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AN EFFICIENT HPLC-UV METHOD FOR THE QUANTITATIVE DETERMINATION OF CEFADROXIL IN HUMAN PLASMA AND ITS APPLICATION IN PHARMACOKINETIC STUDIES

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AN EFFICIENT HPLC-UV METHOD FOR THE QUANTITATIVE DETERMINATION OF CEFADROXIL IN HUMAN PLASMA AND ITS APPLICATION IN PHARMACOKINETIC STUDIES

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□ Cefadroxil is a semi-synthetic first-generation oral cephalosporin used in the treatment of mild to moderate infections of the respiratory and urinary tracts, skin and soft tissue infections. In this work a simple, rapid, economic and sensitive HPLC-UV method is described for the quantitative determination of cefadroxil in human plasma samples using lamivudine as internal standard. Sample pre-treatment was accomplished through protein precipitation with acetonitrile and chromatographic separation was performed with a mobile phase consisting of a mixture of sodium dihydrogen phosphate monohydrate solution, methanol and acetonitrile in the ratio of 90:8:2 (v/v/v) at a flow rate of 1.0 mL/min. The proposed method is linear between 0.4 to 40.0 µg/mL and its average recovery is 102.21% for cefadroxil and 97.94% for lamivudine. The method is simple, sensitive, reproducible, less time consuming for determination of cefadroxil in human plasma. The method can therefore be recommended for pharmacokinetics studies, including bioavailability and bioequivalence studies.

Keywords bioavailability, cefadroxil, HPLC, human plasma, pharmacokinetics, quantification

INTRODUCTION

Cefadroxil is a semi-synthetic first generation oral cephalosporin, similar to cephalixin and cephradine in structure and spectrum of antibacterial activity. It is used in the treatment of mild to moderate infections of the respiratory and urinary tracts, skin and soft tissue infections.^[1,2] Furthermore it has been used in the prophylaxis of recurrent urinary tract infections in children.^[1] Although the microbiological activities of cephalosporins are similar when measured by traditional susceptibility testing

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systems, a study using a kinetic model to simulate the blood concentrations in man showed cefadroxil to be more active than cephalixin and cephradine against *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. This suggests that the more sustained serum and tissue concentrations of cefadroxil improve its microbiological activity in the blood stream.^[2]

Cefadroxil is acid-stable and has a phenyl-glycine side chain that is responsible for almost completely oral absorption. The rate of absorption and serum peak concentration of cefadroxil were not affected when the drug is administered with food and over 90% of the drug is excreted unchanged in urine within 24 hr.^[3,4] The peak serum concentrations are achieved within 1.5–2.0 hr and average about 10–18 µg/mL following a single oral administration 500 mg dose. The plasma half-life elimination of cefadroxil is 1.1–2.0 hr in adults with normal renal function.^[1,3,5,6] Cefadroxil produces higher concentrations in body tissues and fluids, such as sputum, lung, pleura, and skin blisters than cephalixin and cephradine. A dose of 500 mg cefadroxil is sufficient to treat different infections. Cefadroxil is generally well tolerated and adverse effects do not appear to be a serious problem.^[1]

Several methods have been published for quantification of cefadroxil in human plasma, serum, and urine by means of high-performance liquid chromatography with ultraviolet detection (HPLC-UV),^[1,3,5–9] mass spectrometry,^[10] and microbiological assays.^[11,12]

Barbhaiya^[3] reported a simple HPLC-UV for determination of cefadroxil in human plasma, but this method uses a large plasma volume (1000 µL) and does not use an internal standard. Oliveira and colleagues^[10] established a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to determine cefadroxil in human plasma samples which can provide excellent sensitivity and selectivity, but it is not available to all laboratories and researchers due to the high costs associated with the acquisition and maintenance of this equipment. Other methods are somewhat more complex and involve the use of pre-treatment process with several steps,^[7] present long elution time,^[5] high consumption of organic solvent,^[1,6,8,9,13,14] or include additives in the mobile phase.^[14]

As an alternative to existing methods, a HPLC method with simple plasma samples pre-treatment was developed for the determination of cefadroxil. The separation can be performed under the most common experimental conditions, without adding expensive special equipment. Full validation was performed to assess the selectivity, sensitivity, linearity, recovery, accuracy, and precision of the method. The results presented here demonstrate that the method is suitable for analyzing cefadroxil in human plasma and it has been successfully applied to the pharmacokinetic/bioavailability/bioequivalence studies of cefadroxil oral suspension in healthy volunteers.

MATERIALS AND METHODS

Chemicals and Reagents

Sodium dihydrogen phosphate monohydrate analytical grade and methanol and acetonitrile HPLC-grade were obtained from Merck (São Paulo, Brazil). Chromatographic-grade water was produced by a Millipore Milli-Q system (Billerica, MA, USA). Cefadroxil reference standard was purchased from Farmacopéia Brasileira (Rio de Janeiro, Brazil) and lamivudine (internal standard, IS) was kindly supplied by FURP (São Paulo, Brazil).

Stock Standard Solutions

Stock standard solutions of 1 mg/mL of cefadroxil and 100 µg/mL of lamivudine (IS) were prepared in methanol and stored at -20°C .

Calibration Standards and Quality Control Plasma Samples

Stock standard solutions of cefadroxil (1 mg/mL) and IS (100 µg/mL) were diluted with water:methanol solution (90:10, v/v) to produce working stock standard solutions of cefadroxil at concentrations of 400, 320, 240, 160, 80, 40, 10, and 4 µg/mL and working stock standard solution of IS at concentration 10 µg/mL, which were stored at 6°C .

Preparation of calibration standard plasma samples was accomplished daily by spiking known amounts (25 µL) of cefadroxil solutions (4 to 400 µg/mL) to 225 µL of drug-free plasma in 8 mL glass tubes. Effective concentrations of calibration curve for cefadroxil in plasma samples were 0.4; 1.0; 4.0; 8.0; 16.0; 24.0; 32.0, and 40.0 µg/mL.

The quality control (QC) plasma samples were pooled, at concentrations of 1 µg/mL (low), 16 µg/mL (medium), and 32 µg/mL (high), as a single batch at each concentration and then divided into aliquots that were stored in the freezer at -20°C until analysis. The spiked plasma samples (standards and QCs) were processed following the procedure on each analytical batch along with the unknown samples.

Sample Preparation

Portions of 250 µL blank plasma, spiked plasma or pharmacokinetics study plasma were transferred to 8-mL glass test tubes, spiked with 25 µL of IS working stock standard solution (10 µg/mL) and vortex mixed for 5 s. Protein precipitation was accomplished by adding 200 µL of acetonitrile to the mixture. After vortex mixing for 15 s at room temperature and

centrifuging at $10,000 \times g$ for 15 min, the upper layer was filtered through a Durapore membrane ($13 \text{ mm} \times 0.45 \mu\text{m}$) in a clean glass tube and evaporated to dryness using an evaporator at 40°C under a stream of nitrogen. Then, the dried extract was reconstituted in $300 \mu\text{L}$ of mobile phase and $30 \mu\text{L}$ were injected into the chromatographic system.

Instrumental Parameters and Conditions

Analyses were performed on a Shimadzu Scientific Instruments liquid chromatographic system (Kyoto, Japan) composed of a LC-10ADvp pump, DGU-10ADvp degasser, SPD-10ADvp DAD detector, and CTO-10ADvp column oven. The reverse-phase chromatography was performed on an analytical Phenomenex Synergi MAX RP C18 column ($4 \mu\text{m}$, $150 \text{ mm} \times 4.60 \text{ mm}$) (Torrance, CA, USA) protected with a Phenomenex AJO-4287 C18 guard cartridge (Torrance, CA, USA).

Mobile phase consisted of a solution of sodium dihydrogen phosphate monohydrate (20 mmol/L), methanol, and acetonitrile in the ratio of 90:8:2 (v/v/v). Before analysis, the mobile phase was filtered through a $0.22\text{-}\mu\text{m}$ filter, and then degassed ultrasonically for 15 min. The assay was run at 35°C . A flow rate of 1 mL/min was used and the eluted peaks were monitored at 230 nm .

Validation of Bioanalytical Method

The described method was validated in terms of linearity, lower limit of quantification (LLOQ), recovery, selectivity, stability, precision, and accuracy according to international guidelines regarding bioanalytical methods validation.^[15,16]

Selectivity

To evaluate the selectivity of the method, drug free plasma samples were carried through the assay procedure and the retention time of the endogenous compounds in plasma were compared with those of cefadroxil ($0.4 \mu\text{g/mL}$) or IS. Selectivity of the method was assessed to test the matrix influence of six different plasma samples: four normal plasma samples, one hemolized plasma sample and one lipemic plasma sample.

Lower Limit of Quantification and Linearity

The lower limit of quantification (LLOQ) was the smallest analytical concentration that could be measured with accuracy between 80 to 120% and precision lower than 20%.

The linearity was tested by the calibration curve ranging from 0.4 to 40.0 µg/mL in five replicates at each concentration.

Recovery

The analytical recovery was calculated by comparing chromatographic peak areas from unextracted standard samples and from extracted standard samples at three QC concentrations in five replicates.

Accuracy and Precision

Intra assay accuracy and precision evaluations were performed by repeated analysis of cefadroxil in human plasma. The run consisted of a calibration curve plus five replicates of each low, medium, and high quality control samples. Inter assay accuracy and precision were assessed by analysis of samples consisting of a calibration curve and five replicates of low, medium, and high quality control samples for cefadroxil on five separate days. The overall precision of the method was expressed as relative standard deviation (RSD%) and the accuracy was expressed as the percentage ratio between the experimental concentration and the nominal concentration for each sample.

Stability

The stability of plasma samples under different conditions was evaluated through spiked quality control samples at three concentration levels.

The bench-top stability was examined by keeping replicates of plasma quality control samples at room temperature for approximately 4 hr. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 4 hr and refreezing for 12–24 hr. Auto-sampler stability of cefadroxil was tested by analysis of processed and reconstituted plasma quality control samples, which were stored in the auto-sampler for 48 hr at room temperature. Long-term stability of cefadroxil in human plasma was tested after storage at –20°C for 60 d. For each concentration and each storage condition, three replicates were analyzed in one analytical batch. Stability under different conditions was determined by comparison with cefadroxil concentration in freshly prepared samples (0 hr).

Application

The method was applied to determine cefadroxil concentration in plasma samples from 24 healthy volunteers, after oral administration of cefadroxil suspension doses of 450 mg (D1), 480 mg (D2), 500 mg (D3),

and 520 mg (D4). Different doses were administered to each volunteer to simulate products with different bioavailabilities.

The *in vivo* study was performed according to the rules of Good Clinical Practice^[17] and the clinical protocol was submitted and approved by the Ethical Committee of the College of Pharmaceutical Sciences, University of São Paulo in accordance with the Declaration of Helsinki.^[18]

The following pharmacokinetic parameters were calculated using a non-compartmental model: C_{\max} (maximum plasmatic concentration), T_{\max} (time to C_{\max}), and AUC_{0-t} (area under the plasma concentration-time curve from zero to sample time of the last measurable cefadroxil concentration). C_{\max} and T_{\max} were obtained directly from the concentration-time curve and AUC_{0-t} was calculated using the linear trapezoidal method.^[19]

RESULTS AND DISCUSSION

Assay Performance

The HPLC coupled to ultraviolet detection is the most frequently described method to quantify cefadroxil in biological samples. HPLC coupled to mass spectrometry is used with less frequency,^[10] and it is not routinely available in all laboratories, especially in developing countries.

Microbiological assays were described,^[11,12] but nowadays they are not common since they lack sensitivity and specificity.

The sample preparation step is critical for accuracy and sensitivity of the assay method. Several methods for quantification of cefadroxil in plasma are described in literature as protein precipitation (PPT), solid-phase extraction (SPE), and ultrafiltration. PPT is the most widely employed sample preparation method for cefadroxil, using a strong acid solution^[1,5,8] or acetonitrile.^[6,9] PPT often provides higher recovery compared to other methods, especially for compounds having high polarity. This procedure gave a clean chromatogram for a blank plasma sample and yielded the highest recovery for the analytes from the plasma. SPE technique was used by Samanidou and colleagues,^[7] but it involves two steps which makes it very complicated and time-consuming for application in routine assays. Furthermore, it is expensive, especially considering that one cartridge is required per sample. The ultrafiltration method described in the literature^[3] was not validated and did not use an internal standard.

In comparison with previously described sample preparation methods for cefadroxil, the procedure presented in this work is more simple, more economic, and faster. The same group developed a method for quantification of cefadroxil in plasma,^[6] but the results of the new method present better performance in terms of linearity range, recovery, and retention time

for cefadroxil and IS. Furthermore, the previous method used 3 mL of acetonitrile for sample preparation. The use of organic solvents in the extraction procedure in this work was also minimized to only 200 μ L of acetonitrile per sample.

Only two previously reported methods used flow rate in the chromatographic system equal or less than 1 mL/min. Barbhaiya^[3] described a method with a flow rate of 1 mL/min, but retention time of cefadroxil was greater than 5 min. Piotrovskij and colleagues^[5] presented a method with a flow rate of 0.6 mL/min, but the retention time of cefadroxil was 13.4 min. Despite the use of a low flow rate of 1 mL/min in the present method, short retention times for the cefadroxil (3.3 min) and IS (4.6 min) were obtained. Under the present chromatographic conditions, the run time for each sample was 10 min. It was demonstrated that the determination of the analyte was not interfered with by endogenous substances in the plasma in chromatographic separation. Figure 1 represents chromatograms of cefadroxil and IS from human plasma.

The adjustment of pH of the mobile phase is required in several methods.^[1,5,6,8,13] The preparation of the mobile phase presented in this work does not require pH adjustment. In addition we did not include any additives in the mobile phase such as triethylamine, tetrabutylammonium, or ion pairing agents.

The proposed method is linear between 0.4 to 40.0 μ g/mL for cefadroxil and the calibration curve can be described by the equation $y = 0.0739x + 0.0049$ ($r^2 = 0.9993$) (Figure 2). The LLOQ of 0.4 μ g/mL in plasma obtained with 250 μ L of plasma is one of the lowest reported in the literature. Piotrovskij and colleagues^[5] obtained a LLOQ of 0.2 μ g/mL using 500 μ L of plasma sample. Average recovery was 102.21% for cefadroxil and 97.94% for lamivudine (Table 1). The RSD% values were less than 15% and illustrates the good intra- and inter-assay precision of the method. The intra- and inter-assay accuracy ranged from 97.01 to 104.79 (Table 2).

Cefadroxil in plasma samples proved to be stable for a minimum of 4 hr at room temperature. The freeze and thaw cycles did not change the concentration levels of the analyte significantly. The deviation compared to freshly prepared samples (1, 16, and 32 μ g/mL) was found to be -7.70% , $+1.60\%$, and -2.33% , of the nominal value respectively after each freeze-thaw cycle. Forty-eight hour auto-sampler stability results of plasma sample extracts at room temperature indicated no significant change in the concentration levels of the analyte (e.g., more than 15%). Cefadroxil concentrations in plasma samples showed no significant difference after 60 d at -20°C .

This method has several advantages compared to previously reported methods such as a simple and fast procedure for sample pre-treatment, easy

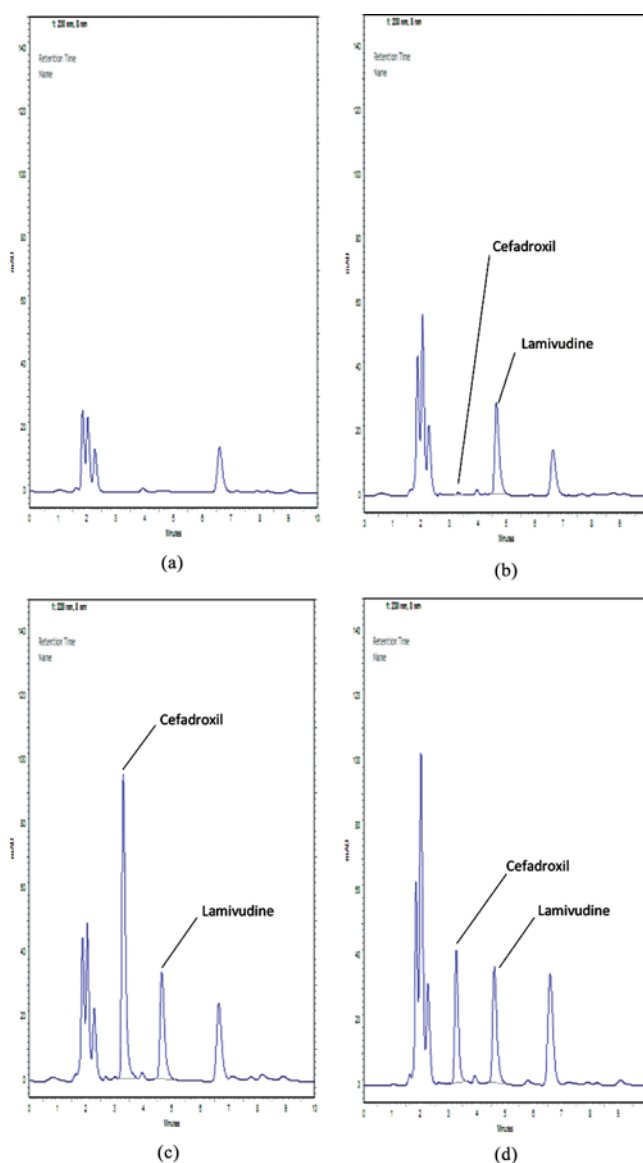


FIGURE 1 HPLC-UV chromatograms obtained for A: blank plasma, B: blank plasma spiked with cefadroxil 0.4 µg/mL (LLOQ) +I.S., C: blank plasma spiked with cefadroxil 32 µg/mL +I.S., and D: plasma from volunteer 1.5 hr after oral administration of 500 mg of cefadroxil +I.S. Retention times are 3.3 min (cefadroxil) and 4.6 min (lamivudine, I.S.). (Color figure available online.)

preparation of the mobile phase, use of a low flow rate in the chromatographic system, and, consequently, decreased use of organic solvent in the mobile phase. The short run time has the advantage of facilitating and enhancing the efficiency of processing large numbers of plasma

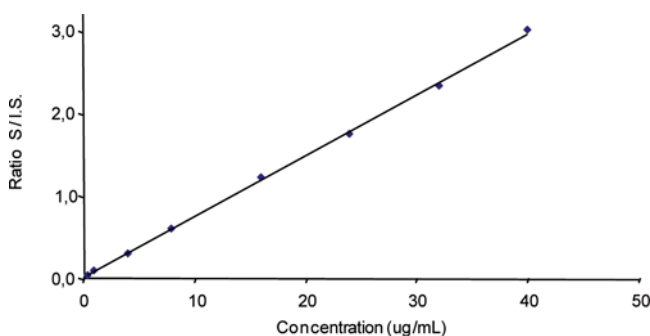


FIGURE 2 Standard calibration curve for cefadroxil quantification in human plasma by HPLC method with UV detection. The equation of calibration curve is $y=0.0739x+0.0049$. (Color figure available online.)

samples obtained from pharmacokinetic/bioavailability/bioequivalence studies in healthy human subjects.

Application

This validated method was applied to monitor the plasma concentration of cefadroxil in healthy volunteers that were administered single oral doses. The lower limit of quantification and good linearity that were obtained using this method were adequate for cefadroxil quantification in human plasma following administration of different doses of cefadroxil. The representative chromatograms obtained from one volunteers at 1.5 hr after drug administration are shown in Figure 1D. The mean plasma concentration–time curve of cefadroxil after oral administration of four different doses to 24 healthy volunteers is shown in Figure 3. The mean C_{\max} results were $D1 = 17.39 \mu\text{g/mL}$, $D2 = 18.53 \mu\text{g/mL}$, $D3 = 19.35 \mu\text{g/mL}$, and $D4 = 19.63 \mu\text{g/mL}$. T_{\max} did not differ significantly between doses and ranged from 1.04 to 1.2 hr, and AUC_{0-t} were $D1 = 48.28 \mu\text{g.h/mL}$, $D2 = 52.88 \mu\text{g.h/mL}$, $D3 = 56.08 \mu\text{g.h/mL}$, and $D4 = 59.41 \mu\text{g.h/mL}$.

TABLE 1 Recovery of Cefadroxil and I.S. from Human Plasma ($n=5$)

Concentration ($\mu\text{g/mL}$)	Recovery % (RSD %)	
	Cefadroxil	Lamivudine (I.S.)
1	86.78 (6.46)	103.59 (1.55)
16	109.91 (7.82)	94.41 (3.92)
32	109.94 (4.67)	95.82 (2.81)
Mean	102.21	97.94

I.S. = Internal Standard.

RSD = relative standard deviation.

TABLE 2 Intra-Day and inter-Day Assay Accuracy and Precision of Cefadroxil; Determination at Three Concentration Levels (n = 5)

	Concentration (µg/mL)	Accuracy (%)	Precision: RSD (%)
Intra-day	1	104.79	11.83
	16	97.01	3.64
	32	99.61	2.50
Inter-day	1	104.15	9.59
	16	104.40	7.40
	32	100.49	3.83

RSD = relative standard deviation.

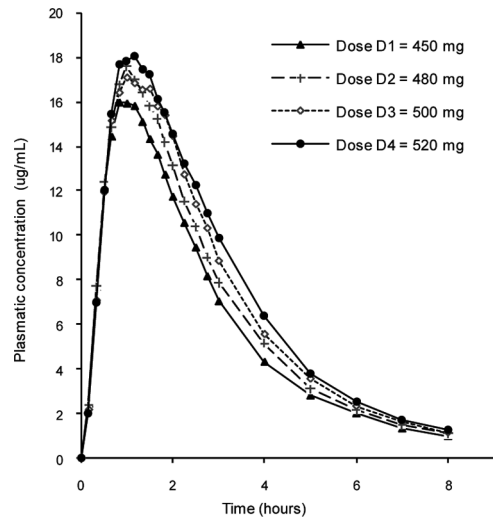


FIGURE 3 Mean plasma concentration-time curve of cefadroxil after oral administration of 450 mg, 480 mg, 500 mg, and 520 mg to 24 healthy volunteers.

CONCLUSION

A simple and efficient isocratic reversed phase HPLC method proposed was found to be accurate, precise, and linear across the analytical range. All the validation parameters for cefadroxil quantification met the criteria of the international guidelines for bioanalytical method validation.^[15,16] The method could therefore be recommended for pharmacokinetics studies, including bioavailability and bioequivalence studies.

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