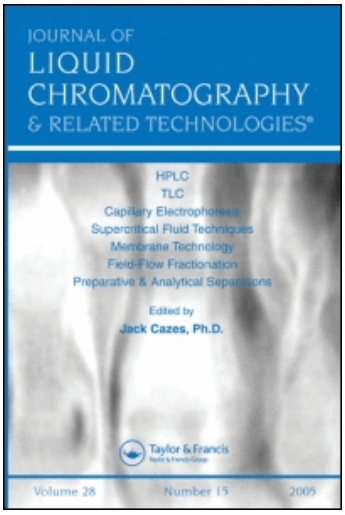

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Sensitive Liquid Chromatographic Determination of Clarithromycin and 14-Hydroxy Clarithromycin in Human Plasma with Tandem Mass Spectrometry

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Abstract: This paper describes a rapid, simple, sensitive, specific, and accurate analytical method for the determination of clarithromycin and 14-hydroxy clarithromycin (metabolite) in human plasma by high performance liquid chromatography with electrospray ionization tandem mass spectrometric detection (LC-MS/MS), using erythromycin as an internal standard (IS). The drug, metabolite, and internal standard were extracted by a precipitation method with the use of acetonitrile as a precipitant and separated on a C_{18} analytical column (5 µ, 4.6 × 100 mm). The mobile phase consisted of acetonitrile: 10 mM ammonium formate buffer (90:10 v/v). Detection was carried out by positive electro spray ionization (ESI+) in multiple reaction monitoring (MRM) mode. The chromatographic separations were obtained within 2.0 min and were linear in the concentration range of 36.5–5066.2 ng/mL for clarithromycin and 28.3–3934.1 ng/mL for 14-hydroxy clarithromycin. The limit of quantification for clarithromycin and 14-hydroxy clarithromycin were 36.5 and 28.3 ng/mL. The average recoveries for clarithromycin, 14-hydroxy clarithromycin, and the IS were 85.6%, 88.8%, and 85.0%, respectively. The results showed that the proposed method is rapid, sensitive, and reproducible to the quantitative determination of clarithromycin and 14-hydroxy clarithromycin in human plasma. Moreover, the proposed method was successfully applied for the bioequivalence study of clarithromycin.

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Keywords: 14-Hydroxy clarithromycin, Clarithromycin, Liquid Chromatography, Macrolide, Precipitation, Tandem mass spectrometry

INTRODUCTION

Chemistry

Clarithromycin is a semisynthetic macrolide antibiotic containing a 14 membered lactone ring[1,2] (Figure 1), which has a unique principle metabolite (14-hydroxy clarithromycin) that has activity equal to or greater than that of the parent drug.[3–5] It has the chemical name 6-(4-dimethylamino-3-hydroxy-6-methyl-tetrahydropyran-2-yl) oxy-14-ethyl-12,13-dihydroxy-4-(5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl)oxy-7-methoxy-3,5,7,9,11,13-hexa-methyl-1-oxacyclotetradecane-2,10-dione. Clarithromycin is the six methyl ether of erythromycin.

The introduction of the 6-O-methyl substituent has been demonstrated to ameliorate gastrointestinal irritation and provide enhanced overall activity relative to erythromycin. Clarithromycin seems to be

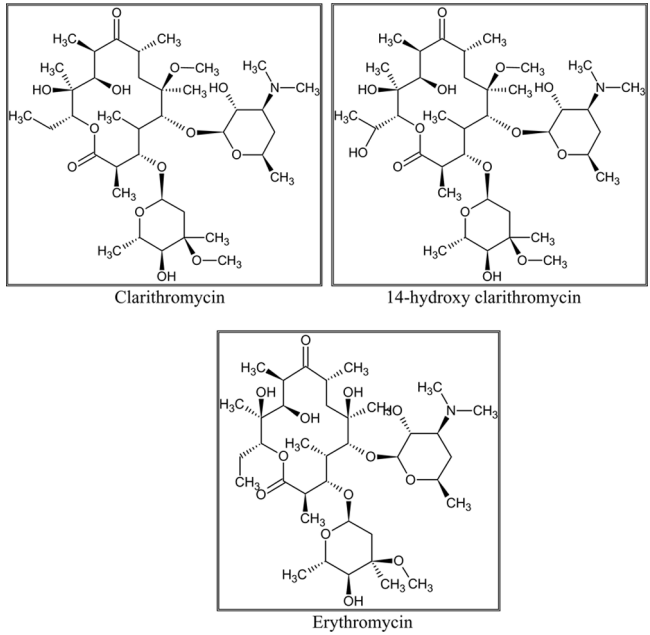

Figure 1. Structure of clarithromycin, 14-hydroxy clarithromycin and erythromycin.

more acid stable than erythromycin and, therefore, has greater oral bioavailablity[1] and less gastrointestinal side effects associated with erythromycin.[6–8] Clarithromycin is active intracellularly, and its action is static or bactericidal, depending on the concentration and the organism. Similar to erythromycin and azithromycin, clarithromycin demonstrated activity against *Mycobacterium avium complex* (MAC).[9–12] The activity of clarithromycin is enhanced by the formation of its active metabolite, 14-hydroxy clarithromycin, and by its extensive distribution into the tissues. Both parent and metabolite has been shown to inhibit the strains of *Haemophilus influenzae* in an additive & synergistic mode.[13,14] Clarithromycin has also been used in the treatment of *Helicobacter pylori*-associated peptic ulcer disease, community acquired infection, sinusitis, and sexually transmitted diseases. It has also been demonstrated to be more effective against *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Moraxella catarrhalis*, *Chlamydia pneumoniae*, *Mycobacterium pneumoniae*, *Toxoplasma gondii*, atypical mycobacteria, and *Mycobacterium leprae*.

Liquid chromatography mass spectrometry (LC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) are regarded as the most popular techniques for separation and quantitative determination of drugs and/or their metabolites and have been used for the determination of clarithromycin.[15,16] Several other methods have been described for the determination of clarithromycin and 14-hydroxy clarithromycin in plasma. Several high performance liquid chromatographic methods have been reported for determination of clarithromycin and/or 14-hydroxy clarithromycin with electrochemical detection.[17–19] Sastre, T.J., and Guchelaar., H.J., devised a method for determination of clarithromycin in human serum using fluorescence detection.[20] These methods exhibit low applicability because of the high detection limit, and/or are time consuming owing to the sample preparation requiring double liquid-liquid extraction, and/or a relatively long chromatographic run time (>7 min). Recently, Wenkui et al.[21] and Jiang, et al.[22] reported on the liquid chromatographic-electrospray tandem mass spectrometric determination of clarithromycin in human plasma. These methods have low applicability in the field of pharmacokinetics and bioequivalence, as the method can be useful only for determination of clarithromycin, but not its equally active metabolite 14-hydroxy clarithromycin.

This paper reports a robust quantification method for simultaneous determination of clarithromycin and 14-hydroxy clarithromycin in human plasma using protein precipitation. This method is not only selective and reliable but also faster than other methods reported till this date.

EXPERIMENTAL

Chemicals and Reagents

Clarithromycin and erythromycin were procured from USP. 14-hydroxy clarithromycin was supplied from the analytical department of Ranbaxy Research Laboratory. Methanol and acetonitrile were of HPLC gradient grade purchased from Sigma-Aldrich (USA). Ammonium acetate was obtained from Fluka (Buchs, Swizerland). Water was purified using a Milli-Q device (Millipore, Moscheim Cedex, France). Drug free human plasma of healthy volunteers was obtained from the Ranbaxy clinical pharmacology unit, Majeedia, New Delhi, India. To ensure the safety during usage, all batches of plasma were screened for Hepatitis B and C, HIV (human immunodeficiency virus) 1 and 2, malaria and syphilis. Human plasma batches, free of significant interference, were used to prepare calibration standards and quality control samples.

Instrumentation

The LC-MS/MS method was performed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan), which consisted of a LC-10ADvp pump, a SCL-10Avp system controller (accompanied by an auto sampler), a CTO-10Avp column oven, an FCV-10ALvp low pressure gradient unit, and DGU-14A degasser. Mass spectrometry was performed with Applied Biosystems API-3000 module (Applied Biosystems, Ontario, Canada), equipped with electrospray ionization interface. The data was collected and processed using Analyst 1.4 software.

Liquid Chromatography Conditions

Chromatographic separation and determination of analytes were carried out on 5 μm (100 mm × 4.6 mm, I.D.), Hypersil HyPURITY Advance column (Thermo). The HPLC system was operated isocratically at controlled temperature, using a mobile phase of acetonitrile: 10 mM ammonium acetate buffer in the ratio of 90:10 (v/v). This was filtered through a 0.2 μm membrane filter (Millipore, Bedford, MA, USA) and run at a flow rate of 0.8 mL/min. The injection volume was 10 μL for both standard and samples.

LC-MS/MS Conditions

The tuning parameters were optimized for clarithromycin, 14-hydroxy clarithromycin, and erythromycin (IS) with ESI and APCI in positive

ion mode, by infusing a solution containing 100 ng/mL of individual analytes at a flow rate of 10 μL/min via an external syringe pump. ESI in positive ion mode was found better as compared to APCI, as in ESI efficiency of ionization in positive ion mode was found higher than that of APCI. Thus, the electrospray ionization was performed in positive mode with the nebulizing gas (N_2), curtain gas, and turbospray set at an arbitrary value of 13, 10, and 70, respectively. The heated nebulizer temperature was set at 375°C. The optimal response was obtained with declustering potential set at 50 V, a focusing potential of 200 V, and entrance potential set at 10 V. The collision gas (N_2) was set at 3, whereas the pause time was set at 5 ms and dwell time at 200 ms.

The Applied Biosystems API 3000 MS/MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transitions $749.1 > 591.5$, $764.7 > 606.6$, and $735.7 > 577.6$ for clarithromycin, 14-hydroxy clarithromycin, and erythromycin (IS), respectively. Figure 2 represented the MSMS spectra for clarithromycin, 14-hydroxy clarithromycin, and erythromycin.

Procedure

Preparation of Stock Solution

The stock solutions of clarithromycin, 14-hydroxy clarithromycin, and erythromycin were prepared by dissolving appropriate amounts of compounds in methanol to give approximate final concentration of 1 mg/mL. Quality control stock solutions were also prepared by dissolving the appropriate amount of compounds in methanol to give the approximate final concentration of 1 mg/mL.

Calibration Standards and Quality Control Samples

A serial eight calibration curve working solution was prepared at a concentration of 1.9 to 194.6 μg/mL for clarithromycin and 1.4 to 145.7 μg/mL for 14-hydroxy clarithromycin by appropriate dilution in 50% methanol aqueous mixture. The quality control working solutions for clarithromycin/14-hydroxy clarithromycin were also prepared at a concentration of 1.7/1.4 μg/mL (LOQ), 4.6/3.7 μg/mL (low), 58.5/47.6 μg/mL (medium), and 172.0/140.0 μg/mL (high) by appropriate dilution of the quality control working solution in 50% methanol aqueous mixture.

The standard and quality control working solutions were spiked in blank pooled plasma to achieve the final concentration of 36.5, 73.0,

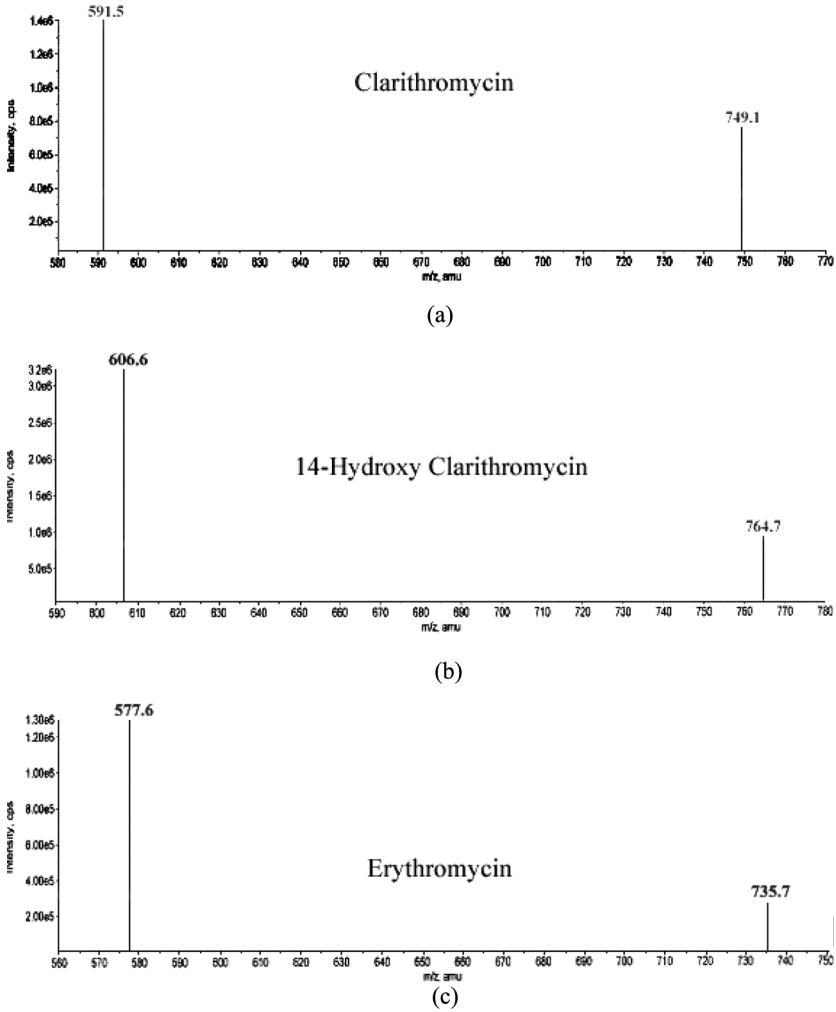

Figure 2. MSMS spectra of clarithromycin, 14-hydroxy clarithromycin, and erythromycin.

182.4, 456.0, 759.9, 1519.9, 3039.7, 5066.2 ng/mL for clarithromycin and 28.3, 56.7, 141.6, 354.1, 590.1, 1180.2, 2360.4, 3934.1 ng/mL for 14-hydroxy clarithromycin or quality control samples of concentration 38.2, 103.2, 1323.0, 3891.2 ng/mL for clarithromycin and 28.6, 77.3, 990.8, 2914.1 ng/mL for 14-hydroxy clarithromycin. All spiked plasma samples were stored below −50°C.

Plasma Extraction Procedure

A total of 200 μL of the spiked plasma was transferred to a microcentrifuge tube, followed by addition of 50 μL internal standard solution (20 μg/mL of erythromycin in acetonitrile). All samples were mixed by vortex agitation for 30 s. Then, 1 mL acetonitrile was added and vortexed for 1 min. The two phases were separated by centrifugation at 14000 rpm for 5 min. The supernatant was transferred into a glass tube and completely evaporated under a nitrogen stream while immersed in a 50°C water bath. The dry residue was reconstituted with 400 μL of acetonitrile and vortex mixed for 30 s. The samples were transferred to autosampler vials and 10 μL was injected into the LC-MS/MS system.

Validation of The Bioanalytical Method

The validation of this procedure was performed in order to evaluate the method in terms of selectivity, linearity of response, sensitivity, accuracy,

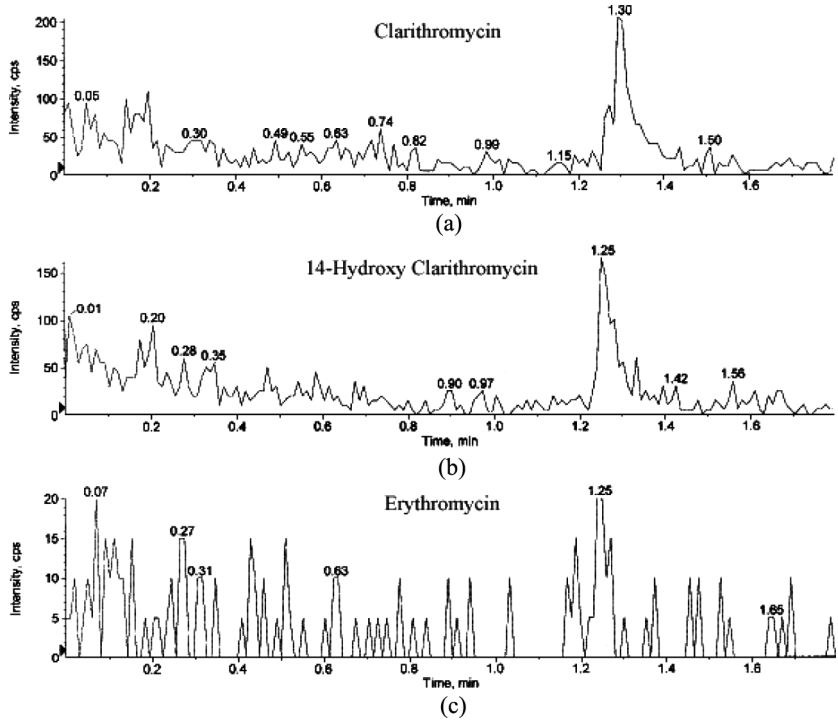

Figure 3. Representative LC-MS profile of a plasma blank.

precision, recovery, stability (in-injector, freeze-thaw, long term, bench top and stock solution), dilution integrity, matrix effect, and ruggedness. The linearity, sensitivity, precision, and accuracy evaluations were performed on three batches of spiked samples.

Pre defined acceptance criteria of ±15% for precision and 85 to 115% for accuracy was followed throughout the validation. Stability studies were performed using six replicates of low and high quality control samples and analyzed against freshly spiked calibration standards. Freeze thaw stability (three cycles), bench top stability (6 hrs), in-injector stability (26 hrs), and long term stability (37 days) was determined for clarithromycin and 14-hydroxy clarithromycin in human plasma.

Recovery was determined at low, middle, and high concentrations (extracted plasma samples) against unextracted (neat aqueous) samples of the same concentration. Matrix effect was determined using six different lots of drug free plasma at low and high concentrations. Dilution integrity was performed by preparing the concentration approximately 1.8 times of ULOQ (upper limit of quantitation), which was further

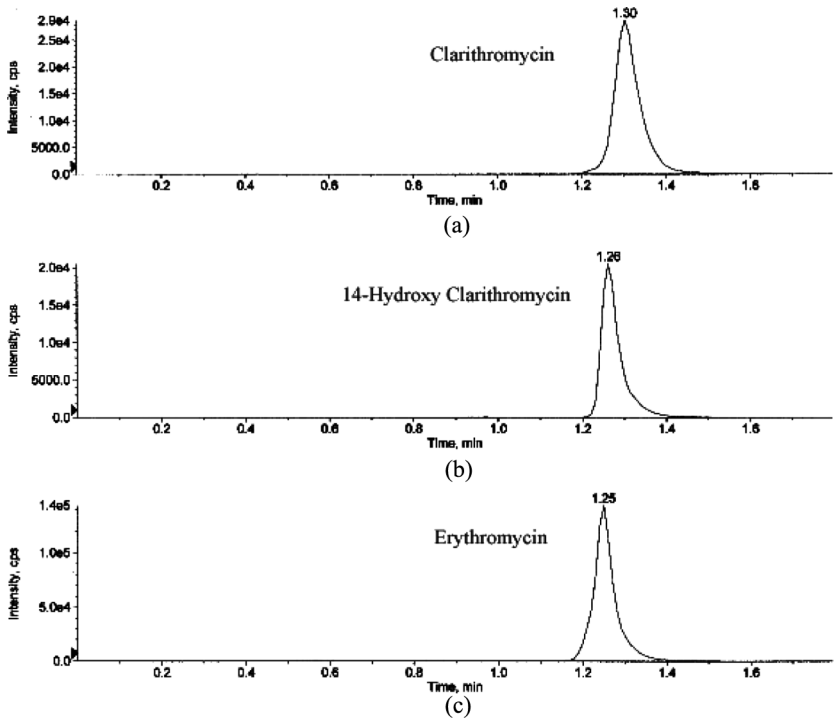

Figure 4. Representative LC-MS profile of a lower limit of quantitative standard.

diluted two and four times with drug free plasma. The two times and four times diluted samples were run against calibration standards and precision and accuracy were determined. Details of each experiment are covered under results and discussions.

Figures 3 and 4 show the representative chromatograms of blank plasma sample and lower limit of quantitative standard with internal standard.

Standardization and Calculation

The chromatographic data were acquired and processed using computer based Analyst software Version 1.4. The best fit lines using weighted ($1/\text{concentration}^2$) linear least square regression analysis were obtained by peak area ratio of clarithromycin and 14-hydroxy clarithromycin to internal standard. The concentration of clarithromycin and 14-hydroxy clarithromycin in unknown plasma samples was calculated using linear regression parameters by the corresponding calibration curve.

RESULTS AND DISCUSSION

Selectivity

Six lots of plasma were used to evaluate the selectivity of the method. An aliquot from each was processed along with six aliquots of the lower limit of quantitation standard sample. None of the blanks showed significant interfering peaks at the retention time of clarithromycin, 14-hydroxy clarithromycin and erythromycin.

Linearity and Sensitivity

The linearity of the clarithromycin and 14-hydroxy clarithromycin was determined by weighted least square regression analysis of the standard plot associated with an eight point standard curve. The calibration was shown to be linear from 36.5–5066.2 ng/mL for clarithromycin and 28.3–3934.1 ng/mL for 14-hydroxy clarithromycin. The r values were consistently 0.99 or greater during the course of validation for clarithromycin and 14-hydroxy clarithromycin. Table 1 refers to the back calculated calibration curve concentrations for clarithromycin and 14-hydroxy clarithromycin.

The limit of quantitation was 36.5 ng/mL for clarithromycin and 28.3 ng/mL for 14-hydroxy clarithromycin. The inter batch precision

Table 1. Back-calculated concentrations of clarithromycin and 14-hydroxy clarithromycin calibration standards and statistics for precision and accuracy from three representative calibration curves

Back calculated calibration curve concentrations for clarithromycin

CC-ID	Nominal concentration (ng/mL)								Slope	Intercept	r
	36.5	73.0	182.4	456.0	759.9	1519.9	3039.7	5066.2			
1	37.3	70.2	176.1	462.9	765.2	1630.3	3020.5	4870.0	0.001	0.003	0.9990
2	37.4	70.3	176.6	446.2	753.5	1606.2	3225.0	4841.7	0.001	0.002	0.9988
3	36.7	72.5	176.0	474.8	725.4	1562.5	3164.3	4891.1	0.001	0.004	0.9992
Mean	37.13	71.00	176.23	461.30	748.03	1599.67	3136.60	4867.60			
S.D (+/−)	0.379	1.300	0.321	14.367	20.455	34.369	105.026	24.787			
C.V. (%)	1.0	1.8	0.2	3.1	2.7	2.1	3.3	0.5			
% Nominal	101.7	97.3	96.6	101.2	98.4	105.2	103.2	96.1			

Back calculated calibration curve concentrations for 14-hydroxy clarithromycin

CC-ID	Nominal concentration (ng/mL)								Slope	Intercept	r
	28.3	56.7	141.6	354.1	590.1	1180.2	2360.4	3934.1			
1	28.9	54.2	143.1	350.8	553.9	1203.1	2430.4	4060.1	0.000	−0.001	0.9992
2	29.2	54.8	132.1	340.1	588.0	1217.3	2496.2	4023.2	0.000	−0.001	0.9988
3	29.0	54.1	139.4	361.0	570.1	1200.0	2439.5	3936.4	0.000	0.000	0.9994
Mean	29.03	54.37	138.20	350.63	570.67	1206.80	2455.37	4006.57			
S.D (+/−)	0.153	0.379	5.597	10.451	17.057	9.224	35.654	63.505			
C.V. (%)	0.5	0.7	4.1	3.0	3.0	0.8	1.5	1.6			
% Nominal	102.6	95.9	97.6	99.0	96.7	102.3	104.0	101.8			

and accuracy at LOQQC (quality control sample at lower limit of quantitation) concentration for clarithromycin using internal standard ratio method was 5.0% and 96.4% and for 14-hydroxy clarithromycin was 4.5% and 109.8%, respectively.

Precision and Accuracy (Tables 2 and 3)

Three precision accuracy batches were run to check intra and inter- day precision and accuracy. Each batch of spiked plasma samples included

Table 2. Intra-day precision and accuracy for clarithromycin and 14-hydroxy clarithromycin determination in human plasma samples

Intra-day precision and accuracy for clarithromycin				
Nominal concentration (ng/mL)	38.2	103.2	1323.0	3891.2
Mean concentration found (ng/mL)[a] for PA[b]1	38.28	95.72	1341.23	3812.88
C.V. (%)	2.5	3.8	3.0	4.1
Accuracy (%)	100.2	92.7	101.4	98.0
Mean concentration found (ng/mL)[a] for PA[b]2	37.07	98.37	1333.5	3909.33
C.V. (%)	4.3	4.5	2.6	2.2
Accuracy (%)	97.0	95.3	100.8	100.5
Mean concentration found (ng/mL)[a] for PA[b]3	35.08	94.2	1282.45	3636.58
C.V. (%)	4.0	2.1	2.6	3.1
Accuracy (%)	91.8	91.3	96.9	93.5
Intra-day precision and accuracy for 14-hydroxy clarithromycin				
Nominal concentration (ng/mL)	28.6	77.3	990.8	2914.1
Mean concentration found (ng/mL)[a] for PA[b]1	32.62	74.85	993.12	2875.78
C.V. (%)	2.7	3.8	1.4	1.7
Accuracy (%)	114.0	96.8	100.2	98.7
Mean concentration found (ng/mL)[a] for PA[b]2	30.93	69.12	1015.87	2941.8
C.V. (%)	4.4	2.4	2.7	3.1
Accuracy (%)	108.2	89.4	102.5	101.0
Mean concentration found (ng/mL)[a] for PA[b]3	30.62	78.2	1003.1	2920.42
S.D (+/−)	1.189	2.898	44.527	135.503
C.V. (%)	3.9	3.7	4.4	4.6
Accuracy (%)	107.1	101.2	101.2	100.2

[a]Mean of six replicates.
[b]Precision and Accuracy.

Table 3. Inter-day precision and accuracy for clarithromycin and 14-hydroxy clarithromycin determination in human plasma samples

Inter-day precision and accuracy for clarithromycin				
Nominal concentration (ng/mL)	38.2	103.2	1323.0	3891.2
Mean concentration found (ng/mL)[c]	36.81	96.09	1319.06	3786.27
C.V. (%)	5.0	3.9	3.3	4.3
Accuracy (%)	96.4	93.1	99.7	97.3
Inter-day precision and accuracy for 14-hydroxy clarithromycin				
Nominal concentration (ng/mL)	28.6	77.3	990.8	2914.1
Mean concentration found (ng/mL)[c]	31.39	74.06	1004.03	2912.67
C.V. (%)	4.5	6.1	3.1	3.3
Accuracy (%)	109.8	95.8	101.3	100.0

[c]Mean of eighteen replicates.

one complete calibration curve (consisting of two blank plasmas, two blank plasmas with internal standards and eight different non-zero concentrations) and six replicate quality control samples (LOQQC, LQC, MQC, and HQC) made up of concentrations corresponding to LOQQC (38.2 ng/mL), LQC (103.2 ng/mL), MQC (1323.0 ng/mL), and HQC (3891.2 ng/mL) for clarithromycin and concentrations corresponding to LOQQC (28.6 ng/mL), LQC (77.3 ng/mL), MQC (990.8 ng/mL), and HQC (2914.1 ng/mL) for 14-hydroxy clarithromycin. LQC, MQC, and HQC are the quality control samples at lower, middle, and higher concentrations.

The intra day accuracy using the internal standard area ratio method ranged from 91.3 to 101.4% and 89.4 to 114.0% for clarithromycin and 14-hydroxy clarithromycin, respectively, and the inter day accuracy ranged from 93.1 to 99.7% for clarithromycin and 95.8 to 109.8% for 14-hydroxy clarithromycin.

The intra day batch precision using the internal standard area ratio method ranged from 2.1 to 4.5% and 1.4 to 4.6% for clarithromycin and 14-hydroxy clarithromycin, respectively, and the total precision ranged from 3.3 to 5.0% for clarithromycin and 3.1 to 6.1% for 14-hydroxy clarithromycin.

Recovery

The percentage recovery of clarithromycin and 14-hydroxy clarithromycin were determined by measuring the peak area response of extracted quality control samples at low, middle, and high levels against the peak area response of un-extracted quality control samples of equivalent concentrations. Percent recovery of clarithromycin and 14-hydroxy

clarithromycin at low, middle, and high levels was found to be consistent (>85.0%). The recovery of internal standard using the same method was 85.0%.

Stability Studies (Table 4)

Freeze Thaw Stability

The freeze thaw stability of the spiked plasma samples was determined during the third freeze thaw cycle by using the replicate number of quality control samples at low and high levels and the degradation was determined against freshly spiked calibration standards. The thawing was performed at room temperature and six replicates at each level were used. Percent stability observed after third freeze thaw ranges between 90.8–95.5% and 93.9–99.2% for clarithromycin and 14-hydroxy clarithromycin, respectively. The precision varies from 3.0–6.1% and 6.3–6.7% for clarithromycin and 14-hydroxy clarithromycin, respectively.

Bench Top Stability

For bench top stability, evaluation involved analysis of replicates of Low and High QC stability samples, which had been kept at room temperature for a designated time (5.0 hour). These samples were processed and run against the freshly spiked calibration standards. The comparative stability ranged from 95.9–98.4% and 99.1–100.9% for clarithromycin and 14-hydroxy clarithromycin, respectively. The precision varies from 2.4–6.0% and 3.2–5.3% for clarithromycin and 14-hydroxy clarithromycin, respectively.

Table 4. Summary of stability of clarithromycin and 14-hydroxy clarithromycin determination in human plasma samples

	Clarithromycin		14-Hydroxy clarithromycin	
Activity	Stability (%)	Precision (%)	Stability (%)	Precision (%)
Freeze thaw stability (Three cycles)	90.8–95.5	3.0–6.1	93.9–99.2	6.3–6.7
Bench top stability (5 hrs)	95.9–98.4	2.4–6.0	99.1–100.9	3.2–5.3
In-injector stability (26 hrs)	93.1–96.2	3.8–6.1	95.3–99.9	4.3–5.2
Long term stability (35 days)	97.7–103.7	1.6–3.0	100.5–102.7	2.0–2.2

In-Injector Stability

In-injector stability of replicate quality control samples was determined. The low and high QC samples kept in an auto injector (10°C) were analyzed after around 26 hours, and the concentration was calculated against the freshly spiked calibration standards. The comparative stability ranged from 93.1–96.2% and 95.3–99.9% for clarithromycin and 14-hydroxy clarithromycin, respectively. The precision varies from 3.8–6.1% and 4.3–5.2% for clarithromycin and 14-hydroxy clarithromycin, respectively.

Stock Solution Stability

Stock solution stability of clarithromycin, 14-hydroxy clarithromycin, and erythromycin was determined for 15 days, respectively. The stocks were kept in a refrigerator (between 1–10°C). After the stability period, fresh stocks of clarithromycin, 14-hydroxy clarithromycin, and erythromycin were prepared. A dilution of each with the same concentration was prepared and six replicate injections were given. Mean response obtained from the stability stock was compared with the response obtained from fresh stock. The percent stability observed was 98.0%, 100.2%, and 98.9% for clarithromycin, 14-hydroxy clarithromycin, and erythromycin, respectively.

Long Term Stability

The long term stability of spiked plasma samples was determined. The low and high QC samples were stored at −50°C in a deep freezer with CPDA as anticoagulants for 35 days. These samples were analyzed against freshly spiked calibration standards. The percent stability ranged between 97.7–103.7% and 100.5–102.7% for clarithromycin and 14-hydroxy clarithromycin, respectively.

Other Validation Parameters

Dilution Integrity (Table 5)

To check the dilution integrity, about 1.8 times ULOQ (upper limit of quantitation) concentration was spiked in plasma and diluted by the factor of 2 and 4 with drug free plasma. Six aliquots of each (dilution factor 2 and 4 times) were processed and run against the calibration curve. The precision observed for two times dilution factor samples was 6.6% and 1.7%; accuracy was 101.0% and 104.2% for clarithromycin

Table 5. Dilution integrity determination for clarithromycin and 14-hydroxy clarithromycin in human plasma samples

	Clarithromycin		14-Hydroxy clarithromycin	
QC	8368.2*	8368.2**	6993.9*	6993.9**
1	7335.5	8579.9	7296.1	7017.7
2	8749.1	9215.5	7262.8	7373.7
3	8469.1	9018.5	7133.7	7484.7
4	8662.5	8755.4	7183.5	7406.2
5	8727.6	8722.4	7480.8	7341.0
6	8761.7	8259.0	7378.3	7554.7
Mean	8450.92	8758.45	7289.20	7363.00
C.V. [%]	6.6	3.8	1.7	2.5
% Nominal	101.0	104.7	104.2	105.3

*2 times dilution
**4 times dilution

and 14-hydroxy clarithromycin, respectively. For four time dilution factor samples, the precision observed was 3.8% and 2.5%; accuracy was 104.7% and 105.3% for clarithromycin and 14-hydroxy clarithromycin, respectively.

Matrix Effect (Table 6)

In the case of mass spectrometry, determination of matrix effect is a must. The matrix effect can be observed across different lots of plasma. To determine the matrix effect, six lots of plasma (free from analyte and internal standard) were chosen. Concentration equivalent to LQC and HQC was spiked in each lot of plasma. At each level, samples in duplicate were processed and run against freshly spiked calibration standards. Precision and accuracy for each plasma at LQC and HQC was checked. Precision of 2.4–4.8% and 4.2–5.9%; accuracy between 92.2–96.6% and 101.7–103.8% was observed for clarithromycin and 14-hydroxy clarithromycin, respectively, thus showing no matrix effect with the proposed extraction method.

Ruggedness

The ruggedness of the extraction procedure and chromatographic method was evaluated by analysis of a batch of six sets of quality control samples and a set of calibration standards by another analyst using a different column of the same make. The precision ranged between 2.0–4.8% and 2.1–9.1%; accuracy ranged between 83.0–98.4% and 90.6–99.5% for clarithromycin and 14-hydroxy clarithromycin, respectively.

Table 6. Matrix effect determination for clarithromycin and 14-hydroxy clarithromycin in human plasma samples

	Clarithromycin		14-Hydroxy clarithromycin	
	LQC (ng/mL)	HQC (ng/mL)	LQC (ng/mL)	HQC (ng/mL)
Matrix ID	103.2*	3891.2*	77.3*	2914.1*
	96.3	3683.9	83.8	3140.1
1	96.8	3656.0	81.8	3155.0
	89.7	3871.7	77.0	3109.0
2	93.1	3814.5	86.3	3133.2
	97.1	3667.9	83.8	3104.6
3	92.7	3766.1	76.6	3037.3
	96.0	3822.2	78.2	2858.8
4	86.4	3910.7	73.6	2863.3
	98.1	3815.0	71.0	2957.3
5	103.7	3773.2	75.0	2791.9
	98.8	3711.2	80.0	3054.6
6	92.9	3622.0	76.1	3099.4
Mean	95.13	3759.53	78.60	3025.38
S.D. (±)	4.525	91.341	4.615	125.950
C.V. (%)	4.8	2.4	5.9	4.2
% Nominal	92.2	96.6	101.7	103.8

*Nominal concentration

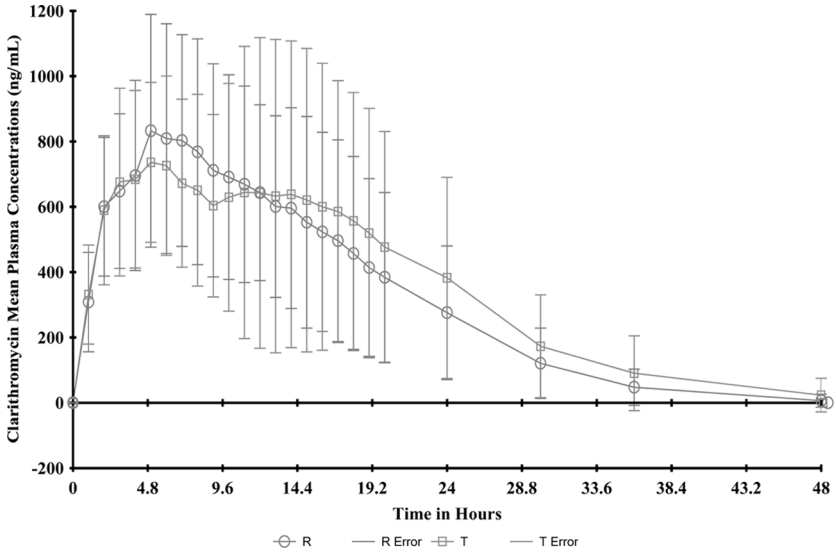

Figure 5. Linear mean plot of clarithromycin plasma concentrations.

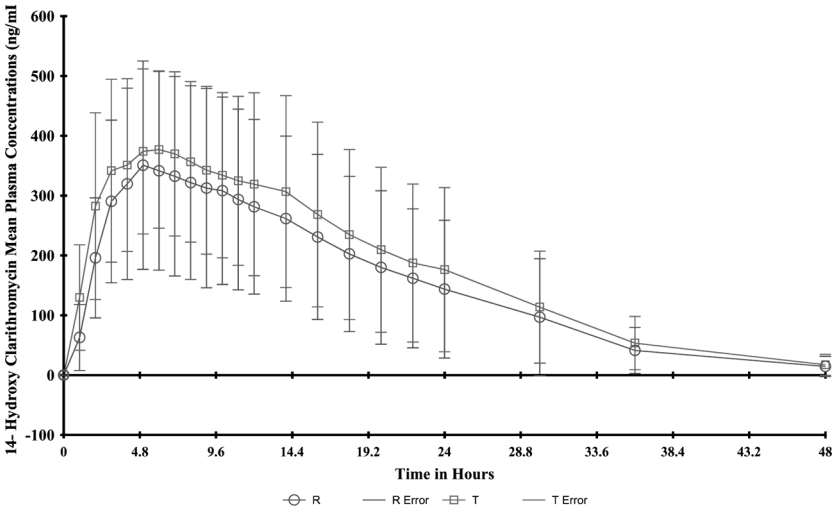

Figure 6. Linear mean plot of 14-hydroxy clarithromycin plasma concentrations.

Application

The validated method has been successfully used to quantify clarithromycin and 14-hydroxy clarithromycin concentration in human plasma samples after the administration of a single 500 mg oral dose of clarithromycin in a bioequivalence study conducted on healthy subjects. Concentration versus time profile was constructed up to 48 hrs. Figure 5 and 6 represents the linear mean plot of clarithromycin and 14-hydroxy clarithromycin plasma concentrations.

CONCLUSION

A rapid, sensitive, and highly selective method for the determination of clarithromycin and 14-hydroxy clarithromycin in plasma was developed and validated using high performance liquid chromatographic separation with tandem mass spectrometric determination. This validated assay method was used in a clinical study in which 40 volunteers were each given a 500 mg oral dose of clarithromycin. The assay method is more sensitive than previously described methods and allows for a much higher sample throughput due to the short chromatographic run time (2.0 min) and simple sample preparation. Robust LC-MS/MS instrument performance was observed, with only slight variation in the instrument

response within batches. A single analytical column was used to chromatograph more than 2500 samples without significant deterioration of column performance. The cost effectiveness, simplicity of the assay using simple acetonitrile precipitation and sample turnover rate of 2.0 min per sample, make it an attractive procedure in high throughput bioanalysis of clarithromycin and 14-hydroxy clarithromycin. Thus, this method is not only more selective and reliable but also faster than other methods reported till date.

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