JOURNAL OF ADVANCEMENT IN
MEDICAL AND LIFE SCIENCES

Journal homepage: http://scienceq.org/Journals/JALS.php

Research Article Open Access

New Improved UPLC-MS/MS Method for Reliable Determination of Clarithromycin in Human Plasma to Support a Bioequivalence Study

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Received: December 28, 2014, Accepted: February 7, 2015, Published: February 7, 2015.

ABSTRACT

An improved, highly sensitive UPLC-MS/MS method has been developed for the determination of clarithromycin in human plasma. For sample preparation, liquid-liquid extraction with *n*-hexane: methyl *tert*-butyl ether (20:80, *v/v*) mixture was carried out using clarithromycin 13C-d3 as the internal standard (IS). Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) analytical column was used for chromatography with methanol-5.0 mM ammonium formate, pH 3.0 (78:22, *v/v*) as the mobile phase under isocratic conditions. The analysis time was 1.5 min. Quantitation of analyte was done by tandem mass spectrometer using electrospray ionization in the positive mode. The precursor → product ion transitions monitored for clarithromycin and IS were *m/z* 748.9 → 158.1 and *m/z* 752.8 → 162.0 respectively. The method was validated over a dynamic concentration range of 0.80-1600 ng/mL with correlation coefficient (r^2) ≥ 0.9998. The mean extraction recovery of clarithromycin was 96.2 % across six quality control levels. Intra-batch and inter-batch accuracy and precision (% CV) ranged from 96.8 to 103.5 % and 1.28 to 4.85 % respectively. Stability of clarithromycin in plasma was evaluated under different conditions like bench top, auto sampler, dry and wet extract, freeze-thaw and long term. The present method was successfully applied to a bioequivalence study in 20 healthy subjects who received single oral dose 250 mg clarithromycin tablet formulation. The reproducibility of the method was investigated by reanalysis of 100 incurred samples.

Keyword: Clarithromycin, clarithromycin 13C-d3, UPLC-MS/MS, human plasma, sensitive, bioequivalence

INTRODUCTION

Clarithromycin (6-O-methylerythromycin, CLA) is a semi-synthetic 14-membered macrolide useful for the treatment of a number of bacterial infections. It is active against several Gram-positive (*Staphylococcus aureus*, *Streptococcus pneumonia*, *Streptococcus pyogenes*) and Gram-negative (*Haemophilus influenza*, *Haemophilus parainfluenzae*, *Moraxella catarrhalis*) bacteria and other microorganisms like *Mycoplasma pneumonia*, *Chlamydia pneumonia*, *Mycobacterium avium* and *Mycobacterium intracellulare* [1,2]. The macrolides are a group of antibiotics whose activity is due to the presence of a large macrocyclic lactone ring which has one or more deoxy sugars, usually cladinose and desosamine [3]. CLA is rapidly absorbed from the gastrointestinal tract after oral administration and has an absolute bioavailability of ~ 50%. It is mainly metabolized by cytochrome P450 (CYP) 3A enzymes (CYP3A4 and CYP3A5) to an active 14-hydroxy metabolite. The plasma protein binding of CLA is about 65-70 % and the maximum plasma concentration is attained within 2-4 h [2, 4].

Literature presents several methods to determine CLA either as a single analyte [5-13], or together with its metabolite and other antibiotics [14-20] in different biological matrices like human plasma [5-7, 10-13, 15, 17, 18], rat plasma [8, 16], human serum [9, 14], human urine [11], gastric juice and gastric tissues [16], horse plasma, epithelial lining fluid and broncho-alveolar cells [19] and dried blood spots [20]. In these methods different analytical techniques like capillary electrophoresis [11], HPLC with electrochemical [5, 15, 16], UV [7, 8], fluorescence [9, 14], and mass spectrometric detection [6, 10, 12, 13, 17-20] have been employed. Amongst these only one UPLC-MS/MS based method has been reported with a linear range of 1.0-3000 ng/mL in human plasma [13]. This method presents a chromatographic run time of 2.0 min with 200 µL plasma volume for processing. A comparative summary of salient features of methods developed in human plasma is presented in Table 1.

In the present work we report an improved UPLC-MS/MS method for determination of CLA in human plasma with respect to the sensitivity, analysis time and plasma sample volume over existing methods. Further, the method employs a deuterated internal standard for better accuracy and precision of the data. The method was applied to a bioequivalence study in 20 healthy subjects using 250 mg clarithromycin tablets.

Table 1 Comparative assessment of chromatographic methods developed for clarithromycin in human plasma

Sr. No.	*Technique; Linear range (ng/ml)*	*Extraction procedure; internal standard*	*Sample volume (µL)*	*Retention time; run time (min)*	*Application*	*Ref.*
1	HPLC-electro-chemical; 100-4000	PP with membrane syringe; roxithromycin	120	15.3; 30.0	Pharmacokinetic study in 4 healthy volunteers with a single oral dose of 500 mg clarithromycin tablet	5
2	HPLC-MS/MS; 2.95-20016	LLE with *n*-hexane-ethyl acetate; roxithromycin	300	1.88; 3.0	Pharmacokinetic study in 9 healthy volunteers with a single oral dose of 500 mg clarithromycin tablet	6
3	HPLC-UV; 31.25-2000	LLE with *n*-hexane-1-butanol and back extraction with acetic acid; norverapamil	1000	8.37; 10.0	Bioequivalence study with different 250 mg tablet formulations of clarithromycin in 14 healthy volunteers	7
4	LC-MS/MS; 10-5000	PP with acetonitrile; telmisartan	50	1.76; 2.4	Bioequivalence study with 500 mg tablet formulations of clarithromycin in 20 healthy volunteers	10
5	LC-MS/MS; 100-5000	PP with acetonitrile in 96 well plate; roxithromycin	25	1.20; 1.75	Pharmacokinetic study in 6 healthy volunteers with 500 mg tablet formulation of clarithromycin	12
6	UPLC-MS/MS; 1-3000	LLE with diethyl ether; roxithromycin	200	< 1.0; 2.0	Pharmacokinetic study in 18 healthy volunteers with 500 mg tablet formulation of clarithromycin	13
7	HPLC-electroche-mical; 100-10000	PP with methanol and acetonitrile followed by SPE; roxithromycin	500	~13.0; 19.0	Study on eluant composition and column temperature for reproducible electrochemical response	15
8[a]	LC-MS/MS; 36.5-5066.2	PP with acetonitrile; erythromycin	200	1.30; 2.0	Bioequivalence study with 500 mg tablet formulations of clarithromycin in 40 healthy volunteers	17
9[b]	LC-MS/MS; 100-10000	PP with methanol and acetonitrile; cyanoimipramine	100	~2.5; 3.5	--	18
10	UPLC-MS/MS; 0.8-1600	LLE with *n*-hexane-methyl *tert*-butyl ether; clarithromycin 13C-d3	100	1.01; 1.50	Bioequivalence study with 250 mg tablet formulations of clarithromycin in 20 healthy volunteers; incurred sample reanalysis with 100 samples	PM

aAlong with 14-hydroxy clarithromycin; bAlong with 14-hydroxy clarithromycin, rifampicin and its metabolite; PP: protein precipitation; LLE: liquid-liquid extraction; SPE: solid phase extraction; PM: present method

EXPERIMENTAL

Chemicals and materials

Reference standards of clarithromycin (99.25 %) and clarithromycin 13C-d3 (IS, 99.58 %) were procured from Clearsynth Labs Pvt. Ltd. (Mumbai, India). HPLC grade methanol was procured from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). Analytical grade ammonium formate, formic acid, methyl *tert*-butyl ether and *n*-hexane were purchased from Merck Specialties Pvt. Ltd. (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20 °C until use.

Optimized liquid chromatographic and mass spectrometry conditions

A Waters Acquity UPLC system (MA, USA) was used for setting the reversed-phase liquid chromatographic conditions. The analysis of CLA and IS was performed on Acquity UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) analytical column maintained at 30°C in a column oven. The mobile phase consisted of methanol-5.0 mM ammonium formate, pH 3.0 (78:22, *v/v*) and was delivered at a flow rate of 0.350 mL/min. The sample manager temperature was maintained at 5°C and the pressure of the system was 6300 psi. Quantitative analysis of CLA and IS was carried out on a Quattro Premier XE (USA) triple quadrupole mass spectrometer from Micro Mass Technologies (MA, USA). It was operated in the positive electro spray ionization mode and the ion transitions monitored for CLA and IS were *m/z* 748.9/158.1 and *m/z* 752.8/162.0 respectively. The optimized mass parameters included cone gas flow: 110 L/h, desolvation gas flow: 630 L/h, capillary voltage: 1.4 kV, source temperature: 120 °C, desolvation temperature: 400 °C and extractor voltage: 4.0 V. The optimum values for cone voltage and collision energy were 35 V and 30 eV for CLA and 35 V and 32 eV for IS respectively. MassLynx software version 4.1 was used for data collection and peak integration and to control all parameters of UPLC and mass spectrometer.

Standard stock, calibration standards and quality control samples

The standard stock solution of CLA (500.0 µg/mL) was prepared by dissolving requisite amount in methanol. Further, intermediate solutions (100.0 µg/mL and 50.00 µg/mL) for spiking was prepared in methanol:water (60:40, *v/v*). Calibration standards

(CSs) were prepared at 0.80, 1.60, 4.00, 12.00, 36.00, 75.00, 200.0, 400.0, 800.0, 1600 ng/mL and quality control (QC) samples were made at six levels, 1400 ng/mL (HQC, high quality control), 600.0/150.0 ng/mL (MQC-1/2, medium quality control), 2.40 ng/mL (LQC, low quality control) and 0.80 ng/mL (LLOQ QC, lower limit of quantification quality control) for CLA. Stock solution (100.0 µg/mL) of the internal standard was prepared by dissolving 1.0 mg of clarithromycin 13C-d3 in 10.0 mL methanol. Its working solution (100 ng/mL) was prepared by appropriate dilution of the stock solution in methanol:water (60:40, *v/v*). All standard stock and working solutions used for spiking were stored at 5 °C, while CSs and QC samples in plasma were kept at -70 °C until use.

Protocol for sample preparation

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 µL of plasma sample, 25 µL of internal standard was added and vortexed for 10s. Further, 2.0 mL of *n*-hexane: methyl *tert*-butyl ether (20:80, *v/v*) solvent mixture was added and vortexed for another 1.0 min. The samples were then centrifuged at 13148 × g for 5 min at 10°C. The organic layer was separated and collected in ria vials, followed by evaporation of the samples at 50 ºC under N_2 gas. Finally the samples were reconstituted with 250 µL mL of mobile phase solution, briefly vortexed and 10 µL was used for injection in the chromatographic system.

Method validation procedures

The method was validated as per the current regulatory requirements to establish the accuracy and precision of the method [21]. The parameters studied were similar to our previous work [22] and are described in brief.

System suitability experiment was performed by injecting six consecutive injections using aqueous standard mixture of CLA and IS at the start of each batch to evaluate the precision for the measurement of area response and retention time. System performance was checked by measuring the signal to noise ratio (S/N) for the lowest concentration of the analyte in the calibration curve. Carryover assessment was carried out through the following sequence of injections, extracted blank plasma, ULOQ sample, two extracted blank plasma samples, LLOQ sample, and extracted blank plasma at the beginning of the batch.

The selectivity of the method was investigated by analyzing eight individual blank plasma sources (5 normal K_3EDTA, 1 heparinized, 1 haemolysed and 1 lipemic). Each blank plasma sample was tested for interference by comparing with spiked plasma samples at LLOQ concentration of the analyte. Method was also assessed for interference of some commonly used medications by human volunteers. This included domperidone, paracetamol, ranitidine, diclofenac, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 µg/ mL) were prepared in methanol:water (60:40, *v/v*), spiked with the sample and analyzed to check for any interference in the quantification of the analyte and IS.

Five calibration curves with ten non-zero concentrations were prepared to determine linearity of the method. Peak area ratio (analyte/IS) response obtained through multiple reaction monitoring *vs.* concentration plot was drawn using linear regression with $1/x^2$ weighting. Intra-batch accuracy and precision were evaluated by measurement of analytes in plasma samples on the same day. The analytical run consisted of a calibration curve and six replicates of QC samples at five concentration levels. The inter-batch accuracy and precision were assessed by analysis of five separate precision and accuracy batches on three consecutive days. The reinjection reproducibility for retention time was also checked by reinjection of one entire analytical batch.

The extraction recovery of CLA and IS from human plasma was evaluated in six replicates by comparing the mean peak area response of pre-extraction spiked samples to those of post-extraction spiked samples [23]. Matrix effect, expressed as matrix factors (MFs) was assessed by comparing the mean area response of post-extraction spiked samples with mean area of solutions prepared in mobile phase solutions (neat standards) with identical concentration. The relative matrix effect was determined by assessment of precision (% CV) values for slopes of calibration lines from eight different plasma lots [24]. For qualitative assessment of matrix effect, post column infusion experiment was performed by infusing a standard solution of CLA at 800 ng/mL concentration into the mobile phase at 10 µL/min post column as reported previously [25].

The stability of CLA in plasma was examined at LQC and HQC levels under different study conditions: bench top at 25 °C, freeze-thaw at -20 °C and -70 °C, wet extract (auto sampler) at 5 °C and 25 ºC, dry extract at 5 °C, and long term stability at -20 °C and -70 °C respectively. The area ratio response (analyte/IS) of stability samples was compared against freshly prepared comparison standards with identical concentration. The procedure also included an evaluation of short term (25 ºC) and long term (5 ºC) stability of CLA and IS in stock and working solutions.

The ruggedness of the method was tested on two different columns of the same make but different batch numbers and also by employing different analysts using two precision and accuracy batches. Further, dilution reliability of the method was studied by analyzing six replicates samples prepared as spiked standard at 2 times ULOQ concentration (3200 ng/mL) after five-/ten-fold dilution respectively. The precision and accuracy for dilution reliability was determined by comparing the results against freshly prepared calibration curve standards.

Bioequivalence study, statistical analysis and incurred sample reanalysis

A randomized single dose study was conducted for the assessment of bioequivalence of a test (250 mg clarithromycin tablets from a Generic Indian Company, India) with a reference (BIAXIN® FILMTAB®, 250 mg clarithromycin tablets from Abbott Laboratories, North Chicago, USA) formulation in 20 healthy subjects under fasting. The subjects were in the age group of 18-45 years, having body mass indices of 19.5- 26.5 kg/m^2. The study was performed according to International Conference on Harmonization and USFDA guidelines [26]. Each subject was judged to be in good health through medical history, physical examination and routine laboratory tests. Subjects were informed of the aims and risks involved in the study and their written consent was obtained. After overnight fasting, the subjects were administered a single dose of test and reference formulations with 240 mL of water after recommended wash out period of 7 days. Blood samples were collected at 0.00 (pre-dose), 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.33, 3.67, 4.0, 4.5, 5.0, 5.5, 6.0, 8.0, 10.0, 12.0, 16.0, 24.0, 36.0, 48.0 and 72.0 h in K_3-EDTA vacutainers. Plasma was separated by centrifugation and kept frozen at -70°C until analysis.

For the purpose of bioequivalence analysis, C_{max}, $AUC_{0\text{-}72h}$ and $AUC_{0\text{-}inf}$ were considered as primary variables. The pharmacokinetic parameters of CLA were estimated by

non-compartmental analysis using WinNonlin® software version 5.3 (Pharsight Corporation, Sunnyvale, CA, USA). The statistical analysis for pharmacokinetic parameters included descriptive statistics, analysis of variance and two one-sided tests for bioequivalence using SAS® software version 9.2 (SAS Institute Inc., Cary, NC, USA). Descriptive statistics involved calculation of least square geometric mean for C_{max}, AUC_{0-72}, and AUC_{0-inf}. To determine whether the test and reference formulations were pharmacokinetically equivalent, C_{max}, AUC_{0-72}, and AUC_{0-inf} and their ratios (test/reference) were assessed using log transformed data; their means and 90% confidence intervals (CIs) were also calculated. The formulations were considered pharmacokinetically equivalent if the difference between the compared parameters was statistically non-significant ($P \geq 0.05$) and the 90% CI for these parameters were within 0.8 to 1.25.

The reproducibility of the assay was assessed by reanalysis of 100 subject samples. The samples selected were mainly near the C_{max} and the elimination phase in the pharmacokinetic profile of the drug. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. According to the acceptance criterion, at least two-thirds of the original results and repeat results should be within 20% of each other [27].

RESULT AND DISCUSSION

Method development

The present work was intended to improve upon the existing UPLC-MS/MS method [13] with respect to the sensitivity, analysis time and plasma sample volume for reliable determination of CLA in human plasma. Mass spectrometric conditions like collision energy, cone voltage and capillary voltage were suitably optimized to obtain maximum sensitivity for CLA and CLA 13C-d3 (IS). Unlike other methods which employed a general IS, a deuterated IS was used for better accuracy and precision of the obtained data. MS/MS analysis was done using electrospray ionization in the positive mode to achieve high selectivity and a good linearity in regression curves. The full scan mass spectra for CLA and IS predominantly contained precursor $[M+H]^+$ ions at *m/z* 748.9 and 752.8 respectively. The most abundant and consistent product ions in Q3 MS spectra for CLA and IS were observed at *m/z* 158.1 and 162.0 respectively (**Figure 1**). These product ions were formed by the breaking up of glycosidic bond to obtain fragments containing tertiary amino group from the protonated precursor ions. A dwell time of 100 ms for both the compounds was sufficient to obtain sufficient to obtain at least 23 data points for quantification. The current UPLC-MS/MS method was more sensitive (0.8 ng/mL) compared to all reported methods for CLA.

Sample preparation is crucial for reliable quantitation of drugs in biological samples. Several reported methodologies have adopted either protein precipitation (PP) [5, 10, 12, 15] or liquid-liquid extraction (LLE) [6, 7, 13] for sample clean-up. Thus, PP was initially tested with acetonitrile and methanol as discussed earlier [10, 15]. However, due to significant matrix effect the recovery of CLA was very poor (40-50 %), especially at LLOQ and LQC levels.

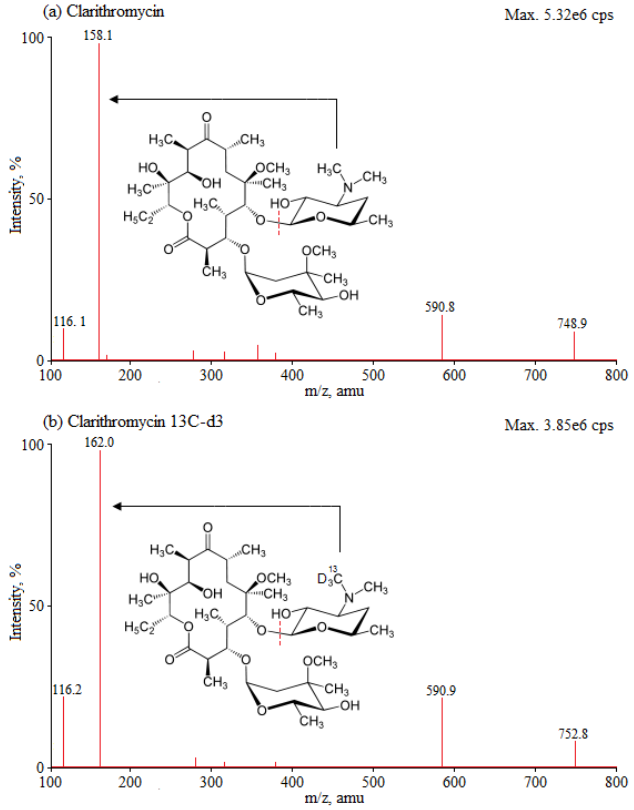

Figure 1 Product ion mass spectra of (a) clarithromycin (*m/z* 748.9 → 158.1) and (b) internal standard, clarithromycin 13C-d3 (*m/z* 752.8 → 162.0) in the scan range 100-800 amu and in the positive ionization mode.

Thus, LLE was tried in different solvents systems like diethyl ether, methyl *tert*-butyl ether, n-hexane and ethyl acetate as the CLA has high logP value of 3.2. Further, in our effort to have an efficient solvent extraction protocol for CLA and IS their combinations were also tested. After several trials it was found that *n*-hexane:methyl *tert*-butyl ether (20:80, *v/v*) was the best extraction solvent system for quantitative and precise recovery of the analyte and IS from plasma samples. Only 100 µL plasma sample was used for processing unlike the other UPLC-MS/MS method [13] which employed 200 µL sample.

The nature of mobile phase and its composition, buffer pH and flow rate was rigorously optimized on UPLC BEH C18 (50 mm × 2.1 mm, 1.7 µm) analytical column so as to have adequate detector response and acceptable peak shape. Initially, organic diluents like acetonitrile/methanol along with acidic buffers (ammonium formate-formic acid/ammonium acetate-acetic acid) in the pH range of 3.0-5.5 were tested. Additionally, the concentration of ammonium formate and ammonium acetate was also tested, including different flow rates (0.250, 0.300, 0.350 and 0.400 mL/min) for adequate response. It was found that methanol and ammonium formate-formic acid buffer provided superior response compared to acetonitrile and ammonium acetate-acetic acid buffer. Thereafter, their ratio was adjusted for adequate retention, response and better peak shape for CLA and IS. The best chromatographic conditions were obtained with methanol and 5.0 mM ammonium formate, pH 3.0 adjusted with 0.1% formic acid (78:22, *v/v*) as the mobile phase at a flow rate of 0.350 mL/min. These conditions afforded a run time of 1.5 min with retention times of 1.01 and 1.02 min for CLA and IS respectively. This analysis time was shorter compared to the existing

UPLC-MS/MS method. The reinjection reproducibility (% CV) of retention times for CLA was ≤ 0.91 % for one entire batch on the same column. Clarithromycin 13C-d3, used as IS in the present work gave excellent results for accuracy and precision at each QC level. The multiple reaction monitoring (MRM) chromatograms illustrated in **Figure 2** of extracted blank plasma (double blank), blank plasma fortified with IS, CLA at 0.8 ng/mL and IS and a subject sample at C_{max} demonstrates the overall performance and selectivity of the method.

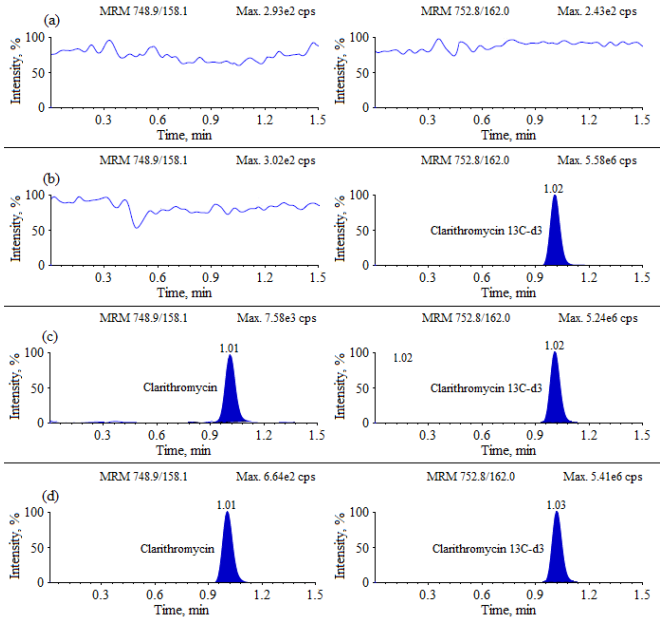

Figure 2 Representative MRM ion-chromatograms of (a) double blank plasma without analyte and IS, (b) blank plasma with working solution of clarithromycin 13C-d3 (*m/z* 752.8 → 162.0), (c) clarithromycin (*m/z* 748.9 → 158.1) at LLOQ and IS, (d) clarithromycin in real subject sample at C_{max} and IS after administration of 250 mg dose of clarithromycin.

Method validation results

The precision (% CV) of system suitability test was observed in the range of 0.26 to 0.51 % for the retention time and 0.84 to 1.28 % for the area response for CLA and IS, while the signal to noise ratio for system performance was ≥ 20. Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. The column and auto-sampler carry-over evaluation showed negligible carry over in blank plasma (≤ 0.38 % of LLOQ sample) after subsequent injection of ULOQ sample (chromatograms not shown) at the retention time of CLA and IS.

There was adequate linear correlation between the CLA concentration and the response over the concentration range of 0.80-1600 ng/mL with correlation coefficient (r^2) ≥ 0.9998. The mean linear equation for the calibration curves was $y = (0.001632 \pm 0.000004)x - (0.000013 \pm 0.000011)$. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 97.7 to 101.7 % and 1.03 to 2.90 % respectively. The analytical method was shown to be selective based on absence of any analytical signals at the retention time of CLA and IS in ten different batches of blank plasma. The intra-batch and inter-batch precision and accuracy results were within the stipulated range of ±15 % of the nominal concentration and < 15 % CV of the mean values as shown in Table 2.

Table 2 Intra-batch and inter-batch precision and accuracy data for clarithromycin

Nominal concentration (ng/mL)	Intra-batch (n = 6; single batch)			Inter-batch (n = 30; 6 from each batch)		
	Mean conc found (ng/mL)	% CV	% Accuracy	Mean conc found (ng/mL)	% CV	% Accuracy
HQC (1400)	1405.6	2.80	100.4	1393.0	1.28	99.5
MQC-1 (600.0)	613.2	3.23	102.2	585.6	3.59	97.6
MQC-2 (150.0)	147.5	2.91	98.3	154.3	2.84	102.9
LQC (2.40)	2.484	2.89	103.5	2.374	2.94	98.9
LLOQ QC (0.80)	0.774	4.85	96.8	0.825	3.68	103.2

CV: coefficient of variation; HQC: high quality control; MQC: medium quality control; LQC: low quality control; LLOQ QC: lower limit of quantitation quality control

Table 3 Extraction recovery and matrix factor for clarithromycin

QC level	Area response (replicates, n = 6)			% Extraction recovery, (B/A)		Matrix factor		
	A (post-extraction spiking)	B (pre-extraction spiking)	C (neat samples in mobile phase)	Analyte	IS	Analyte (A/C)	IS	IS-normalized
LQC	2309	2205	2127	95.5	97.0	1.085	1.077	1.007
MQC-2	148124	142127	136189	96.0	96.5	1.087	1.082	1.004
MQC-1	586876	566554	549738	96.5	97.4	1.067	1.071	0.996
HQC	1371549	1328753	1295364	96.9	95.9	1.058	1.067	0.991

IS: Internal standard; LQC: low quality control; MQC: medium quality control; HQC: high quality control.

Matrix effect can be attributed to some undesirable effects that originate from a biological matrix. These components may result in ion suppression/enhancement, decrease/increase in sensitivity of analyte over a period of time, increased baseline, imprecision of data, drift in retention time and distortion or tailing of a chromatographic output. It is suggested that evaluation of matrix factors (MFs) can help to assess the matrix effect. Further, matrix effect needs to be checked in lipemic and haemolysed plasma samples in addition to normal K_3EDTA plasma. MFs can be determined from the peak area response for the analyte and IS separately, while the ratio of the two factors yields IS-normalized MF. The IS-normalized MFs using stable-isotope labelled IS should be close to unity because of the similarity in the chemical properties and elution times for the analyte and IS. The extraction recovery and matrix factors for CLA are presented in Table 3. The mean extraction recovery for CLA and IS was 96.2 and 96.7 % respectively. The relative matrix effect in eight different plasma sources was evaluated by calculation of precision (% CV) of the slopes of calibration lines (Table 4).

Table 4 Relative matrix effect in eight different lots of human plasma for clarithromycin

Plasma lot	Slope
Lot-1 (K_3EDTA)	0.001651
Lot-2 (K_3EDTA)	0.001615
Lot-3 (K_3EDTA)	0.001648
Lot-4 (K_3EDTA)	0.001618
Lot-5 (K_3EDTA)	0.001653
Lot-6 (heparinized)	0.001608
Lot-7 (haemolysed)	0.001658
Lot-8 (lipemic)	0.001606
Mean	0.001632
± Standard deviation	0.000022
% Coefficient of variation	1.36

SD: Standard deviation; CV: coefficient of variation

Further, qualitative assessment of matrix effect through post-column infusion experiment showed no ion suppression or enhancement at the retention time of CLA and IS in the chromatograms (Figure 3)

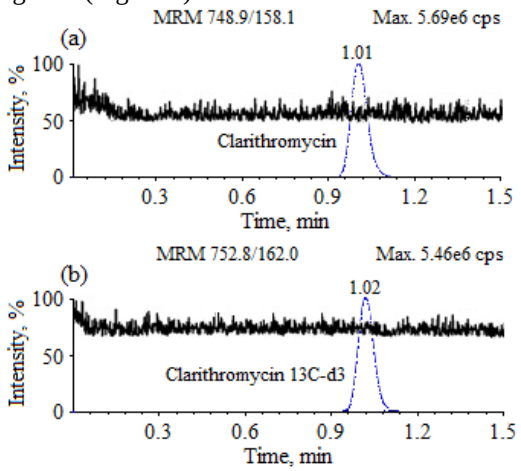

Figure 3 Injection of extracted blank human plasma during post column infusion of (a) clarithromycin at 800 ng/mL and (b) clarithromycin 13C-d3 at100 ng/mL.

The stability of analyte and IS in human plasma and stock solutions was examined under different storage conditions. Stock solutions for short term stability of CLA and IS were stable at room temperature up to 24 h and between 2-8 °C for a minimum period of 30 days. CLA in control human plasma (bench top) at room temperature was stable for at least 20 h at 25°C and for minimum of five freeze and thaw cycles. Autosampler (processed sample) stability of the spiked quality control samples was determined up to 52 h. Similarly, dry and wet extract stability of the samples was ascertained up to 24 h and 48 h respectively. Long term stability of the spiked quality control samples remained unchanged up to 176 days. The % change values for different stability experiments at LQC and HQC levels in plasma are shown in Table 5.

Table 5 Stability results of clarithromycin in plasma under various conditions (n = 6)

Storage conditions	QC Level	Nominal conc. (ng/mL)	Mean stability sample (ng/mL) ± SD	Change (%)
Bench top stability at 25 °C, 20 h	HQC	1400	1404.80 ± 37.07	0.34
	LQC	2.40	2.407 ± 0.070	0.31
Freeze-thaw stability at -20 °C	HQC	1400	1410.60 ± 38.93	0.76
	LQC	2.40	2.409 ± 0.037	0.39
Freeze-thaw stability at -70 °C	HQC	1400	1391.80 ± 37.85	-0.59
	LQC	2.40	2.390 ± 0.046	-0.42
Autosampler stability at 4°C,52 h	HQC	1400	1397.60 ± 39.68	-0.39
	LQC	2.40	2.382 ± 0.057	-0.72
Dry extract stability at 2-8°C, 24 h	HQC	1400	1408.80 ± 37.79	0.63
	LQC	2.40	2.386 ± 0.042	-0.57
Wet extract stability at 2-8°C, 48 h	HQC	1400	1389.00 ± 40.70	-0.79
	LQC	2.40	2.411 ± 0.039	0.49
Long term stability at -20 °C, 176 days	HQC	1400	1392.60 ± 32.78	-0.53
	LQC	2.40	2.404 ± 0.031	0.18
Long term stability at -70 °C, 176 days	HQC	1400	1406.60 ± 24.26	0.47
	LQC	2.40	2.394 ± 0.026	-0.22

SD: standard deviation; n: number of replicates; HQC: high quality control;

LQC: low quality control

$$\%\text{Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$$

The precision (% CV) and accuracy values for two different columns ranged from 2.12 to 3.34 % and 96.1 to 103.9 % respectively at all five quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 1.64-3.41 % and 97.6 to 100.1 % respectively at these levels. The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. The precision and accuracy values for 1/2th and 1/10th dilution ranged from 2.97 to 4.57 % and 96.9-101.3 % respectively for CLA.

Application of the method in healthy human subjects

The validated method was successfully applied for the assay of CLA in 20 healthy Indian males. **Figure 4** shows the plasma concentration vs. time profile of clarithromycin after oral administration of 250 mg dose under fasting.

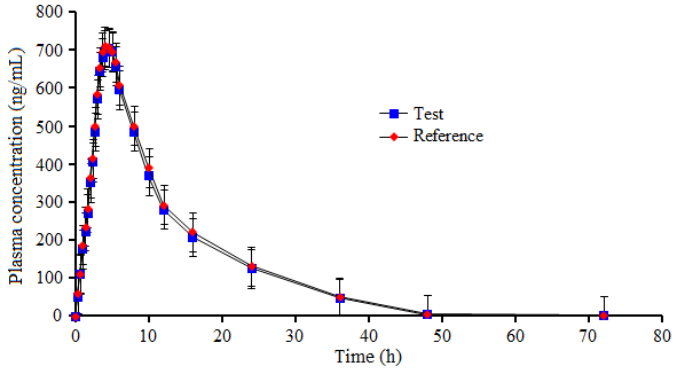

Figure 4 Mean plasma concentration-time profile of clarithromycin after oral administration of test (250 mg tablets from a Generic Indian Company, India) with a reference (BIAXIN® FILMTAB®, 250 mg tablets from Abbott Laboratories, USA) formulation to 20 healthy subjects.

Approximately 900 samples including the calibration and QC samples with volunteer samples were run and analyzed during a period of 5 days and the precision and accuracy for calibration and QC samples were well within the acceptable limits. The important pharmacokinetic parameters, maximum plasma concentration (C_{max}), area under the plasma concentration-time curve from 0 to 72 h (AUC_{0-72}), area under the plasma concentration-time curve from zero hour to infinity (AUC_{0-inf}), time point of maximum plasma concentration (T_{max}), half life of drug elimination during the terminal phase ($t_{1/2}$) and elimination rate constant (K_{el}) were calculated for the test and reference formulations and are presented in Table 6. The T_{max} and $t_{1/2}$ values were in agreement with a reported study in healthy Indian subjects [17].

Table 6 Mean pharmacokinetic parameters after oral administration of 250 mg clarithromycin tablet formulation in 20 healthy Indian subjects under fasting.

Parameter	Test (Mean ±SD)	Reference (Mean ±SD)
C_{max} (ng/mL)	700.15 ± 223.27	711.09 ± 214.82
AUC $_{0\text{-}72\ h}$ (h. ng/mL)	8844.2 ± 345.54	9077.3 ± 356.18
AUC $_{0\text{-}inf}$ (h. ng/mL)	8915.5 ± 399.35	9152.2 ± 389.79
T_{max} (h)	4.07 ± 1.02	4.01 ± 1.09
$t_{1/2}$ (h)	9.01 ± 2.15	9.39 ± 2.78
Kel (1/h)	0.172 ± 0.005	0.170 ± 0.007

SD: standard deviation

Further, the 90 % confidence interval (CI) of individual ratio geometric mean for test/reference was within 80-125 % for AUC_{0-72}, AUC_{0-inf} and C_{max} (Table 7). These observations confirm the bioequivalence of the test sample with the reference product in terms of rate and extent of absorption.

Table 7 Treatment ratios and 90% CIs of natural log (Ln)-transformed parameters of test and reference tablet formulations of clarithromycin in 20 healthy subjects under fasting.

Parameter	Ratio (test/reference),%	90% CI (Lower – Upper)	Power	Intra subject variation, % CV
C_{max}	98.5	95.1-102.8	0.9999	3.49
AUC $_{0\text{-}72\ h}$	97.4	95.2-100.5	0.9995	5.72
AUC $_{0\text{-}inf}$	97.2	94.7-102.2	0.9998	4.25

CV: coefficient of variation

The incurred sample reanalysis (ISR) results showed % change values to be within ±13 % for CLA for selected samples. This confirms the reproducibility of the proposed method.

CONCLUSIONS

A highly sensitive and rapid UPLC-MS/MS method for the determination of CLA in human plasma has been developed and fully validated. The method was shown to be selective and free from matrix interference as evident from the results of post-column infusion, IS-normalized matrix factors and relative matrix effect in different plasma lots. The validation results indicate good linearity, accuracy and precision, recovery and stability of CLA in human plasma. With twofold dilution reliability, it is possible to extend the upper limit of quantitation to 3200 ng/mL. The developed method was successfully applied to analyze CLA concentration in pharmacokinetic study. Further, an incurred sample reanalysis of 100 selected samples confirms the reproducibility of the proposed method, which is not reported in any previous method.

REFERENCES:

1. D. H. Peters, S. P. Clissold, Clarithromycin: a review of its antimicrobial activity, k pharmacokinetic properties and therapeutic potential, Drugs 44 (1992) 117-164.
2. BIAXIN® FILMTAB® (clarithromycin tablets), Prescribing Information, June 2012. Manufacture by Abbott Laboratories, North Chicago, IL 60064, USA. http://www.drugs.com/ pro/biaxin.html
3. M. Ogrendik, Antibiotics for the treatment of rheumatoid arthritis, Int. J. Gen. Med. 7 (2014) 43-47.
4. A. D. Rodrigues, E. M. Roberts, D. J. Mulford, Y. Yao, D. Ouellet, Oxidative metabolism of clarithromycin in the presence of human liver microsomes. Major role for the cytochrome P4503A (CYP3A) subfamily, Drug Metab. Dispos. 25 (1997) 623-630.
5. S. J. Choi, S,B. Kim, H. Y. Lee, D. H. Na, Y. S. Yoon, S. S. Lee, J. H. Kim, K. C. Lee, H. S. Lee, Column-switching high-performance liquid chromatographic determination of clarithromycin in human plasma with electrochemical detection, Talanta 54 (2001) 377-382.

6. 6. G. F. V. Rooyen, M. J. Smit, A. D. D. Jager, H. K. L. Hundt, K. J. Swart, A. F. Hundt, Sensitive liquid chromatography-tandem mass spectrometry method for the determination of clarithromycin in human plasma, J. Chromatogr. B 768 (2002) 223-229.
7. 7. H. Amini, A. Ahmadiani, Sensitive determination of clarithromycin in human plasma by high-performance liquid chromatography with spectrophotometric detection, J. Chromatogr. B 817 (2005) 193-197.
8. 8. W. Li, H. Jia, K. Zhao, Determination of clarithromycin in rat plasma by HPLC-UV method with pre-column derivatization, Talanta 71 (2007) 385-390.
9. 9. G. Bahrami, B. Mohammdi, Determination of clarithromycin in human serum by high-performance liquid chromatography after pre-column derivatization with 9-fluorenylmethyl chloroformate: Application to a bioequivalence study, J. Chromatogr. B 850 (2006) 417-422.
10. 10. Y. Jiang, J. Wang, H. Li, Y. Wang, J. Gu, Determination of clarithromycin in human plasma by liquid chromatography-electrospray ionization tandem mass spectrometry, J. Pharm. Biomed. Anal. 43 (2007) 1460-1464.
11. 11. X. Peng, Z. Wang, J. Li, G. Le, Y. Shi, Electrochemiluminescence detection of clarithromycin in biological fluids after capillary electrophoresis separation, Anal. Lett. 41 (2008) 1184-1199.
12. 12. J. Shin, D. F. Pauly, J. A. Johnson, R. F. Frye, Simplified method for determination of clarithromycin in human plasma using protein precipitation in a 96-well format and liquid chromatography-tandem mass spectrometry, J. Chromatogr. B 871 (2008) 130-134.
13. 13. X. Lu, L. Chen, D. Wang, J. Liu, Y. Wang, F Li, Quantification of clarithromycin in human plasma by UPLC-MS-MS, Chromatographia 68 (2008) 617-622.
14. 14. J. S. Torano, H. J. Guchelaar, Quantitative determination of the macrolide antibiotics erythromycin, roxithromycin, azithromycin and clarithromycin in human serum by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection, J. Chromatogr. B 720 (1998) 89-97.
15. 15. A. Pappa-Louisi, A. Papageorgiou, A. Zitrou, S. Sotiropoulos, E. Georgarakis, F. Zougrou, Study on the electrochemical detection of the macrolide antibiotics clarithromycin and roxythromycin in reversed-phase high-performance liquid chromatography, J. Chromatogr. B 755 (2001) 57-64.
16. 16. J. I. D. Wibawa, P. N. Shaw, D. A. Barrett, Quantification of clarithromycin, its 14-hydroxy and decladinose metabolites in rat plasma, gastric juice and gastric tissue using high-performance liquid chromatography with electrochemical detection, J. Chromatogr. B 783 (2003) 359-366.
17. 17. S. Gurule, P. R. P. Verma, T. Monif, A. Khuroo, P. Partani, Sensitive liquid chromatographic determination of clarithromycin and 14-hydroxy clarithromycin in human plasma with tandem mass spectrometry, J Liq. Chromatogr. & Relat. Technol. 31 (2008) 2955-2973.
18. 18. F. D. Velde, J. W. C. Alffenaar, A, M. A. Wessels, Ben Greijdanus, D. R. A. Uges, Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography-tandem mass spectrometry, J. Chromatogr. B 877 (2009) 1771-1777.
19. 19. S. Ostwald, J. Peters, M. Venner, W. Siegmund, LC-MS/MS method for the simultaneous determination of clarithromycin, rifampicin and their main metabolites in horse plasma, epithelial lining fluid and broncho-alveolar cells, J. Pharm. Biomed. Anal. 55 (2011) 194-201.
20. 20. D.H. Vu, R.A. Koster, M.S. Bolhuis, B. Greijdanus, R.V. Altena, D.H. Nguyen, J.R.B.J. Brouwers, D.R.A. Uges, J.W.C. Alffenaar, Simultaneous determination of rifampicin, clarithromycin, and their metabolites in dried blood spots using LC-MS/MS, Talanta 121 (2014) 9-17.
21. 21. Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER). Centre for Veterinary Medicine (CVM), May 2001.
22. 22. P. Sharma, D.P. Patel, M. Sanyal, H. Berawala, S. Guttikar, P.S. Shrivastav, Simultaneous analysis of oxybutynin and its active metabolite N-desethyl oxybutynin in human plasma by stable isotope dilution LC-MS/MS to support a bioequivalence study, J. Pharm. Biomed. Anal. 84 (2013) 244-255.
23. 23. B. K. Matuszewski, M. L. Constanzer, C. M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019-3030.
24. 24. B.K. Matuszewski, Standard line slopes as a measure of relative matrix effect in quantitative HPLC-MS bioanalysis, J. Chromatogr. B 830 (2006) 293-300.
25. 25. R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah. Mechanistic investigation of ionization suppression in electrospray ionization, J. Am. Soc. Mass Spectrom. 11 (2000) 942-950.
26. 26. Guidance for Industry, ICH E6 Good Clinical Practice, U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), 1996.
27. 27. M. Yadav, P. S. Shrivastav, Incurred sample reanalysis (ISR): a decisive tool in bioanalytical research, Bioanalysis 9 (2011) 1007-1024.

Citation: Pranav S. Shrivastav, et al. (2015). New Improved UPLC-MS/MS Method for Reliable Determination of Clarithromycin in Human Plasma to Support a Bioequivalence Study. J. of Advancement in Medical and Life Sciences. V2I3. DOI: 10.15297/JALS.V2I3.03