Journal of Pharmaceutical Analysis 2013;3(4):285–291

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

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ORIGINAL ARTICLE

Simultaneous pharmacokinetic assessment of cefadroxil and clavulanic acid in human plasma by LC–MS and its application to bioequivalence studies

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Received 4 August 2012; accepted 4 February 2013
Available online 26 February 2013

KEYWORDS

Cefadroxil;
Clavulanic acid;
LC–MS;
APCI;
Pharmacokinetics;
Bioequivalence

Abstract A simple, rapid and selective liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) assay method has been developed and fully validated for the simultaneous quantification of cefadroxil (CF) and clavulanic acid (CA) in human plasma. Analytes and internal standard (IS) were extracted from human plasma by solid-phase extraction (SPE) technique using Sam prep (3 mL, 100 mg) extraction cartridge. The extracted samples were chromatographed on a reverse phase C_{18} column using a mixture of methanol: acetonitrile: 2 mM ammonium acetate (pH 3.5) (25:25:50, v/v/v) as the mobile phase at a flow rate of 0.8 mL/min. Quantification of the analytes and IS were carried out using single quadrupole LC–APCI–MS through selected-ion monitoring (SIM) at m/z 362 and m/z 198, for CF and CA, respectively. Method validation was performed as per the FDA guidelines and the results met the acceptance criteria. Plasma concentration of CF and CA followed by the oral administration of CF/CA (500/125 mg) pill to healthy male volunteers ($n=12$) was measured. Area under plasma concentration–time curve from 0 to 12 h ($AUC_{0-12\ h}$) and 0 h extrapolated to infinity ($AUC_{0-\infty}$) were calculated. The ratio of $AUC_{0-12\ h}/AUC_{0-\infty}$ was found to be >85% for all the subjects, as recommended by the FDA guidelines.

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1. Introduction

Cefadroxil (CF) (8-[2-amino-2-(4-hydroxyphenyl)-acetyl]amino-4-methyl-7-oxo-2thia-6-azabicyclo-[4.2.0]oct-4-ene-5-carboxylic acid, $C_{16}H_{17}N_3O_5S$, MW 363.3), is a broad-spectrum first generation cephalosporin, used in the treatment of urinary tract infections. It has significant activity against both

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Peer review under responsibility of Xi'an Jiaotong University.

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http://dx.doi.org/10.1016/j.jpha.2013.02.003

gram-positive and gram-negative bacteria. Clavulanic acid (CA) ((*Z*)-(2*R*, 5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]-heptane-2-carboxylate, $C_8H_9NO_5$, MW 199.2), is a beta-lactamase inhibitor. Plasmid mediated beta-lactamases are responsible for transferred drug resistance to beta-lactam antibiotics, such as penicillin and cephalosporin's. CA covalently binds to a serine residue in the active site of the beta-lactamase, and inhibits them. This inhibition restores the antimicrobial activity of beta-lactam antibiotics against lactamase-secreting resistant bacteria.

A number of analytical methods have been reported for quantification of CF [1–17] and CA [18–22] individually or with other drugs in biological samples. To the best of our information, only one method has been reported for the simultaneous determination of CF and CA [23]. To date, no liquid chromatography mass spectrometry (LC–MS) method has been reported for the simultaneous determination of CF and CA in human plasma. The present work describes a simple, selective and sensitive method, which employs a simple solid-phase extraction (SPE) technique for sample preparation and LC–MS for the simultaneous quantitation of CF and CA in human plasma. The application of this assay method to a clinical pharmacokinetic study in healthy male volunteers following oral single pill administration of CF and CA is described.

2. Experimental

2.1. *Chemicals and reagents*

Working standards of CF (99.9% pure), potassium clavulanate (99.9% pure), chloramphenicol (99.9% pure) (Internal standard, IS) and CF/CA (500/125 mg) pills were obtained from Macleods Pharmaceuticals Pvt. Ltd. (Mumbai, India). Water used for the LC–MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). HPLC grade methanol and acetonitrile were procured from Merck (Mumbai, India). All other chemicals and solvents of analytical grade were purchased from Merck (Mumbai, India). Sam prep (3 mL, 100 mg) extraction cartridge was purchased from Ranbaxy fine chemicals Ltd. (New Delhi, India). Pooled plasma was collected from healthy, drug-free volunteers and stored at -20 ± 2 °C until analysis.

2.2. *Preparation of calibration standards and quality control samples*

Calibration samples were prepared by spiking 950 μL of control human plasma with the appropriate working standard solution of the each analyte (25 μL dilution of CF and 25 μL of CA). Calibration curve standards consisting of a set of eight non-zero concentrations ranging from 5.0 to 30000.0 ng/mL for CF and 2.0 to 12000.0 ng/mL for CA were prepared.

Quality control samples (QCs) at a minimum of three concentrations (lower quality control (LQC), middle quality control (MQC), and high quality control (HQC)) were prepared by spiking the working standard solution into a pool of drug free human plasma. The QCs prepared for each analyte are 15.0 (LQC), 15000.0 (MQC) and 25000.0 ng/mL (HQC) for CF and 10.0 (LQC), 5000.0 (MQC) and 9000.0 ng/mL (HQC) for CA. All the samples were stored at -20 ± 2 °C for subsequent use.

2.3. *Sample processing*

A 500 μL aliquot of human plasma sample was mixed with 500 μL of the IS working solution (100 μg/mL) and vortexed for 10 s. The sample mixture was loaded onto a Sam prep (3 mL, 100 mg) extraction cartridge that was pre-conditioned with 1.0 mL of methanol followed by 1.0 mL water. The extraction cartridge was washed with 2.0 mL of water. CF, CA and IS were eluted with 0.5 mL of mobile phase. Aliquot of 20 μL of the extract was injected into the LC–MS.

2.4. *LC–MS analysis*

Shimadzu LCMS-2010A (Shimadzu Corporation, Kyoto, Japan) consisting of Phenomenex C_{18} column (150 mm × 4 mm i.d., 5 μm), LC-10 AD-Vp solvent delivery system (pump), SIL 10 AD-Vp auto injector, CTO 10 Vp column oven, DGU 14AM de gasser was used for the study. Aliquots of the processed samples (20 μL) were injected into the column, which was kept at 20 °C. The isocratic mobile phase, a mixture of methanol: acetonitrile: 2 mM ammonium acetate (pH 3.5) (25:25:50, v/v/v) was delivered at 0.8 mL/min into the atmospheric pressure chemical-ionization (APCI) chamber of the mass spectrometer. Quantitation of CF, CA and IS was performed on the single quadrupole mass spectrometer in the negative ion mode. The ion source temperature, desolvation temperature, and the capillary voltage were set at 400 °C, 250 °C, and 1.3 kV, respectively. Detection of the ions was carried out in the selected-ion monitoring (SIM) mode, at m/z 362 for CF, m/z 198 for CA, and m/z 322 for the IS. The analysis data obtained were processed by LC–MS solutions data station.

2.5. *Method validation*

Method validation of CF and CA in human plasma was performed as per the US FDA guidelines [26]. The assay was validated for specificity, linearity, sensitivity, accuracy, precision, extraction recovery, matrix effect and stability.

Specificity of the method was determined by analyzing six different lots of human plasma samples. The responses of the interfering substances or background noises at the retention time of CF and CA are acceptable if they are less than 20% of the response of the lowest standard curve point or lower limit of quantification (LLOQ).

Linearity was tested for CF and CA in the concentration range of 5.0–30000.0 and 2.0–12000.0 ng/mL, respectively. For the determination of linearity, standard calibration curves containing eight points (non-zero standards) were plotted and checked. The acceptance limit of accuracy for each of the back-calculated concentrations is $\pm 15\%$ except LLOQ, where it is $\pm 20\%$. For a calibration run to be accepted at least 67% of the standards, the LLOQ and upper limit of quantification (ULOQ) are required to meet the acceptance criterion, otherwise the calibration curve was rejected. Six replicate analyses were performed on each calibration standard. Sensitivity was established from the background noise or response from six spiked LLOQ samples. The six replicates should have a precision of $\pm 20\%$ and an accuracy of $\pm 20\%$.

Intra-, inter-day assay precision and accuracy were determined by analyzing six replicates at three different QCs levels on three independent days. The acceptance criteria included

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