Validation of a Method for Determination of Ampicillin in Human Plasma using LC–DAD

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Abstract

We describe the validation data of a simple but selective chromatographic method for determination of ampicillin in human plasma using liquid chromatography–diode array detector. Blank plasma free of drugs was transferred to eppendorf's tubes and spiked with ampicillin stock solution to obtain quality control samples at 1.00, 2.50, 5.00, and 10.00 µg/mL. Extraction of ampicillin and cephalexin (internal standard) from plasma samples (250 µL) was investigated using three different methods: precipitation with perchloric acid, ultra-filtration and solid-phase extraction. Chromatographic separation was achieved using a Shimpak C_{18} column (300 mm × 4.6 mm i.d.; 5 µm), and detection was done at 215 nm with a diode array UV–Vis detector. The mobile phase consisted of dihydrogen phosphate (pH 3.5)–acetonitrile (87.5:12.5, v/v) delivered at a flow rate of 1.00 mL/min. Selectivity was evaluated with different pools of human plasma. Perchloric acid precipitation showed an excellent selectivity for normal plasma. The precipitation method presented recoveries above 84.0 ± 3.3% and 82.0 ± 1.6%, (n = 3) for ampicillin and cephalexin, respectively. The method has a limit of detection of 0.15 µg/mL and is linear in the range of 0.30 to 100.00 µg/mL. Standardized residue analysis demonstrated normality and homocedasticity. Inter-day precision was 4.5%, and accuracy was 11.1% (n = 9). Stability studies demonstrated instability of β-lactamics in human plasma at 20 and 2°C after 6 and 360 h of storage, respectively.

Introduction

β-Lactam antibiotics (i.e., penicillins and cephalosporins) are probably the most widely used class of medicines to treat respiratory tract infections, prostatitis, urinary tract infections, skin, and soft tissue infections of sensitive bacteria. β-Lactam antibiotics are also widely used in the treatment of dairy cattle for several infections (1). Ampicillin is a β-lactam antibiotic that belongs to the group of penicillins. The basic structure of the penicillins, 6-aminopenicillanic acid, consists of a thiazolidine ring fused to a β-lactam ring with a side chain where ampicillin present a primary amine group nonexistent in all other penicillins except epicillin and becampicillin. Ampicillin is extremely active against both gram-positive and gram-negative organisms, including several pathogenic enteric organisms. Ampicillin is used to treat infections caused, for example, by Escherichia coli, Salmonella, Proteus, and Klebsiella in spite of any species resistant to this drug (2). Cephalexin is a semi-synthetic drug from the class of α-aminocephalosporins. Cephalexin is used to treat many different types of bacterial infections, such as bronchitis, tonsillitis, ear infections, skin infections, and urinary tract infections. Children and adults also take this antibiotic for mild acne and other infections. Cephalexin is also used for the treatment of heart diseases due to its enhanced oral activity (3). β-lactamics are well-known for their instability in acids and alkalines medium. Penicillins also present degradation in methanol and suffer degradation from resistant microorganisms (4).

When extracting analytes from plasma samples, it is assumed that this matrix will require some degree of pre-treatment primarily due to binding of drugs by the plasma proteins albumin, α-acid glycoprotein, lipoproteins, and τ-globulins (5). There are reports in the literature of plasma precipitation with several precipitating agents to obtain clean extracts with recoveries greater than 70% (6). This precipitation method is preferable when it is necessary for the extraction of many drugs of the same therapeutic group, as candidates to therapeutic use, and when there is the need for fast development of a bioanalytic method. One disadvantage of this convenient method is drug loss due to occlusion of the drugs by the precipitating proteins (7). Nowadays, this method has been applied also for co-extraction of polar drugs (8).

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An automated protein precipitation method was reported with recoveries of 80% for salbutamol at 15 ng/mL (9) and a recovery of 78% for ICL670 at 1 µg/mL (10). Solid-phase extraction (SPE) was applied for β-lactam antibiotics in samples of wastewater, bovine milk, foodstuff, plasma, serum, and animal tissues. In these cases, the recovery rates ranged from 70–100% (11–18). SPE was also used for chlortetracycline with recoveries between 59–83% (19). Online SPE has been also used for the determination of amoxicillin and ampicillin (20).

There is a great variety of detectors coupled to liquid chromatography (LC) used for determination of β-lactam antibiotics in human biofluids. LC–UV is used for determination of cephalosporins (7,21–23) and penicillins (20,24–26) with derivatization (27–28). LC-chemiluminescense method has been used (29) as well as mass spectrometry (30–34).

The purpose our work was to validate a simple yet selective and accurate chromatographic method for the determination of ampicillin in human plasma using LC–diode array detector (DAD) with a simple extraction method using perchloric acid precipitation.

Experimental

Reagents and chemicals

Ampicillin USP (Ferjinsá, Mexico), cephazolin USP (US Pharmacopeia), amoxicillin, and cephalexin reference standards (Brazilian Pharmacopeia) were used throughout the study. Perchloric acid, phosphoric acid, potassium dihydrogen phosphate, and HPLC-grade acetronitrile were purchased from Merck (Darmstadt, Germany). Water was purified with a reverse-osmosis system connected to an ion exchange unit (Gehaka, Brazil). All other reagents were of analytical reagent-grade. A Microcon Ultracel-YM ultra filtration device with 10,000.00 nominal molecular weight limit was used and purchased from Millipore (Billerica, MA). SPE was done using polypropylene cartridges (3 mL) containing C18 sorbent (500 mg) from Supelco/Sigma Aldrich (St. Louis, MO).

Chromatographic system

The HPLC system consisted of an LC-10AD pump, a column oven (model CTO-10AS), a DAD (SPDM10A), an autosampler SIL-10AD, and a controller module SCL-10A (Shimadzu, Tokyo, Japan) coupled to a personal computer running the software Shimadzu Class VP for data acquisition. A high-speed REVAN centrifuge (Tecnal, São Paulo, Brazil) was used to centrifuge the spiked plasma with the drugs. A vortex apparatus from Quimis (São Paulo, Brazil) was used to homogenize the plasma samples. A 24-port SPE Visiprep Vacuum Manifold from Supelco/Sigma Aldrich was used for clean-up and concentration steps of drugs into the plasma.

LC–DAD conditions

The mobile phase consisted of 0.1 M potassium dihydrogen phosphate (pH 3.5)–acetonitrile (87.5:12.5, v/v). The phosphate buffer pH was adjusted with 4% phosphoric acid. Separation was achieved at 25ºC using a Shimpak C18 column (300 mm × 4.6 mm i.d., 5 µm) fitted with a Phenomenex Security guard C_{18} column (4.0 mm × 3.0 mm i.d., Torrance, CA). The flow rate was set at 1.0 mL/min, and a discrete channel on the DAD was configured to acquire data at 215 nm. Samples were introduced using the autosampler, and the injection volume was 40 µL.

Sample preparation

Fresh blank plasma was donated from the Hemotherapy Center of Paraíba (João Pessoa, Paraíba, Brazil) after approval from the research ethics committee. The plasma was kept in freezer at –20°C. The stock solutions of ampicillin, amoxicillin, cephalexin, and cephazolin were prepared in ultrapure water at 5.00 mg/mL concentration and diluted to quality control's concentrations of 1.00, 5.00, and 10.00 µg/mL. For validation studies, plasma samples (250 µL) were spiked with ampicillin and cephalexin stock solutions into 50:1 plasma to stock solution ratio. Ampicillin and its internal standard were evaluated in three different methods of extraction to obtain the best conditions of recovery and selectivity. Amoxicillin and cephazolin were evaluated only in the precipitation method with perchloric acid. All extractions methods are described later.

Ultra-filtration

Spiked plasma (500 µL) with ampicillin and cephalexin at 10.00 µg/mL were transferred to Microcon filtering unit allocated in ependorff tubes, which was submitted to clean-up process by centrifugation for 10 min at 7444 g (9000 rpm). The ultra filtrate (200 µL) was transferred to autosampler vials for HPLC analysis.

SPE

Spiked plasma (300 µL) with ampicillin and cephalexin at 10 µg/mL were submitted to SPE process. The C_{18} SPE sorbent was first conditioned and equilibrated with HPLC-grade methanol (2000 µL) and ultrapure water (2000 µL), respectively. Then spiked plasma (300 µL) was treated with 25% acetic acid (30 µL) and applied to the cartridges. The cartridges were then washed with ultrapure water (1000 µL) two times and finally were eluted with HPLC-grade methanol (3000 µL). The extract was evaporated to dryness on a stream of nitrogen. and the residue was redissolved in 300 µL of mobile phase (35).

Perchloric acid precipitation

Two hundred fifty microliters of spiked plasma with ampicillin, amoxicillin, cephalexin, and cephazolin were transferred to ependorff tubes and submitted to precipitation with 20 µL of 60% perchloric acid. The eppendorff tubes were vortexed and then centrifuged for 6 min at 7244 g (9000 rpm). The supernatant (150 µL) was placed in the autosampler vials to be injected.

Spiked plasma was also deproteinized with 20 µL of 40% perchloric acid, then were vortexed and centrifuged as decribed earlier. The supernatant was neutralized with 40 µL acetate buffer 2.5M (pH 4.5) and transferred for autosampler vials for HPLC analysis.

Development and validation of the method

Adjustment of retention time and choice of internal standard

The percentage of acetonitrile was evaluated to obtain information about time of analysis as well as selectivity and resolution

between the drugs and the plasma interferents. Acetonitrile percentage was modified between 10% and 15%. Amoxicillin, cephalexin, and cephazolin were tested as internal standards for ampicillin. In this step, these drugs were assayed only in drastic conditions using 60% perchloric acid.

Recovery and selectivity

Recovery was assessed by spiking plasma samples with both ampicillin and cephalexin in quality control samples (2.50, 5.00, 10.00, and 20.00 µg/mL). The absolute recovery was calculated by comparing the response of ampicillin or cephalexin in the extracted samples to those obtained for freshly prepared solutions of each standard in deionized water.

Selectivity of ampicillin and cephalexin from plasma interferents was investigated by using two batches of normal plasma from the hemotherapy center of João Pessoa and six plasma batches (four normal, one lipemic and one hemolyzed plasma) from the outpatient center of the University Hospital (Federal University of Paraíba).

Linearity, precision, and accuracy

Intra-day and inter-day precision and accuracy were evaluated for both ampicillin and cephalexin. Ampicillin and cephalexin precision and accuracy were evaluated by spiking plasma samples with the drugs at different concentrations samples at the range of 0.30–100.00 µg/mL. Three samples at each concentration were prepared daily by taking 250 µL aliquots from a 1000-µL volume of spiked plasma, and the analyses repeated for three consecutive days. Calibration curves were prepared daily using the range of 0.30–100.00 µg/mL for ampicillin and cephalexin using both internal and external calibration.

Limit of detection and limit of quantification

The limit of quantification (LOQ) was determined as the lowest concentration of ampicillin and cephalexin, which could be quantified with a value of bias below 20% and a signal-to-noise ratio of at least 5. The limit of detection (LOD) was considered as the concentration of each analyte producing a signal-to-noise ratio of 3 (36).

Stability studies

The stability of ampicillin and cephalexin was evaluated in stock solutions and in plasma samples extracted with perchloric acid precipitation and stored at 2ºC and 20ºC for 15 days. The stability was evaluated by comparing the responses of ampicillin and cephalexin in stored samples with those of a freshly prepared sample at the same concentration level. An upper and lower limit of 10% of the original concentration was established as acceptance criteria.

Results and Discussion

Adjusment of retention time and choice of internal standard

Mobile phase compositon, mainly the acetonitrile percentage, had a direct effect on the retention time of ampicillin and internal standard candidates. Ampicillin, cephalexin and cephazolin presented a sharp reduction in retention time (k') with the increasing acetonitrile percentage in mobile phase, but this did not occur with amoxicillin. Acetonitrile percentage influenced the resolution between ampicillin and other drugs. Amoxicillin and cephalexin reduced their resolution in relation to ampicillin as acetonitrile percentage in mobile phase increased, while cephazolin increased its resolution. With 12.5% of acetonitrile, ampicillin and cephalexin showed good resolution (resolution = 3.30–3.80) with a tailing factor of 1.12 and 1.10, respectively for ampicillin and cephalexin. Ampicillin and cephalexin had retention times of 9.3 and 10.8 minutes, respectively, and a total analyses time of 12.0 min.

After precipitation of spiked plasma samples with 60% perchloric acid, there was a marked decrease in the responses to amoxicillin and cephazolin, but ampicillin and cephalexin kept the signal intensity similar to that observed in stock solution samples (Figures 1A and 1B). Amoxicillin demonstrated a low stability in biological fluids, whereas cephazolin strongly binds to plasma proteins with about 89% of protein binding (1). These considerations led to cephalexin being chosen as the internal standard for the validation steps.

Sample preparation

Extraction of ampicillin and cephalexin were tested using three different methods of extraction and cleanup. The ultrafiltration method demonstrated to be unfavorable for cleanup and quantitation of ampicillin and cephalexin in spite of the good recovery (88.70%), which was due to problems of coelution with

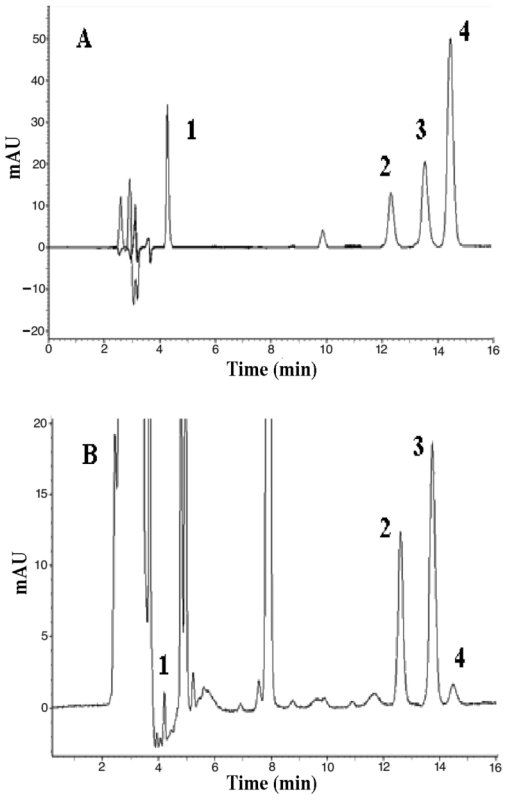

Figure 1. Chromatograms of β-lactamics in stock solution (A) and spiked plasma (B). Amoxicillin, 1; ampicillin, 2; cephalexin, 3; and cephazolin, 4, all at a concentration of 10 µg/mL. Mobile phase, ACN: 0.1 M potassium dihydrogen phosphate pH 3.1 (12.5:87.5, v/v).

interferents and low resolution for analytes (Figure 2).

The SPE method resulted in good cleanup and recovery (87.31 ± 0.60% and 88.80 ± 1.80% for ampicillin and cephalexin, respectively, $n = 3$). However, it was a tedious and lengthy procedure, thus not adequate in the offline mode for a large number of samples. Also, the SPE cartridges cost may be prohibitive for some laboratories (Figure 2).

The third method of extraction for the β-lactamics was the precipitation with 60% perchloric acid. We detected degradation or biologics instabilities for amoxicillin and cephazolin in acidic conditions (Figure 1B). A detailed analysis was done to check and eliminate the possibility of sample degradation during the running time (Figure 3A). We monitored the stability of ampicillin and cephalexin towards acidic conditions because β-lactamics are known to have low stability in acid and alkaline conditions. Ampicillin demonstrated degradation after 20 µL of 60% perchloric acid was added to a stock solution of ampicillin and cephalexin (at 5 µg/mL for both). This fact was observed after six consecutively injections while cephalexin remained stable in the same conditions. To avoid ampicillin degradation, we reduced the perchloric acid concentration to 40% and buffered the medium with 2.5 M acetate buffer (pH 4.5) after the centrifugation step. Using this strategy, ampicillin degradation was lower

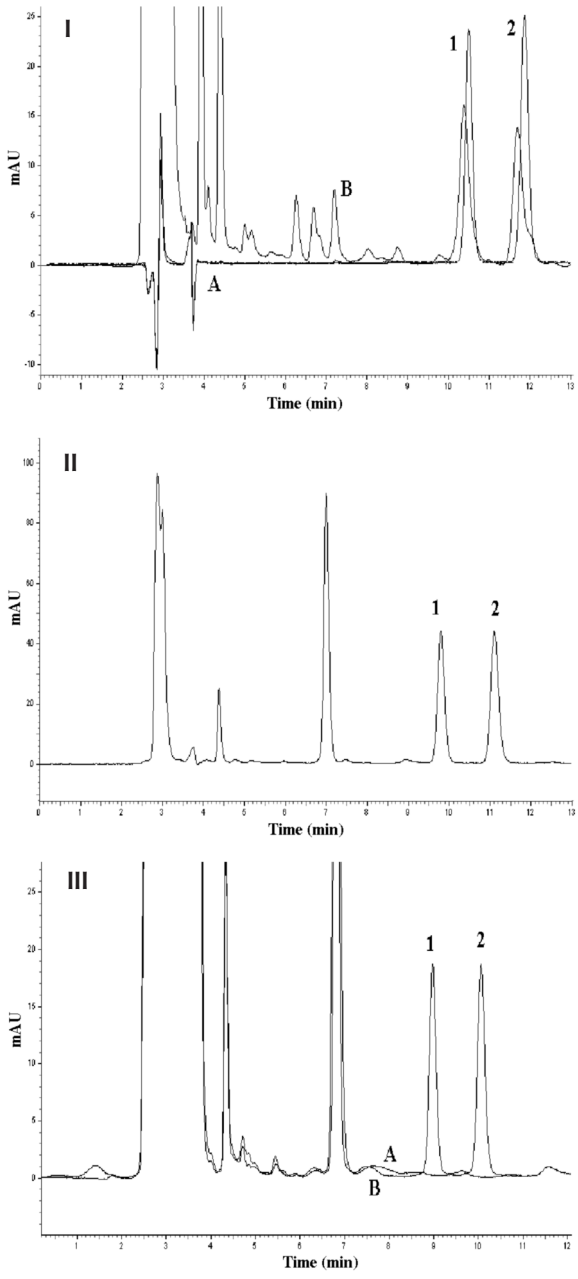

Figure 2. Chromatograms of stock solution (A) and plasma (B) after ultrafiltration (I) and SPE off line (II) treatment for ampicillin (10 µg/mL), 1; and cephalexin (10 µg/mL), 2. Chromatograms of drug-free plasma (A) and plasma spiked (B) after precipitation treatment (III) with 40% perchloric acid and buffering acetate buffer pH 4.5 with ampicillin (5 µg/mL) and cephalexin (5 µg/mL).

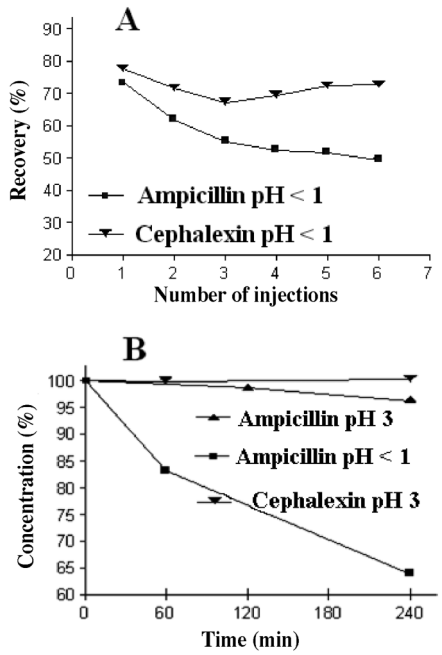

Figure 3. Stability study in acidic conditions for ampicillin and cephalexin. Stability of stock solutions in 60% perchloric acid (each injection had a running time of 16 min), A; Stability study in 40% perchloric acid using buffering with acetate buffer (pH 4.5) after centrifugation step, B.

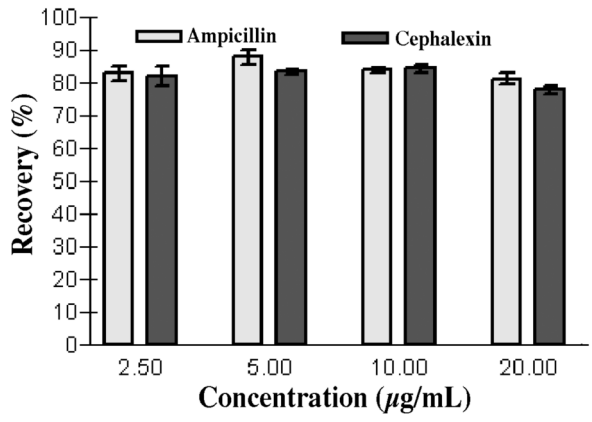

Figure 4. Recovery of ampicillin and cephalexin in human plasma after precipitation method with 40% perchloric acid followed by buffering with 2.5 M acetate buffer pH 4.5.

than 5% at 4 h (Figure 3B). The method showed good selectivity and stability for the analytes in acid conditions (Figure 2).

Recovery and selectivity

Ampicillin and cephalexin presented a percentage of recovery of (84.0 ± 3.3% and 82.0 ± 1.6%, respectively, n = 12) after precipitation with 40% perchloric acid followed by buffering with sodium acetate 2.5M (pH 4.5) post-centrifugation. The buffering step had the inconvenience of diluting the sample and decreasing the accuracy and sensibility of the method (Figure 4).

Selectivity studies in different types of human plasma showed selectivity of bioanalytic method for the four normal human plasma samples, but interferences occurred in specimens of lipemic plasma and hemolyzed plasma with coelution, which affected the accuracy of the cephalexin peak. This fact was considered during the collection of biologic specimens and the selection of healthy volunteers.

Linearity

Linearity of the method was evaluated using both external and internal standard calibration. Good correlation coefficients (r^2) were obtained for both methods (Table I). But the slope of cephalexin calibration curves presented significant variation in relative standard deviation (RSD) when external calibration was used (RSD = 22.5% and 3.2% for external and internal standard calibration, respectively). Ampicillin presented acceptable variations in slope using internal standard and external calibration models (RSD = 4.1% and 3.9%).

The straight line behavior was verified for ampicillin and cephalexin by projecting the standardized residue as a function of error frequency in a gaussian curve form. In this case, we evaluated the homocedasticity, independence and normality of calibration curve (Figure 5). In spite of large variations in slope, it was verified that both ampicillin and cephalexin responses were normally distributed. The internal standard calibration model was chosen since it had residue values 1.0 point below external standard calibration. Standardized residue analyses have been used as an important tool in the validation step of bioanalytic methods (37).

Precision and accuracy

Table II shows the values of precision and accuracy for ampicillin and cephalexin. The method presented good accuracy (< 11.0% for ampicillin and < 3.0% for cephalexin), and an intra-day precision values for ampicillin (< 5.0%) and cephalexin (< 9.0%) were in the acceptance limits. But the method presented an intermediary precision of lower rank to 20.0% for cephalexin and 5.0% for ampicillin, respectively, at 1.0 mg/mL concentration.

The report by Chiap (38) showed the relationship between a number of replicates and accuracy and precision of the method, especially due to limited recovery obtained in this method. The ideal number of replicates to obtain good values of precision into the acceptance limits established by regulatories agencies is five (39–41).

LOD and LOQ

Ampicillin and cephalexin were tested to evaluate their LOQ and LOD in the concentration range of 0.15–0.50 µg/mL. Table III show precision and accuracy values for ampicillin and

Table I. Linearity Parameters for Ampicillin and Cephalexin

	Internal Standard		External Calibration	
	Least squares fit	R^2	Least squares fit	R^2
Ampicillin				
Inter-Day 1	0.0453 + 0.0706x	0.998	–705.1 + 39599x	0.999
Inter-Day 2	0.0232 + 0.0682x	0.999	6475.6 + 36780x	0.999
Inter-Day 3	0.1376 + 0.0650x	0.974	16845 + 39071x	0.999
Cephalexin				
Inter-Day 1	0.0054 + 0.0946x	0.999	–739.7 + 42968x	0.999
Inter-Day 2	0.0274 + 0.0681x	0.999	8965.5 + 41878x	0.999
Inter-Day 3	0.0310 + 0.1079x	0.998	–2266.3 + 40318x	0.999

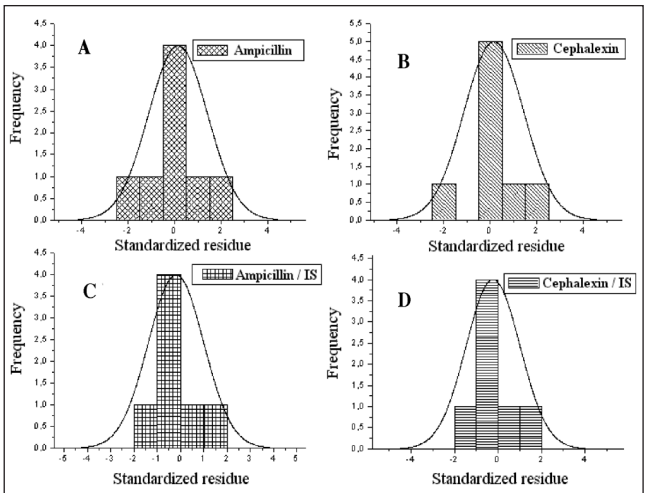

Figure 5. Histogram of standardized residues for ampicillin (A), cephalexin (B), ampicillin/IS (C), and cephalexin/IS (D). Graph A and B reflect external calibration; graph C and D reflect internal standard calibration.

Table II. Intra-day and Inter-day Precision and Accuracy for Ampicillin and Cephalexin

Samples	Precision (average ± SD; RSD%)				Accuracy	
QC	Intra-day			Inter-day	(%)*	Bias %
Ampicillin						
QC_L†	0.93 ± 0.02; 2.15	0.89 ± 0.03; 3.40	0.85 ± 0.01; 1.20	0.89 ± 0.04; 4.50	88.90	11.10
QC_M‡	5.21 ± 0.17; 3.26	4.86 ± 0.18; 3.70	5.03 ± 0.13; 2.60	5.03 ± 0.17; 3.40	100.60	0.60
QC_H§	10.05 ± 0.12; 1.20	9.78 ± 0.09; 0.93	10.23 ± 0.43; 4.20	10.02 ± 0.22; 2.20	100.20	0.20
Cephalexin						
QC_L	1.11 ± 0.06; 5.40	0.75 ± 0.06; 8.00	1.06 ± 0.03; 2.83	0.97 ± 0.19; 19.60	97.00	–3.00
QC_M	5.11 ± 0.23; 4.50	4.77 ± 0.04; 0.83	5.02 ± 0.02; 0.40	4.97 ± 0.17; 3.42	99.40	–0.60
QC_H	10.16 ± 0.32; 3.15	9.81 ± 0.36; 3.70	10.07 ± 0.19; 1.90	10.01 ± 0.18; 1.80	100.10	0.10

* % of nominal concentration = (average measured value/nominal value) × 100.
Bias % = [(measured value – true value)/true value] × 100.
† QC_L = Quality control low, 1.0 µg/mL. ‡ QC_M = Quality Control median, 5.0 µg/mL.
§ QC_H = Quality Control high, 10.0 µg/mL. Each value is expressed as mean ± SD of three determinations.

cephalexin, respectively.

The data of accuracy and intra-day precision revealed a LOQ of 0.30 µg/mL for both drugs analyzed into the approval limits (≤ 20%) for precision and accuracy according to AOAC standardization. Intra-day precision at the concentrations below 0.30 µg/mL were outside of the limits. LOQs were also evaluated by signal-to-noise ratio and were superior to 5:1. LODs for drugs were also estimated by signal-to-noise ratio of 3:1 at the concentration level 0.15 µg/mL.

For the β-lactamics, the therapeutic range with efficacy concentration was cited as 3.0–5.0 µg/mL. Thus, concentration was much more elevated than the LOQs described for this method (1).

Table III. Determination of LOQ and LOD for Ampicillin

Conc. (µg/mL)	Precision (average ± SD; RSD%)				Accuracy	
	Intra-day		Inter-day		(%)*	Bias %
Ampicillin						
0.50	0.47 ± 0.00; 0.0	0.51 ± 0.02; 3.9	0.50 ± 0.02; 4.0	0.49 ± 0.02; 4.1	98.5	–1.5
0.40	0.41 ± 0.01; 2.4	0.40 ± 0.01; 2.5	0.42 ± 0.08; 19.0	0.41 ± 0.01; 2.4	102.3	2.3
0.30	0.28 ± 0.02; 7.1	0.30 ± 0.01; 3.3	0.33 ± 0.01; 3.0	0.31 ± 0.02; 6.4	101.6	1.6
0.20	0.17± 0.01; 5.9	0.21 ± 0.01; 5.5	0.20± 0.06; 30.0	0.19 ± 0.02; 10.5	96.9	–3.1
0.15	0.08 ± 0.01; 12.5	0.18 ± 0.01; 5.5	0.11 ± 0.06; 54.5	0.12 ± 0.05; 41.7	82.4	–17.8
Cephalexin						
0.50	0.41 ± 0.01; 2.4	0.45 ± 0.03; 6.7	0.49 ± 0.01; 2.0	0.45 ± 0.04; 8.9	90.3	–9.7
0.40	0.36 ± 0.02; 5.5	0.37 ± 0.02; 5.4	0.42 ± 0.07; 16.7	0.39 ± 0.04; 10.2	97.1	–2.9
0.30	0.23 ± 0.04; 17.4	0.29 ± 0.03; 10.3	0.30 ± 0.01; 3.3	0.27 ± 0.04; 14.8	90.8	–9.2
0.20	0.17± 0.01; 5.9	0.15 ± 0.02; 13.3	0.19± 0.05; 26.3	0.17 ± 0.02; 11.8	84.9	–15.1
0.15	0.10 ± 0.01; 10.0	0.12 ± 0.01; 8.3	0.10 ± 0.04; 40.0	0.11 ± 0.01; 9.1	72.3	–27.7

* % of nominal concentration = (average measured value/nominal value) × 100. Bias %= [(measured value–true value)/true value] × 100. Each value is expressed as mean ± SD of three determinations.

Table IV. Stability Study in Stock Solution for Ampicillin and Cephalexin at 20°C*

Time (h)	Concentration maintained (%)					
	Ampicillin			Cephalexin		
	2.5 mg/mL	10.0 mg/mL	50.0 mg/mL	2.5 mg/mL	10.0 mg/mL	50.0 mg/mL
0	98.1 ± 0.9	93.0 ± 1.4	100.6 ± 0.5	98.2 ± 1.9	91.3 ± 1.0	100.3 ± 1.7
6	95.5 ± 1.9	94.3 ± 1.2	100.0 ± 1.2	96.9 ± 1.6	92.2 ± 1.0	100.6 ± 1.7
12	108.5 ± 3.7	109.1 ± 0.4	105.7 ± 0.1	98.8 ± 2.2	99.1 ± 0.5	99.3 ± 0.1
24	95.0 ± 1.0	94.6 ± 0.2	96.2 ± 0.1	97.2 ± 0.8	97.6 ± 0.1	97.9 ± 0.1
72	105.0 ± 1.0	94.6 ± 0.1	100.7 ± 0.2	99.5 ± 0.8	96.6 ± 0.5	96.7 ± 0.0
120	101.0 ± 0.7	96.8 ± 0.2	102.1 ± 0.1	100.5 ± 1.3	100.8 ± 1.0	102.6 ± 0.2
360	67.1± 0.1	52.9 ± 0.1	82.8 ± 0.2	60.0 ± 0.7	71.2 ± 0.2	80.1 ± 0.1

* Each value is expressed as media ± SD of three determinations.

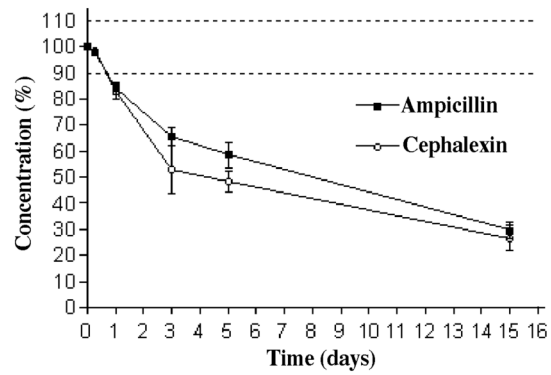

Figure 6. Stability study of ampicillin and cephalexin in human plasma at 20ºC. The values were calculated as the median ± SD of three determinations of the quality control samples.

Stability studies

In the stock solutions, ampicillin and cephalexin did not present degradation up to 120 h of storage at 20°C. The median values between the quality control samples did not exceed the 10% limits of variation during the five days (Table IV). After this time, ampicillin and cephalexin presented degradation that was observed in 360 h or 15 days of storage. Stock solutions of ampicillin and cephalexin (5 mg/mL) were stored in a refrigerator without problems of degradation or precipitation for five days.

The stability of ampicillin and cephalexin spiked in human plasma revealed an elevated degradation of the drugs after 6 h of storage at 20°C. In 24 h of storage, ampicillin and cephalexin presented degradation above the limit of 10%. In contrast to the stability studied in stock solution at 20°C, ampicillin and cephalexin presented a percentage of degradation close to 70% as compared to the 30% in stock solution during 15 days of storage (Figure 6). Ampicillin and cephalexin spiked in human plasma were submitted to stability studies at 2°C, and we observed degradation closed to 10% and 40% during five and 15 days of studying, respectively.

We could observe that plasma contituents have accelarated degradation of ampicillin and cephalexin for both stability studies in human plasma at 2°C and 20°C when compared with stability studies in stock solution. These data demostrated that the β-lactamic antibiotics studied require special conditions for storage before the start of sample processing and analytical running in bioavailability or bioequivalence studies.

Conclusion

The analytical conditions for determination of ampicillin in human plasma were considered satisfactory with special attention to its stability. Recoveries above 80% for both ampicillin and cephalexin were possible with the protein precipitation method. Inter-day precision was better than 5% and accuracy was 11.1%

RSD (n = 9). The LOD for ampicillin was 0.15 µg/mL. Stability studies demonstrated instability of β-lactamics in human plasma at 20ºC, which means the samples should be processed in a period shorter than 6 h. For cephalexin, attention must be paid to selectivity parameters during the choice of health volunteers to avoid lipemic and hemolyzed plasma. The method described here presents a simple yet selective and accurate alternative for the quantitation of ampicillin in relation to previously published methods and can potentially be applied to a large number of bioanalytical problems.

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