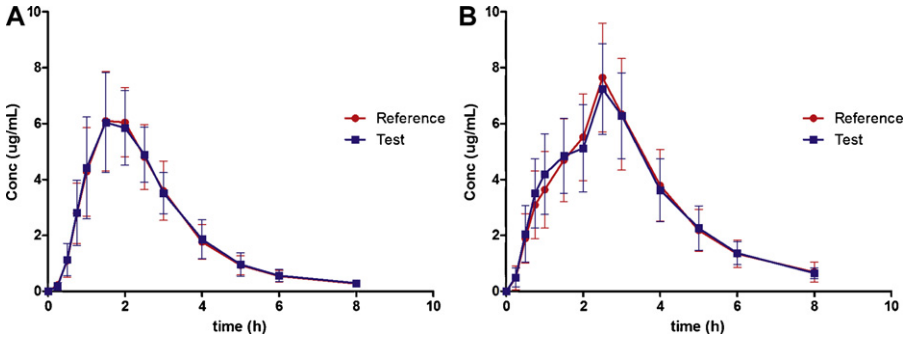

Fig. 2. Mean plasma concentration–time profiles of amoxicillin (A) and sulbactam (B) after single oral administration of 1.0 g test and reference formulations to healthy Chinese male volunteers.

Table 3
Pharmacokinetic parameters (mean ± SD) of amoxicillin and sulbactam after single oral administration of 1.0 g test or reference formulations to healthy Chinese male volunteers.

Parameters	Amoxicillin		Sulbactam	
	Test formulation	Reference formulation	Test formulation	Reference formulation
t_{max} (h)	1.8 ± 0.4	1.8 ± 0.4	2.4 ± 0.4	2.4 ± 0.4
C_{max} (μg/mL)	6.5 ± 1.3	6.6 ± 1.4	7.5 ± 1.4	8.0 ± 1.5
AUC_{0-8} (μg h/mL)	17.6 ± 3.2	17.5 ± 3.4	25.0 ± 4.5	25.0 ± 5.1
$AUC_{0-\infty}$ (μg h/mL)	18.1 ± 3.4	17.9 ± 3.5	26.2 ± 4.7	26.2 ± 5.5
$t_{1/2}$ (h)	1.3 ± 0.4	1.3 ± 0.4	1.5 ± 0.3	1.5 ± 0.3

study were similar to those of published studies [1,14,22]. The 90% confidence intervals for the ratios of test drug to reference drug in terms of AUC_{0-t} and C_{max} lay within the reference ranges of 80–125% and 70–143%, respectively, which is required to establish bioequivalence [29].

4. Conclusions

A sensitive, rapid and specific HPLC-DAD-based method has been developed for the simultaneous determination of amoxicillin and sulbactam in human plasma. The method has been successfully applied to bioequivalence study of two combination formulations of amoxicillin and sulbactam pivoxil in healthy male volunteers.

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