Original Research Article

Validated High Performance Liquid Chromatography Method for Analysis of Cefadroxil Monohydrate in Human Plasma

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Abstract

Purpose: To develop a simple, rapid and sensitive high performance liquid chromatography (HPLC) method for the determination of cefadroxil monohydrate in human plasma.
Methods: Schimadzu HPLC with LC solution software was used with Waters Spherisorb, C18 (5 µm, 150mm × 4.5mm) column. The mobile phase was sodium dihydrogen phosphate buffer pH 4.0 and methanol in a ratio of 96:4. Flow rate was 1.5 ml/min and injection volume was 100 µl. Peak response was detected at 260 nm.
Results: System suitability results revealed that the coefficient of variation (CV) for retention time, peak response, tailing factor and resolution of six replicate injections was < 3 %. The method was selective to determine cefadroxil in plasma because there was no peak interference of plasma with cefadroxil at its retention time (7.792 min). Linearity was in the range of 0.5 - 30 µg/ml with slope and intercept of 41694.53 and 22614.87, respectively (R^2 = 0.9953). Limit of detection (LOD) and lower limit of quantification (LLOQ) of the method were 0.03 and 0.06 µg/ml, respectively. Absolute recovery of cefadroxil from plasma was in the range 71 - 90.4 %, while inter-day and intra-day analysis showed satisfactory precision and accuracy; thus, the method was reproducible with the range of CV, i.e., 0.35 - 4.01 and 1.88 - 7.9 % for interday and intraday precision, respectively.
Conclusion: The developed method being simple, rapid, reproducible can be suitably employed in pharmacokinetic and bioequivalence studies of cefadroxil monohydrate.

Keywords: Validation, Cefadroxil monohydrate, Human plasma, Pharmacokinetics Bioequivalence

INTRODUCTION

Cephalosporins are bactericidal antibiotics that inhibit cell wall synthesis of bacteria. These antibiotics are derived from cephalosporin C which was for the first time isolated from the cultures of *Cephalosporium acremonium* in 1948 by an Italian scientist, Giuseppe Brotzu. The first agent was cephalothin, discovered in 1964. Now four generations of cephalosporins are available in pharmaceutical dosage forms [1].

Cefadroxil is a parahydroxyl derivative of cephalexin. Its molecular formula is $C_{16}H_{17}N_3O_5S.H_2O$ and structural formula is given in Figure 1. It is an oral cephalosporin successfully used in the treatment of mild to moderate infections of skin, soft tissues, urinary

tract and upper respiratory tract. It is used in doses of 500 mg to 1000 mg in single or divided doses. It is effective against gram-positive and gram-negative species including *Staphylococcus aureus*, *Streptococcus pneumonia*, *Streptococcus pyogenes*, *Moraxella catarrhalis*, *Escherichia coli*, *Klebsiella* spp, *Proteus mirabilis*. Cefadroxil is approximately 100 % absorbed through gastrointestinal tract and protein binding is just 20 %. Plasma half-life is 1.5 h. Most of the drug is excreted unchanged in urine. Side effects of cefadroxil include diarrhea, nausea, vomiting, abdominal discomfort and rarely allergic reactions [2].
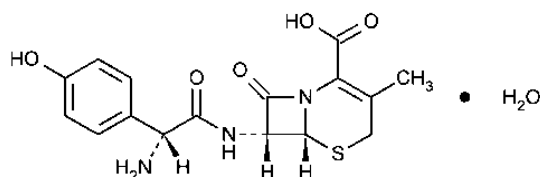

Fig 1: Structure of cefadroxil monohydrate

Cefadroxil monohydrate is available in tablets, capsules and dry powder for oral suspension, manufactured by local and multinational companies. It is an official monograph in United States Pharmacopeia (USP) [3]. Cefadroxil monohydrate has been quantitatively determined in biological fluids, including plasma, serum, and urine [4-16]. Many analytical methods were reported to determine cefadroxil in combination with other cephalosporin. Spectroscopic determination was also reported to quantitatively determine cefadroxil [13]. For bioequivalence and pharmacokinetic studies, cefadroxil has to be estimated in plasma or serum, for which high performance liquid chromatography (HPLC) method is a better choice. The aim of the present study was to develop and validate a simple, rapid, cost-effective and reproducible HPLC method for bioequivalence and pharmacokinetic studies of cefadroxil monohydrate in human subjects.

EXPERIMENTAL

Chemicals

Pure standard of cefadroxil monohydrate (CEF) was gifted by Bio Pharm (PVT) Ltd, Pakistan. Methanol (HPLC grade), trichloroacetic acid, potassium dihydrogen orthophosphate (analytical grade) and orthophosphoric acid (analytical grade) were obtained from Merck, Darmstadt, Germany.

Instruments

Schimadzu HPLC with LC solution software was used with Waters Spherisorb C18 (5 µm, 150 mm × 4.5 mm) column. Centrifuge, sonicator and analytical balance (Kern) were also used.

Chromatographic conditions

The column was C18 and detection was at 260 nm wavelength. Mobile phase was sodium dihydrogen phosphate buffer (pH 4.0) and methanol in the ratio of 96:4 with flow rate of 1.5 ml/min. Column temperature was 25 ^{o}C and injection volume was 100 µl.

Preparation of buffer solution

Sodium dihydrogen phosphate (6.8 g) was dissolved in 1000 ml deionized water and was adjusted to pH 4.0 with orthophosphoric acid (2M).

Preparation of standard solution

The stock solution of CEF was prepared in buffer by dissolving 25 mg of CEF in 25 ml of buffer (pH 4.0). The stock solution was diluted with buffer as required.

Preparation of plasma sample

Drug-free plasma was obtained from the local blood bank. CEF was spiked in plasma by adding the required quantity of stock solution to make 500 µl and then made up to volume with plasma in Eppendrof microcentrifuge tubes. Proteins were precipitated by adding 500 µl of 6 % trichloroacetic acid, vortexed for 30 s and centrifuged at 6000 rpm for 5 min [17].

Method validation

All the procedures were carried out according to United States Pharmacopeia (USP-31), Center for Drug Evaluation and Research (FDA) CDER 1994 and International conference on harmonization (ICH-1996) [3,29,30]. The method was validated for suitability, selectivity, linearity, accuracy, sensitivity, precision, reproducibility and stability.

Specificity of the method

Selectivity of the method is the property of method to identify CEF in presence of other indigenous substances of plasma. System selectivity was determined by analyzing plasma spiked with CEF and plasma matrix without it so as to observe the influence of pure matrix on

separation of active drug. It was determined by injecting three different concentrations (5, 10 and 30 µg/ml) of CEF into plasma and also blank plasma. Chromatograms were recorded and compared with that of matrix.

System suitability

System suitability is studied to observe the tolerable resolution and reproducibility of the chromatographic systems including column for the assay of CEF. The limits for system suitability were according to USP monograph of CEF. The six replicates of CEF in plasma (30 µg/ml) were injected to assess system suitability. Peak area, peak height, retention time, tailing factor and theoretical plates were observed.

Linearity

Linearity was also investigated by preparing serial dilutions of CEF in plasma from its stock solution. The concentration of six serial dilutions ranged from 30 to 0.5 µg/ml. Peak response was plotted verses concentration to observe the linear relationship. Three calibration curves were used to assess correlation coefficient (R^2). Mean, standard deviation (SD), % coefficient of variation (CV) and % accuracy were also determined by backward calculation of these concentrations from calibration curves.

Precision and accuracy

Intra-day and inter-day precision and accuracy was evaluated by analyzing three samples of three different concentrations which were injected at three different times of the same day and at three alternative days. Concentrations of CEF in plasma were 30, 10 and 5 µg/ml.

Limit of detection (LOD) and lower limit of quantification (LLOQ)

Limit of detection (LOD) and lower limit of quantification (LLOQ) was investigated by preparing serial dilutions of CEF in plasma as follows: 10, 5, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, 0.007 and 0.004 µg/ml. Signal to Noise ratio was calculated to detect LLOQ and LOD.

Analytical recovery

Percent recovery from plasma was calculated by noting the peak response of low, medium and high concentrations of cefadroxil in plasma and comparing it with peak response of the same concentration in buffer. Concentrations were 30, 10 and 5 µg/ml.

Stability parameters

Stability parameters were also validated. Nine samples of two concentration level of CEF in plasma (30 and 1 µg/ml) were prepared and stored at -20 °C. Three samples of the two concentrations were taken out, thawed and caused to freeze again. After three freeze-thaw cycles, these were analyzed by comparing similar concentration freshly prepared in plasma and CV% was determined. The remaining samples were analyzed for long term stability after storing at -20 °C for six weeks.

Statistical analysis

Standard regression curve analysis was performed by use of Statistical Package for Social Sciences (SPSS 20.0) without forcing through zero ($p < 0.05$). Linearity graphs were plotted using Microsoft Excel 2007.

RESULTS

The above stated method was validated according to International Conference on Harmonization (ICH), United States Pharmacopeia (USP) and Center for Drug Evaluation and Research-Food and Drug Administration (FDA-CDER) in terms of selectivity, suitability, linearity, precision, accuracy and stability. The coefficient of variation (% CV) for retention time, peak response, tailing factor and theoretical plates was < 3 % (Table 1). The method was selective to determine cefadroxil monohydrate in plasma because there was no peak interference of plasma with cefadroxil monohydrate at its retention time (i.e., 7.792 min). Linearity was in the range of 0.5 to 30 µg/ml with slope and intercept of 41694.53 and 22614.87, respectively ($R^2 = 0.9953$).
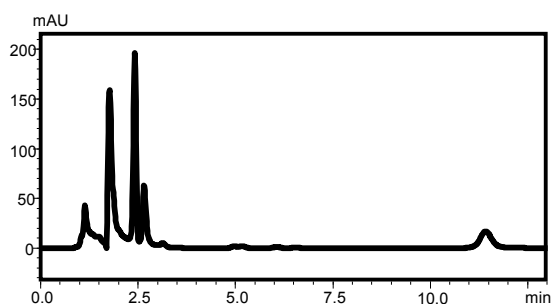

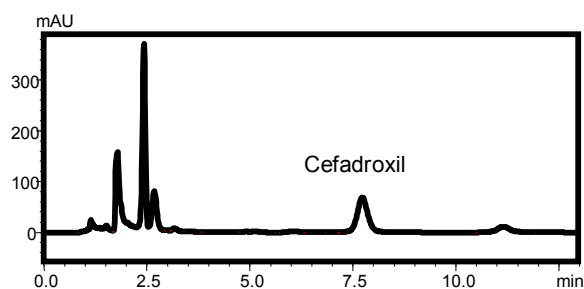**Fig 2:** Chromatogram of blank human plasma

Fig 3: Chromatogram of plasma spiked with cefadroxil monohydrate (30 µg/ml)

Limit of detection (LOD) and lower limit of quantification (LLOQ) of the above method were 0.03 and 0.06 µg/ml, respectively. Percentage absolute recovery of cefadroxil monohydrate from plasma was in the range of 71 - 90.4 %. Intraday and interday precision and accuracy was determined by injecting three different concentrations at three different times of the same day and these same concentrations on three different days. These results are as reported in Table 1 and they show satisfactory precision and accuracy; hence the method is reproducible with very low CV of 0.35 - 4.01 %, and 1.88 - 7.90 % for inter-day and intra-day precision, respectively.

Table 1: Intra- and inter-day precision and accuracy data

Concentration (µg/ml)	Mean ± SD	CV (%)	Accuracy (%)
Intra-day (n = 3)			
30	29.397 ± 0.553	1.88	98
10	10.332 ± 0.821	7.9	103
5	4.238 ± 0.197	4.65	84.8
Inter-day (n = 3)			
30	27.4 ± 0.721	2.63	91.33
10	10.44 ± 0.362	0.35	104.4
5	6.407 ± 0.257	4.01	128

Stability studies of plasma spiked with cefadroxil was conducted by storing plasma spiked with cefadroxil monohydrate at high and low concentrations (i.e., 30 and 1 µg/ml) and at -20 ^{o}C. Plasma samples after three freeze thaw cycles were analyzed by comparing with freshly prepared plasma sample of same concentration levels. Long term stability was determined by storing the plasma sample at -20 ^{o}C and analysis was done after the sixth week (Table 2). Cefadroxil monohydrate was stable at -20 ^{o}C and hence analysis of volunteer's plasma could be carried out within one month.

DISCUSSION

The method was developed and validated in order to be employed satisfactorily in pharmacokinetic or bioequivalence studies of cefadroxil monohydrate. This method is simple, rapid, and easy. Single step plasma sample preparation makes it simple and quick. The extraction of plasma protein was adopted from a previous study [17]. This is a modification of the high performance liquid chromatographic (HPLC) method of analysis as documented in cefadroxil monohydrate monograph in USP 31 [3]. The organic solvent was methanol instead of acetonitrile. Although similar mobile phase was used by other researchers [5,8], the least volume ratio of methanol was used in the above method. The column selected for the study was C18 Waters Spherisorb (USA), also used by other researcher [12].

The developed method can be used for bioavailability/bioequivalence studies of cefadroxil monohydrate and it is cost effective. Simple mobile phase, reduced ratio of organic solvent, ultra violet detection and HPLC instead of liquid chromatography-mass spectroscopy (LC-MS) make the method cost effective. Even though the detection limit and quantification limit was very low which was usually achieved by

Table 2: Freeze-thaw and long-term stability studies of plasma spiked with cefadroxil monohydrate

Conc. (µg/ml)	Freeze and thaw stability				Long-term stability		
	Freshly spiked plasma (n=3)	After three freeze thaw cycle	Mean ± SD	%CV	After 6 weeks storage at-20ºC	Mean ± SD	%CV
30	29.31	27.99 28.63 28.91	28.51±0.47	1.66	27.11 28.38 27.98	27.82±0.65	2.33
1	0.98	0.91 0.83 0.87	0.87 ± 0.04	4.6	0.76 0.81 0.79	0.79±0.025	3.18

complex and expensive methods like, post-column derivatization and LC-MS [7,11]. The LOQ result obtained in this study is comparable with LOQ of HPLC methods adopted in many bioavailability or bioequivalence studies conducted in different parts of the world [4,12,16,17,20].

CONCLUSION

The developed method can be efficiently used to detect and quantify very low concentration of cefadroxil monohydrate in human plasma such as that encountered during pharmacokinetic or bioequivalence studies. Its simplicity and low-cost makes this HPLC method of determination of cefadroxil monohydrate in human plasma very attractive.

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