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Rapid Determination of Clarithromycin in Human Plasma by LCMS/MS Assay

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Abstract

A rapid liquid chromatographic tandem mass spectrometric (LC-MS/MS) assay for the measurement of clarithromycin level in human plasma was developed and validated using erythromycin as internal standard (IS). Analysis was performed at room temperature using a reversed phase Atlantis dC_{18} (2.1 × 100 mm, 3 µm) column. The components of interest were detected in the positive ion mode of electrospray ionization using transition 749 → 158.4 and 719.3 → 158.2 for clarithromycin and the IS, respectively. Quantification and detection limits were 5 and 2 ng/ml, respectively. Mean extraction recovery was ≥ 86% for clarithromycin and 99% for the IS. Relationship between clarithromycin concentration and peak height ratio of clarithromycin to the IS was linear (R^2 ≥ 0.9833) in the range of 0.005-4.0 µg/ml, and the intra- and inter-day coefficient of variations were 2.9% to 13.1% and 2.5% to 9.6%, respectively. Clarithromycin in human plasma was stable for at least 24 hours at room temperature (≥ 83%) or 14 weeks at -20°C (≥ 93%), and after three freeze-thaw cycles (≥ 83%). The method was successfully used to determine clarithromycin levels in human plasma samples obtained from a healthy volunteer.

Keywords: Clarithromycin; Erythromycin; Human plasma; LC-MS/MS

Abbreviations: API: Atmospheric Pressure Ionization; CAS: Chemical Abstract Number; CV: Coefficient of Variation; eV: Electron Voltage; FT: Freeze-Thaw; HPLC: High Performance Liquid Chromatography; IS: Internal Standard; kV: Kilo Voltage; L/hr: Liters/Hour; LCMS/MS: Liquid Chromatography-Tandem Mass Spectrometry; m/z: Mass to Charge Ratio; ng/ml: Nanogram/Milliliter; QC: Quality Control; RT: Room Temperature; SD: Standard Deviation; UV: Ultra-Violet; USP: United State Pharmacopeia; µg/L: Microgram/Liter.

Introduction

Clarithromycin (CAS: 81103-11-9) is a broad-spectrum semi-synthetic macrolide antibiotic used in the treatment of various bacterial infections [1]. Its absolute bioavailability is about 55%, with a peak plasma concentration of 2.41 to 2.85 µg/ml at 2-3 hours after the ingestion of a 500 mg therapeutic dosage [2,3].

A thorough literature review of clarithromycin assays revealed a number of high performance liquid chromatography (HPLC) methods using ultraviolet (UV) [4-6], fluorescence [7,8], electrochemical [9-13], or liquid chromatography-mass spectrometry (LC-MS/MS) detection [14-16]. Some of these methods require laborious multi-step, extraction procedures [4,5,6], derivatization [6-8], or require large sample volumes [7,8].

Most of the reported assays for the determination of clarithromycin in biological matrix employed electrochemical detection, since the molecule lacks a suitable chromophore to be detected by UV. Some HPLC-UV assays used a wavelength of ≤ 210 nm, where interference from sample matrix usually occurs [4,5]. Clarithromycin was also measured by fluorescence detection after sample treatment with 9-fluorenylmethyl chloroformate or 9-fluorenylmethyloxycarbonyl chloride [7,8]. The reported LC-MS/MS methods used various compounds as internal standard including stable isotope-labelled clarithromycin [14-16].

In the present study, we describe a simple, precise, and rapid LCMS/MS assay for the quantitative determination of clarithromycin in human plasma using erythromycin as internal standard. The method requires 0.20 ml human plasma and simple liquid extraction. It was used to determine the stability of clarithromycin under various clinical laboratory conditions and to determine clarithromycin level in human plasma samples obtained from a healthy volunteer.

Materials and Methods

Instrumentation

LC-MS/MS analysis was performed on Waters Alliance HPLC 2695 Separation module consisting of quaternary pump, autosampler, column thermostat, and Micromass Quattro micro API bench-top triple quadruple mass spectrometer interfaced with a Z-spray electro-spray ionization probe. Data acquisition and analysis were performed using Mass Lynx 4.0 software with Quan Lynx program (Waters Associates Inc., Milford, MA, USA).

Chemicals and reagents

All chemicals were of analytical grade unless stated otherwise. Clarithromycin and erythromycin were purchased from USP reference standard Rockville, MD, USA. Triethylamine, phosphoric acid, *tert.* butyl methyl ether, and acetonitrile (HPLC grade) were purchased from Fisher Scientific, NJ, USA. Water for HPLC was prepared by reverse osmosis and further purified by using synergy water purification system (Millipore, Bedford, MA, USA). The study was approved by

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the Research Ethics Committee, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia.

Chromatographic conditions

Analysis was performed on a reversed phase Atlantis dC_{18} (2.1× 100 mm, 3 µm) column proceeded by Symmetry C_{18} (3.9 × 20 mm, 5 µm) guard column. The mobile phase, containing 0.05% triethylamine (pH=4.0, adjusted with phosphoric acid) and acetonitrile (65:35, v:v), was filtered through a 0.22 µm membrane filter (Millipore Corporation, Bedford, MA, USA), degassed, and delivered at a flow rate of 0.25 ml/min. Mass Lynx software working under Microsoft Windows XP professional environment was used to control the instruments, data acquisition, peak integration, peak smoothing, and signal-to-noise ratio measurements. The electrospray ionization source was operated in the positive-ion mode at a capillary voltage of 4.0 kV and a cone voltage of 30 V. Nitrogen was used as nebulizing and desolvation gas at a flow rate of 60 and 600 L/hr, respectively. Argon was used as the collision gas at a pressure of 1.28×10^{-3} mbar. The optimum collision energy for clarithromycin and erythromycin (internal standard, IS) was 25 eV. The ion source and the desolvation temperatures were maintained at 125 and 350°C, respectively.

Standards and controls

Clarithromycin and IS stock solutions were prepared in methanol (100 µg/ml). Calibration standards at nine different concentrations (0.005-4.0 µg/ml) and quality controls at four concentrations: 0.005, 0.015, 2.0, and 3.8 µg/ml were prepared in human plasma. IS working solution was prepared in methanol (1.0 µg/ml). Standard and control solutions were vortexed for one minute, and 200 µl aliquots were transferred into 7 ml glass culture tubes and stored at -20°C until used.

Sample preparation

50 µl of the IS working solution was added to 200 µl plasma sample, calibration standard, or quality control (QC) samples in a 7 mL culture tubes and vortexed. 4.0 ml *tert*. butyl methyl ether was added, vortexed for one minute, and centrifuged at 6000 rpm for 10 minutes at room temperature. The clear supernatant layer was transferred to a clean culture tube and dried under gentle steam of nitrogen at 40°C. The residue was reconstituted in 100 µl mobile phase. 5 µl of the clear solution was injected into the LC-MS/MS system.

Results and Discussion

LCMS/MS condition optimization

Although mass spectrometry has been recognized as a technique for quantification since its inception, the greatest impetus to its use in the field of quantitative measurement of organic compound has come from its coupling with liquid chromatography (LC). In order to optimize LC conditions, we initially used ammonium acetate buffer in combination with acetonitrile in range of 40-60%. No satisfactory results were obtained. However, after replacing ammonium acetate with triethylamine, we found consistently satisfactory results. Triethylamine in the mobile phase facilitated the generation of ion in the electrospray ionization mode and allowed completing the analysis within 3.0 minutes. Therefore, detection and quantification of clarithromycin were optimized using a mobile phase composed of 0.05% triethylamine (pH=4.0, adjusted with phosphoric acid) and acetonitrile (65:35, v:v) at a flow rate 0.25 ml/min.

Mass spectra: precursor ions of clarithromycin and IS and their corresponding product ions were determined from spectra obtained during the infusion of standard solutions into the mass spectrometer. Clarithromycin and IS produced (m+1) ion peak at *m/z* 749 and 719, respectively. The product ion transitions were quantitatively measured as peak height at *m/z* 749 → 158.4 for clarithromycin and 719.3 → 158.2 for the IS in multiple reaction mode. Figure 1 depicts the precursor and product spectrum of clarithromycin and IS, whereas Figure 2 depicts a LC-MS/MS chromatograms of plasma spiked with 1.0 ng/ml IS and clarithromycin at three concentrations (0.015, 2.0 and 3.6 µg/ml), respectively.

Extraction recovery: The absolute recovery of clarithromycin was assessed by comparing absolute peak height of spiked plasma and mobile phase samples, using five replicates of four QC samples (0.005, 0.015, 2.0 and 3.6 µg/ml). Similarly, the recovery of the IS was determined by comparing the peak height of the IS in 5 aliquots of human plasma spiked with 1.0 ng/ml IS with the peak height of equivalent samples prepared in mobile phase. The extraction recoveries were 86-101% for clarithromycin and 99% for the IS. The results are presented in Table 1.

Method validation

The procedures used for validation were according to the US Food and Drug Administration (FDA) bioanalytical method validation guidance [17].

Specificity: To evaluate assay specificity, we screened six different batches of human plasma and eight frequently used medications namely: acetaminophen, ibuprofen, aspirin, ranitidine, nicotinic acid, ascorbic acid, caffeine, and omeprazole for potential interference. None was found to interfere with the quantification of clarithromycin or the IS.

Linearity and limit of quantification: Linearity of the assay was evaluated by analyzing a series of nine standards over the range of 0.005-4.0 µg/ml. Corresponding peak height ratio and concentrations were subjected to regression analysis. The mean equation obtained from eight standard curves was y=0.0048+1.8704 x, with a mean coefficient of correlation (SD) of 0.9941 (0.007). The detection and quantification limits were established as 2 ng/ml and 5 ng/ml, respectively.

Accuracy and precision: Accuracy and precision were determined by measuring levels of clarithromycin in four QC samples (0.005, 0.015, 2.0 and 3.8 µg/ml). The intra-and inter-day imprecision and bias were determined over three different days. Intra-day (n=10) imprecision and bias were ≤ 13.1% and ≤ 9.0%, respectively. Inter-day (n=20) imprecision and bias were ≤ 9.6% and ≤ 12.2%, respectively (Table 2).

Robustness: The robustness of the current assay was evaluated by altering the pH of triethylamine (±0.5), and the amount of acetonitrile (±2.0%) in the mobile phase. No significant effects were observed.

	Human plasma (n=5)		Mobile phase (n=5)		*Recovery (%)
	Mean	SD	Mean	SD	
Clarithromycin (µg/ml) 0.005	120	12.1	122	4.3	98
0.015	325	8.0	322	4.6	101
2.0	34299	1028	38439	1188	89
3.6	58253	997	67845	1601	86
IS 1.0 (µg/ml)	17024	453	17260	838	99

Table 1: Extraction recovery of clarithromycin and erythromycin (IS). Data represent mean and standard deviation of peak height.*Mean peak height in human plasma divided by mean peak height in mobile phase × 100. SD, standard deviation.

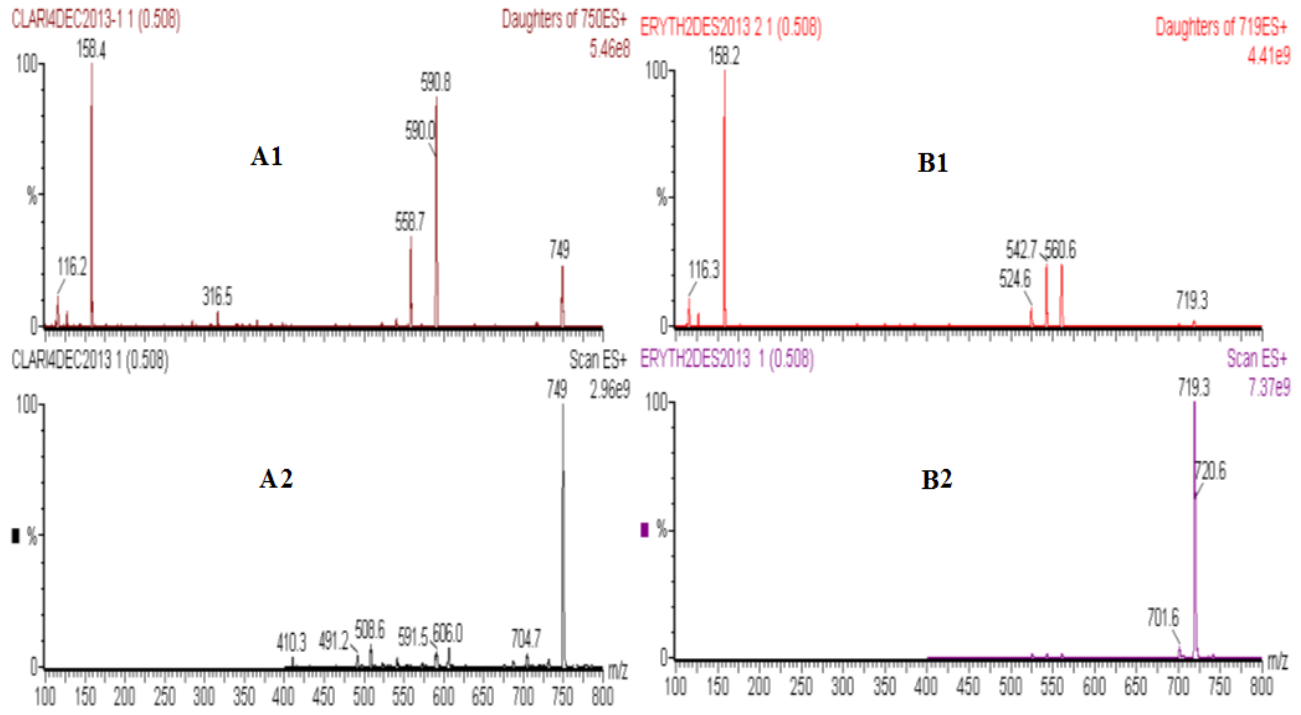

Figure 1: Precursor and product ion spectrum of clarithromycin (A1 and A2) and erythromycin, IS (B1 and B2), respectively.

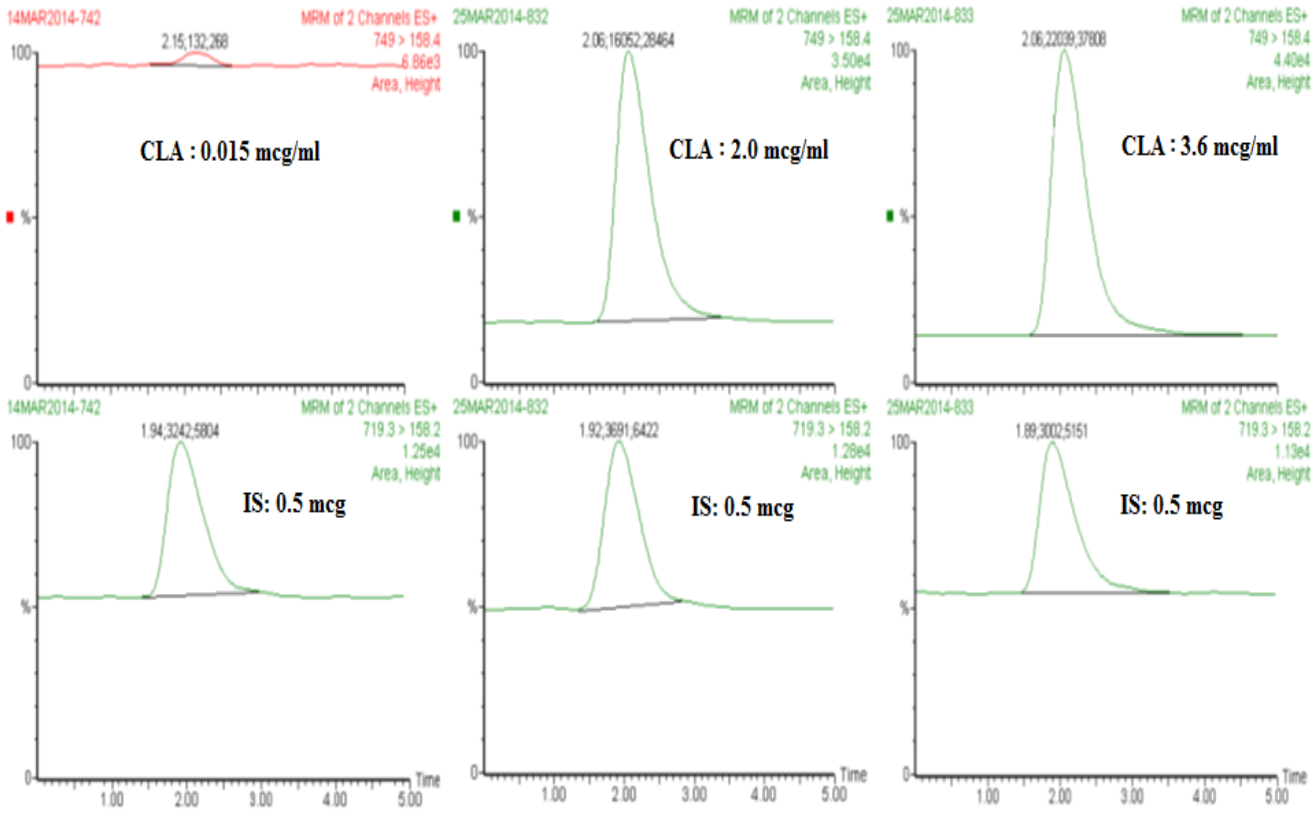

Figure 2: Quality control samples of human plasma spiked with erythromycin (IS) and clarithromycin at three concentrations (0.015, 2.0 and 3.6 µg/ml).

Stability

Stability of clarithromycin in processed and unprocessed plasma samples was investigated. Clarithromycin in processed samples (0.015 and 3.6 µg/ml) was found to be stable for 24 hours at room temperature (≥ 98%) and 48 hours at -20°C (≥ 97%). Clarithromycin in unprocessed plasma samples was stable for at least 24 hours at room temperature (≥ 83%), 14 weeks at -20°C (≥ 93%), and after three freeze-and thaw cycles (≥ 83%). Data are summarized in Table 3. Clarithromycin in stock solution (1 mg/ml in methanol) was stable for 24 hours at room temperature (≥ 92%) and at least two weeks at -20°C (≥ 96%). Further, no significant change in chromatographic behavior of clarithromycin or the IS was observed under any of the above conditions.

Application to volunteer's sample

The method was used to determine clarithromycin levels in bioequivalence study (results are not shown). Figure 3 depicts a representative chromatogram of samples collected from a volunteer

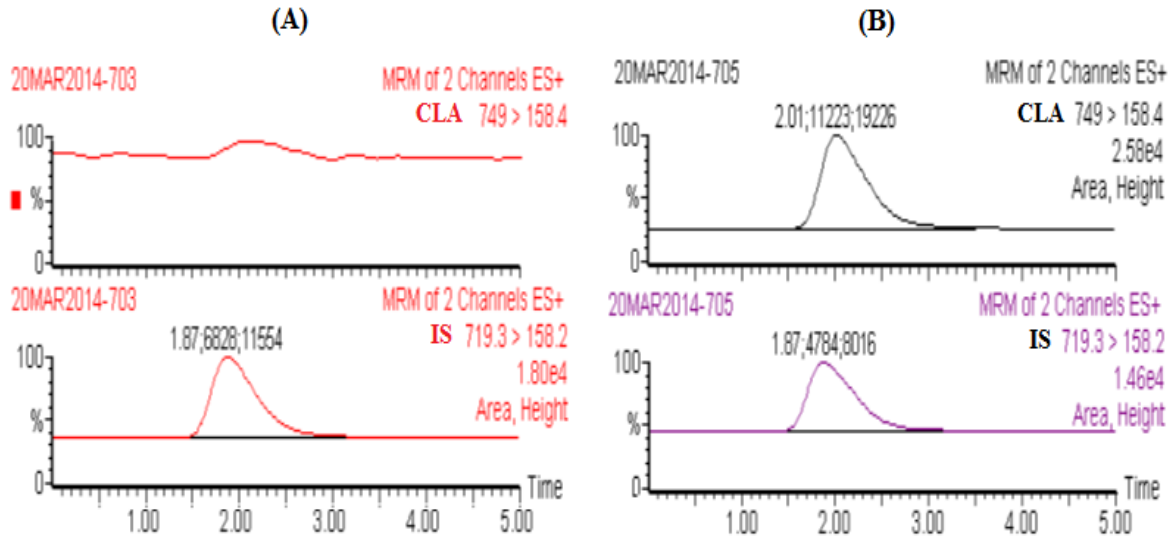

Figure 3: Multiple reaction monitoring chromatogram of plasma samples obtained from a healthy volunteer before (A) and after 2 hours after (B) single oral 500 mg clarithromycin dose.

Nominal level (µg/ml)	Intra-day (n=10)				Inter-day (n=20)			
	Mean	SD	CV (%)	Bias (%)	Mean	SD	CV (%)	Bias (%)
0.005	0.0053	0.0007	13.1	5.1	0.0047	0.0004	9.5	-5.9
0.015	0.0164	0.0018	10.7	9.0	0.0171	0.0016	9.6	12.2
2.0 3.6	1.8219 3.3252	0.0647 0.0953	3.6 2.9	-9.0 -7.8	1.8699 3.3878	0.0529 0.0854	2.8 2.5	-7.0 -6.3

Table 2: Intra- and inter-run precision and accuracy of clarithromycin assay. Data represent measured levels (µg/ml). SD, standard deviation. CV, coefficient of variation as a measurement of imprecision=standard deviation divided by mean measured concentration × 100. Bias, measured level - nominal level divided by nominal level × 100.

Storage condition	Nominal level µg/ml	Measured level* µg/ml (SD)	Stability (%)
Baseline	0.015 3.6	0.150 (0.001) 3.394 (0.056)	
Processed samples 24 hr. (RT)	0.015 3.6	0.015 (0.002) 3.343 (0.056)	100 98
48 hr. (-20°C)	0.015 3.6	0.015 (0.002) 3.291 (0.060)	104 97
Unprocessed samples 24 hr. (RT)	0.015 3.6	0.012 (0.001) 3.421 (0.175)	83 101
14 wks. (-20°C)	0.015 3.6	0.153 (0.002) 3.342 (0.506)	94 93
FT cycle-1	0.015 3.6	0.012 (0.001) 3.306 (0.144)	83 99
FT cycle-2	0.015 3.6	0.015 (0.002) 3.163 (0.229)	104 95

Table 3: Stability of clarithromycin in human plasma under different storage conditions. *Data represent mean and standard deviation (SD). Stability (%)=Mean measured level (n=5) at the indicated time divided by mean measured level at base line × 100. RT, room temperature (22°C). FT, Freeze-thaw cycle; samples were frozen at -20°C and thaw at RT.

before and 2.0 hours after the ingestion of a single dose of 500 mg clarithromycin. Measured levels of clarithromycin were zero and 2.4 μg/ml, respectively.

Conclusion

The described LC-MS/MS method, consisting of liquid-liquid extraction, reversed phase LC, erythromycin as IS, electrospray ionization, and MS/MS detector, is simple, precise, and accurate for rapid measurement of clarithromycin level using 0.2 ml human plasma. The assay was used to measure clarithromycin stability under various condition encountered in the clinical laboratory. Further, it was successfully applied to determine clarithromycin level in human plasma samples obtained from a healthy volunteer.

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