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Bioanalysis and *in-vivo* studies of clarithromycin modified release solid dosage formulations by liquid chromatography-mass spectroscopy

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ABSTRACT

The current study was undertaken to develop the new bioanalytical method and validation for determining Clarithromycin by LC-MS Method and as well as to conduct *in vivo* studies. Princeton octadecyl silane column (10 cm x 4.6 mm id, 5μm) used as adsorbent and cyanomethane: 0.5 % Methanoic acid was treated as the eluent for the separation of the analyte from the biological fluid in an isocratic mode having the ratio 60:40 % v/v and 0.5 ml/min as flow rate, and injection volume was set as 20 μl. APCI and the mass detected of Clarithromycin and Azithromycin (act as internal standard) was detected at 748.45 and 749.70, respectively. Developed bioanalytical methods have been used to quantify the Pharmacokinetic parameters like C_{max}, T_{max}, AUC0-t & AUC0-∞, K_{eli}, and $t_{1/2}$ studied and the values for reference formulation (3.382μg/ml, 7.333 h, 114.429μg.h/ml, 131.435μg.h/ml, 0.031 h^{-1}, and 23.397h respectively) and the test formulation (3.847 μg/ml, 7.417 h, 132.318 μg.h/ml, 151.388 μg.h/ml, 0.031 h^{-1}, and 23.187 h, respectively) were compared and found to be bioequivalent. Based on our study, the test formulation of Clarithromycin modified-release formulation containing 500 mg of Clarithromycin is Bioequivalent to that of the reference. Compare to our method (LC-MS) is simple, sensitive, precise as well as comparable with the reference formulation of the modified release product of clarithromycin 500 mg.

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INTRODUCTION

Clarithromycin is a good example of broad-spectrum macrolide antibacterial drugs (Langtry and Brogden, 1997; Jelić and Antolović, 2016), and the chemical structure of Clarithromycin is obtained from erythromycin-A (Dinos *et al.*, 2003). It is used to treat a wide variety of bacterial infections, and it can be taken orally. Clarithromycin is also prescribed along with anti-ulcer drugs as a combination. Clarithromycin is available as immediate-release formulation as well as modified-release formulations. Whenever they want to release the product in the market, the concerned company should study bioequivalence studies. Bioequivalence studies (BE) (Yu and Li, 2014) are conducted to investigate the pharmacokinetic parameters of two pharmaceutical formulations of the same drug and to demonstrate the equivalence of their pharmacokinetic parameters. BE studies are accessed via whole blood/plasma/serum /urine data using the following parameters: AUC or the cumulative amount of drug excreted in the urine, C_{max}, or the rate of drug excretion in urine and T_{max}. The present study was undertaken to establish the new method for

the quantification of Clarithromycin in blood plasma and validated the developed method as per the US FDA guidelines as well as after validation to perform in vivo studies (Alkhalidi *et al.*, 2008; Samiullah *et al.*, 2017) to prove the equivalence of test Clarithromycin concerning the reference Clarithromycin modified release dosage form.

Eluent and chemicals:

Cyanomethane and Methanoic acid HPLC quality obtained from Merck, Ammonium acetate was procured from SD fine chem and HPLC quality aqua obtained from a Milli-Q Reverse Osmosis system. The working standard of Clarithromycin obtained from Neopharma, Abu Dhabi.

MATERIALS AND METHODS

Instruments

To perform the bioanalytical study of Clarithromycin, Shimadzu 2010A LC-MS has been used.

Separation conditions

LC Conditions

Stationary phase: Princeton 10 cm octadecyl silane column (with 4.6 mm, i.e., 5 μm) was used and Cyanomethane: 0.5 % Methanoic acid, considered as eluent to separate analyte in an isocratic mode having the ratio of 60:40 % v/v respectively. The flow rate of the eluent was fixed:0.5 ml/min, and the volume of Injection in the system was 20 μl/L using the Autoinjector.

MS Conditions

Interface: APCI and SIM mode having positive polarity with ambient probe temperature, CDL & Block Temperature 250º C & 200º C, respectively. The mass detected of Clarithromycin and Azithromycin (act as internal standard) was detected at 748.45 and 749.70, respectively. The entire analysis was conducted at ambient conditions.

Preparation of Clarithromycin standard stock solution

Constituted 1 mg/ml solution by weighing 100mg of Clarithromycin and dissolved in 100ml of Cyanomethane. Labeled the container and stored below 8°C.

Standard solution for Calibration Curve

Constituted 10 ml of each of 0.3, 0.5, 1, 2, 3, 4, 5, and 6 μg/ml of standard solutions for calibration curve obtained from Clarithromycin standard stock solutions by using eluent and finally stored at –20±2 ºC until analysis.

Standard solution for Quality Control

Constituted 10 ml each of 0.3, 3, and 6 μg/ml of Clarithromycin standard solutions for quality control obtained from Clarithromycin standard stock solution and eluent and stored at –20±2ºC until analysis Figures 1, 2 and 3.

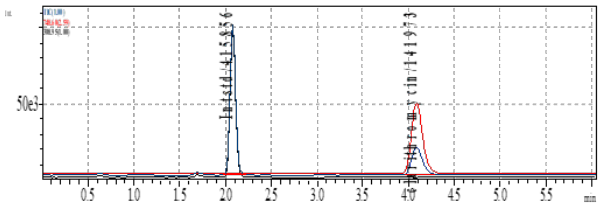

Figure 1: Typical chromatogram of standard solution

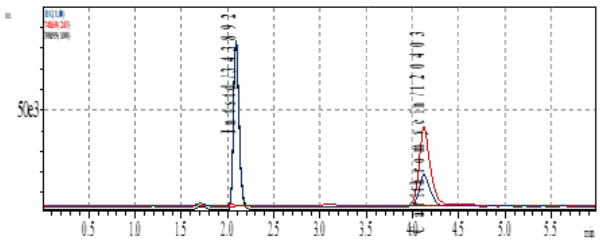

Figure 2: Typical chromatogram of sample solution

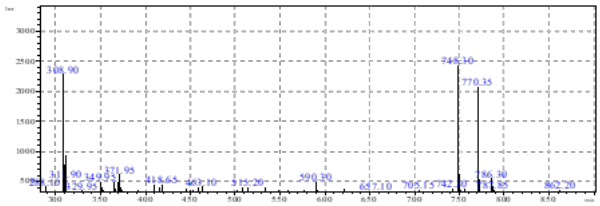

Figure 3: Mass spectrum of sample solution

Preparation of stock and Calibration curve samples

Constituted 10.0 ml each of 0.6, 1, 2, 4, 6, 8, 10, and 12 μg/ml of clarithromycin calibration curve samples using clarithromycin standard stock solution with blank human plasma. After the dilution calibration curve samples are transferred into different 2.0 ml centrifuge tubes and stored at –70±2 ºC until processing.

Preparation of Quality Control Samples

Constituted 10.0 ml each of 0.6, 6.0, and 12.0 μg/ml of Clarithromycin Calibration curve samples from of Clarithromycin standard stock solution (0.5 ml) and made up the volume with blank plasma, transfer into different 2.0 ml centrifuge tubes and stored at–70±2 ºC until processing.

Preparation of plasma samples

Table 1: Recovery studies

Level	The concentration of drug added μg/ml	Amount of drug recovered (μg/ml) in the plasma sample	Recovery (%)	Amount of Drug recovered (%) in Mobile phase	Relative Recovery (%)
Level-I	0.30	0.28 $\pm$ 0.23	Mean: 96.72 CV: 1.86 N:6	Mean: 98.31 CV: 2.02 N: 6	98.38
Level-II	3.0	2.91 $\pm$ 1.24	Mean: 97.43 CV: 1.01 N: 6	Mean: 98.57 CV: 1.83 N: 6	98.84
Level-III	6.0	5.97 $\pm$ 0.88	Mean: 98.81 CV: 35 N: 6	Mean: 98.49 CV: 2.38 N: 6	100.30

Table 2: Linearity studies

The concentration of clarithromycin (μg/ml)	Concentration of Azithromycin (μg/ml)	Response Factor (RSD)
0.30	100	0.0043
0.50	100	0.0084
1.0	100	0.0168
2.0	100	0.0254
3.0	100	0.0505
4.0	100	0.0673
5.0	100	0.0842
6.0	100	0.1019

Plasma samples were drawn from the deep freezer and kept in the ambient temperature and allowed to thaw. 0.5 ml of the plasma sample pipetted into a 2.0 ml centrifuge tubes with this 50 μl of azithromycin solution (100 μg/ml) and 1.0 ml of the cyanomethane (as precipitating agent) was added. The resulting solution was vortexed for 5 minutes and centrifuged at 4,000 rpm for 10 min. Supernatant liquid from the above solutions separated and used for the analysis.

Order of Analysis

Injected 10 μl of each sample in the following order: The standard solutions, linearity samples, quality control samples, and plasma sample solutions injected with the above separation conditions and recorded the chromatograms. The quantification achieved as per the guidelines.

Validation Studies

Accuracy and Precision

Accuracy and precision of the method achieved as per the USFDA guidelines. Precision studies were undertaken in three levels (Low QC, Middle QC, and High QC) at nine times and three different occasions. The results are tabulated in Table 1.

Selectivity

Blank plasma samples were harvested from six different volunteers and analyzed for selectivity. The chromatograms were obtained after the analysis and compared with the standard chromatograms for the interference studies. No interferences found in the chromatograms.

Linearity and Range

0.3, 0.5, 1, 2, 3, 4, 5, and 6 μg/ml of Clarithromycin analyzed, and the peak areas and response factors were determined as per the guidelines. Stability Studies, Limit of Detection, Limit of Quantification, Robustness, and ruggedness have been studied as per the USFDA guidelines given in Table 2.

System suitability

The results of column efficiency, peak symmetry factor, resolution factor, and capacity factor for the standard Clarithromycin solutions were computed in Table 3.

***In-Vivo* Study**

In vivo study performed by recruiting the healthy

Table 3: Stability studies of Clarithromycin in plasma

	Nominal Concentration (μg /mL)		
Freeze and Thaw	LQC	MQC	HQC
	0.3000	3.0000	6.0000
Cycle 1	0.2724	2.7234	5.6809
Cycle 2	0.2939	2.8169	5.7223
Cycle 3	0.3086	2.9235	5.8971
Mean	0.2916	2.8213	5.7668
S.D (+/-)	0.0182	0.1001	0.1148
C.V. (%)	6.24	3.55	1.99
% Nominal	97.21	94.04	96.11
n	3	3	3
	Nominal Concentration (μg /mL)		
Plasma at Ambient Temperature (Short Term)	LQC	MQC	HQC
	0.3000	3.0000	6.0000
After 1 hr	0.2648	2.8746	5.789
After 2 hrs	0.2706	2.9134	5.8137
After 3 hrs	0.2815	2.9357	5.9321
Mean	0.2723	2.9079	5.8449
S.D (+/-)	0.0085	0.0309	0.0765
C.V. (%)	3.11	1.06	1.31
% Nominal	90.77	96.93	97.42
n	3	3	3
	Nominal Concentration (μg /mL)		
Plasma Sample at	LQC	MQC	HQC
- 70º C (Long Term)	0.3000	3.0000	6.0000
After 1 week	0.2567	2.6389	5.2391
After 2weeks	0.2715	2.7145	5.8222
After 4 weeks	0.2946	2.8033	5.0164
Mean	0.2743	2.7189	5.3592
S.D (+/-)	0.0191	0.0823	0.4161
C.V. (%)	6.96	3.03	7.76
% Nominal	91.42	90.63	89.32
n	3	3	3
	Nominal Concentration (μg /mL)		
Standard Stock solutions	LQC	MQC	HQC
	0.3000	3.0000	6.0000
After 3 hrs	0.2891	2.9164	5.8224
After 6 hrs	0.2978	2.9376	5.5981
After 3 Weeks	0.3091	2.9588	6.0012
Mean	0.2987	2.9376	5.8072
S.D (+/-)	0.0100	0.0212	0.2020
C.V. (%)	3.36	0.72	3.48
% Nominal	99.56	97.92	96.79
n	3	3	3

Table 4: System suitability data

S.No	Parameters	Azithromycin	Clarithromycin
1	Theoretical Plate	58312	45067
2	Resolution factor	1.52	
3	Asymmetric factor	1.12	1.00
4	LOD(ng/ml)	1.00	3.0
5	LOQ(ng/ml)	3.00	10.0

Table 5: Summary of results (n=24)

	Clarithromycin 500 mg Modified Release formulations			
S.No	Pharmacokinetic parameters	Test formulation	Reference formulation	% RATIO
1.	AUC0-24 (μg.h/ml)	132.318± 26.581	114.429± 24.445	115.63
2.	AUC0-inf (μg.h/ml)	151.388± 39.156	131.435± 34.655	115.18
3.	Cmax (μg/ml)	3.847± 5.23	3.382± 597.538	113.74
4.	tmax (h)	7.417±0.93	7.333±1.40	101.14
5.	keli(h-1)	0.031± 0.005	0.031± 0.006	100.00
6.	$t_{\frac{1}{2}}$ (h)	23.187± ±5.305	23.397± 4.637	99.10

human volunteers by using crossover study with randomization, single-dose of Clarithromycin under fasting conditions. After recruiting the volunteers, 15 blood samples were harvested at dosing. Blood samples were harvested through an indwelling cannula placed in a forearm vein. The pre-dose blood sample was harvested within one hour before dosing, and post-dose samples were collected within 2-minutes of the scheduled time. The blood samples were collected in vacutainers containing sodium citrate as the anticoagulant. Plasma separated and divided into two portions while storing the containers labeled and stored at -70 ºC till analysis and study are conducted as per the prior approval from the institutional ethics committee (JSSCP/HEC/LOA/ 077).

Study Design

After a single oral dose of the Clarithromycin in 24 (+2 standby) healthy male human volunteers in a randomized, two-way, two-period, crossover design. In each dosing session, volunteers received either the Reference or Test formulation of respective formulation as a single-dose, only on the study day, as per the randomization code at a fixed time.

Blood Sampling Schedule

Blood samples were obtained from the volunteer at specific time points from 0 (before drug administration), 30, 60, 90, 120, 150, 180, 240, 300, 360, 480, 720, 1440, 2160, 2880, 3600, and 4320 minutes with 10 days washout period between two periods.

RESULTS AND DISCUSSION

Validation of the developed method as per USFDA guidelines

Selectivity and Sensitivity, Accuracy, and Precision studies

Selectivity and sensitivity, accuracy, and precision studies observed as per the USFDA guidelines. All the values fall within the criteria. Accuracy studies details have been present in Table 1

Linearity Studies

Linearity and range studies Figure 4 have been studied by using different concentrations of standard solutions of the study drugs prepared and analyzed along with Azithromycin as an internal standard. After the analysis, the response factor evaluated. As per the USFDA guidelines, the linearity and range calculated, and it has shown the R^2 value within the range mentioned in Table 2.

Stability Studies

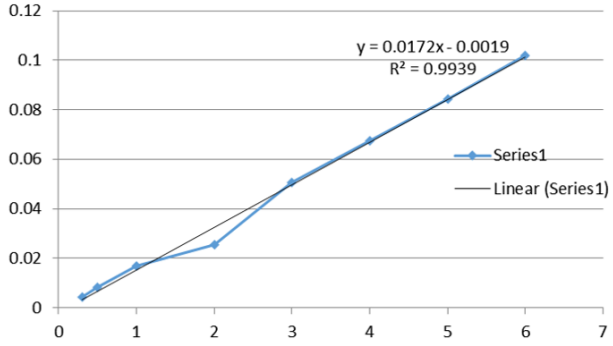

Figure 4: Calibration curve of clarithromycin

Stability studies of plasma samples spiked with Clarithromycin subjected to three freeze-thaw cycles, Short term stability at room temperature for 3 hours, and long term stability at – 70 ºC over three weeks. The stability studies data presented in Table 3 No degradation was found in both short term and long term stability studies.

System suitability

The parameters, namely the Asymmetric factor, resolution factor, and theoretical plate for the standard solutions calculated. The determination of the LOD and LOQ computed based on the signal-to-noise ratio. The data demonstrates that the developed methods have adequate sensitivity. The values obtained demonstrated the suitability of the system for the analysis of Clarithromycin in plasma in Table 4.

Ruggedness and Robustness

Robustness and ruggedness studies established as per the US-FDA guidelines by changing the pH, flow rate, and it fell within the prescribed criteria.

Pharmacokinetics Data

Pharmacokinetic parameters such as Peak plasma concentration (C_{max}), Time to peak concentration (T_{max}), Area under the plasma concentration-time curve (AUC_{0-t} & $AUC_{0-\infty}$), elimination rate constant (K_{eli}), Elimination half-life ($t_{1/2}$) calculated separately and the blood level data of the Reference product and the Test product compared.

Volunteers have taken the dosage forms by orally after the administration of the Reference formulation, and the test formulation in the fasting state exhibited measurable Clarithromycin blood levels in all the volunteers from 0.50 hr. Onwards Based on the Pharmacokinetic data namely, AUC_{0-t}, T_{max}, C_{max}, K_{eli}, Half-life and AUC_{0-inf} of the reference and the test formulations and their statistical analysis concluded that the Test formulation of Clarithromycin containing 500 mg of clarithromycin is Bioequivalent to that of Reference formulation and which is exhibited in Table 5.

The improved validated analytical method represents an accurate, precise, linear, selective, and sensitive method for the quantification of Clarithromycin. Based on the Pharmacokinetic data namely, C_{max}, T_{max}, AUC0-t & AUC0-∞, K_{eli}, and $t_{1/2}$ studied and the values for reference formulation (3.382μg/ml, 7.333 h, 114.429μg.h/ml, 131.435μg.h/ml, 0.031 h^{-1}, and 23.397h respectively) and the test formulation (3.847 μg/ml, 7.417 h, 132.318 μg.h/ml, 151.388 μg.h/ml, 0.031 h^{-1}, and 23.187 h, respectively) were compared and found to be bioequivalent.

CONCLUSIONS

The developed and validated LCMS method is specific, precise, accurate, robust, and rugged. This method adopted to analyze the modified-release formulation of clarithromycin in biological fluids.

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