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Article

Determination and Stability Assessment of Clarithromycin in Human Plasma using RP-LC with Electrochemical Detection

M.F. Zaater[1]*, Y.R. Tahboub[1] and E. Ghanem[2]

[1]Department of Applied Chemistry, Jordan University of Science and Technology, Irbid, Jordan, and [2]International Pharmaceutical Research Centre, Amman, Jordan

*Author to whom correspondence should be addressed. Email: mzaater@just.edu.jo

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A selective, sensitive and stability-indicating reversed-phase high-performance liquid chromatography method was developed and validated for the determination of clarithromycin antibiotic in human plasma. Liquid chromatography was performed on a 5-μm (100 × 4.6 mm) C8 column at 40°C. The mobile phase consisted of acetonitrile with 0.045M H_3PO_4 (37:63, v/v) adjusted to pH 6.7 and pumped at a flow rate of 1.2 mL/min. Detections were monitored on an electrochemical detector operated at a potential of 0.85 V with glassy carbon electrode against Ag/AgCl reference electrode. Each analysis required 13 min and quantification over the range of 0.05–5.0 μg/mL of plasma was linear, as indicated by a correlation coefficient (R^2), 0.9999.

The method was validated according to international guidelines. Data with respect to accuracy, within-run and between run, were close to 100% with 4% precision. Absolute recovery was 95%. The limit of quantification was 0.05 μg/mL. Neither endogenous substances nor commonly used drugs were found to interfere with the retention times of analytes. Stock solutions and calibration standards of the drug and quality control preparations were demonstrated to be stable at room temperature and −20°C for long and short periods of time. Eventually, the proposed method was successfully applied to quantify clarithromycin in spiked human plasma and real samples from healthy volunteers, indicating the utility and throughput of this method for clinical and bioavailability studies.

Introduction

Clarithromycin (CLA), Biaxin or Klaricid are the approved or common names of a semisynthetic 14-membered macrolide drug with two deoxy sugar moieties known as 6-0-methylerythromycin. It has a formula of $C_{38}H_{69}NO_{13}$, as demonstrated in Figure 1 (1–5).

Clarithromycin has been approved by the Food and Drug Administration (FDA) since the early 1990s and went generic in the market after 2000 as an acid-stable, potent and broad-spectrum antibiotic (1, 5), Thus, it is indicated for the treatment of bacterial infection associated with sinusitis, tonsillitis, pneumonia, acne (vulgari) and the advanced stage of HIV infections in AIDS patients (1, 5–7). CLA in combination with amoxicillin and a proton inhibitor drug is of particular interest because it is being used effectively in duodenal ulcer treatment to eradicate helicobacter pylori in a short period of time (1–3, 5, 7–10). CLA *in vitro* undergoes rapid transformation into a 14-HO-metabolite that is more effective than its precursor (4, 6).

Currently, drug level determination is increasingly significant and represents a stimulating analytical challenge in routine patient care, quality control and bioassay studies (9). Nevertheless, analysis of CLA is complicated because it shows only a nonspecific and weak absorption radiation below 220 nm. Moreover, it provides low concentration in plasma after administration. Thus, its analysis needs to be selective and sensitive (4, 10).

In this context, various methods and techniques have been reported for determination of CLA in different matrices (7, 9–11). Among those reported are liquid chromatography (LC) with ultraviolet (UV) detection (12–14), LC–fluorometry (10, 15–17), capillary electrophoresis (18), LC–mass spectrometry (MS) and ultra-performance LC with tandem MS (ULC–MS-MS) and LC–nuclear magnetic resonance (NMR) (19–25). Other reported methods have been high-performance liquid chromatography (HPLC) with electrochemical detection (ECD) (26–30). In our search for CLA determination in human plasma, the lack of suitable chromophores in the drug structure, together with the complexity and lengthy procedures associated with fluorometric derevatization and the unavailability of expensive MS and NMR high-tech techniques that are not commonly present in academic labs, restricted its adoption and encouraged the use of a convenient, sensitive and fast response ECD. Various methods in this context with different sample preparation, extraction procedures and chromatographic conditions along with ECD utilization have been reported for CLA alone or in combination with other analytes (26–30). However, many of the published methods are lengthy (9, 29), suffer from low recovery (28) or high detection limit (5, 19, 28) or apply a high column temperature (10, 17, 29, 30). Additionally, some

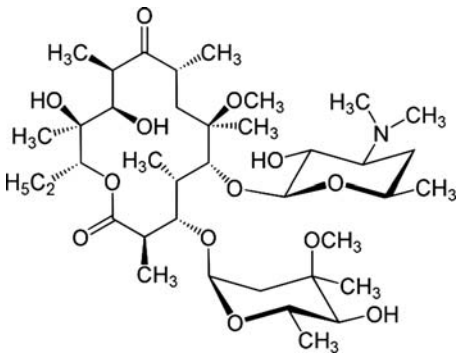

Figure 1. Chemical structure of Clarithromycin (CLA).

methods were assigned for analysis of the drug in rat plasma and tissue cells, where its concentration is much higher than in blood (5, 18, 26). Therefore, it was relevant to modify and validate a reliable, simple and sensitive procedure. Emphasis was geared to circumvent most of the previously mentioned shortcomings and to provide a low limit of quantitation that permits a reliable analysis of CLA in clinical, forensic toxicology and bioavailability studies.

Experimental

Instrumentation

Analyses of CLA were carried out on a RP-HPLC–ECD system consisting of LC-1017 DVP pump, SCL-system controller and CTO-oven kept at 40°C. An electrochemical detector, L-ECD-6A, operated at 0.85 V with glassy carbon electrode versus Ag/AgCl reference electrode and a software processor, VP-5 (version 5.03) were all from Shimadzu (Tokyo, Japan). A Rheodyne manual injector and a symmetry C8 column (4.6 × 100 mm; 3.5 μm) were from Waters (Milford, MA).

Reagents and materials

Acetonitrile and *tert*-butylmethyl ether used were of HPLC-grade (Merck, Darmstadt, Germany). Sodium hydroxide and sodium carbonate were of analytical grade; Deionized water was prepared locally using Pure Link RO and an Easy Pure UV system. The mobile phase was a combination of acetonitrile and 0.045M H_3PO_4 (37:63, v/v), adjusted to pH 6.7 with 5M NaOH solution and pumped isocratically at a flow rate of 1.2 mL/min.

Standard solutions

Stock solutions of CLA and azithromycin as an internal standard (IS) were prepared separately in acetonitrile at a concentration of 1.00 mg/mL each. Working standards of the analytes were prepared by diluting 1,000.0 μL of CLA stock solution and 200.0 μL of the IS stock solution with deionized water up to 50 mL to produce concentrations at 20.0 and 4.0 μg/mL for CLA and IS, respectively.

Standard solutions of CLA at different concentrations were prepared in 10-mL volumetric flasks via appropriate dilutions of the working standards with nine batches of drug-free plasma to afford eight (nonzero) concentrations labelled as 0.00, 0.05, 0.10, 0.20, 0.50, 1.0, 2.0, 3.0 and 5.0 μg/mL. The individual solutions were vortexed for 5 min, and then aliquots of 0.5 mL of each concentration were transferred to a 10.0-mL glass tubes and reserved for validation.

Quality control (QC) solutions for regular runs were prepared from CLA working standard in 25.0-mL volumetric flasks at concentrations of 0.05, 0.5, 2.5 and 4.0 μg/mL of plasma. Samples of 0.5 mL of each QC standard were placed separately into 10-mL glass tubes and similarly kept for validation.

Sample preparation and measurement

Aliquots of 50 μL of the IS working solution (4.0 μg/mL) were added to 0.5 mL of CLA QC and calibration standards. Each combination was vortexed for 30 s, followed by an addition of 250 μL of 0.1M Na_2CO_3, vortexed again for 30 s and centrifuged for 5 min at a speed of 3,000 rpm. Eventually, to each preparation a volume of 6.0 mL *tert*-butylmethyl ether was added for extraction. Organic layers following vortex mixing and centrifugation were transferred to 10-mL glass tubes and evaporated almost to dryness at 55°C. Residues were then reconstituted in 200 μL of the mobile phase, vortexed for 30 s and transferred to 0.75-mL eppendorf tubes and centrifuged for 2 min at a speed of 13,000 rpm.

Aliquots (100 μL) of the prepared samples were injected into the chromatographic system, which was thermostated at 40°C. Calibration curves were constructed from the respective chromatograms by plotting drug peak area to that of IS versus drug nominal concentration. Finally, the best fit regression line equations were estimated and used later to backcalculate standard concentrations and volunteers' unknown sample concentrations.

Method Validation

To check the overall performance of this assay, the method was evaluated in accordance with International Council on Harmonization (ICH) guidelines (31) as described in the following.

Specificity and selectivity

The specificity of the method was performed by screening chromatograms of many batches of drug free plasma (blank) and plasma spiked with different levels of CLA together with a fixed amount of the IS, as shown in Figures 2A and 2B. However, selectivity was tested by screening chromatograms of the mobile phase solutions fortified with the commonly used drugs; namely, aspirin, acetaminophen, ascorbic acid, ibuprofen, caffeine and nicotine. None of the tested drugs *in vitro* were detected or found to interfere at the studied retention times.

Linearity

Calibration curves of CLA standards containing a fixed amount of IS over the range of 0.05–5.0 μg/mL were constructed at six replicates each. The best fit regression line equations and correlation coefficients were deduced and averaged to give a representative general equation, $Y = 0.7426X - 0.0005$; $R^2 = 0.9999$, which was used to backcalculate tested concentrations (Table I).

Accuracy and precision

Evaluation of the pertinent accuracy and precision was conducted over one and three consecutive days on samples of low, medium and high QC levels of CLA in plasma. Replicates of each QC concentration were tested and back calculated for the intra-run and inter-run accuracy and precision. Results are included in Table II. [Accuracy and precision were assessed on the basis of percent bias and percent coefficient of variation (CV%), respectively].

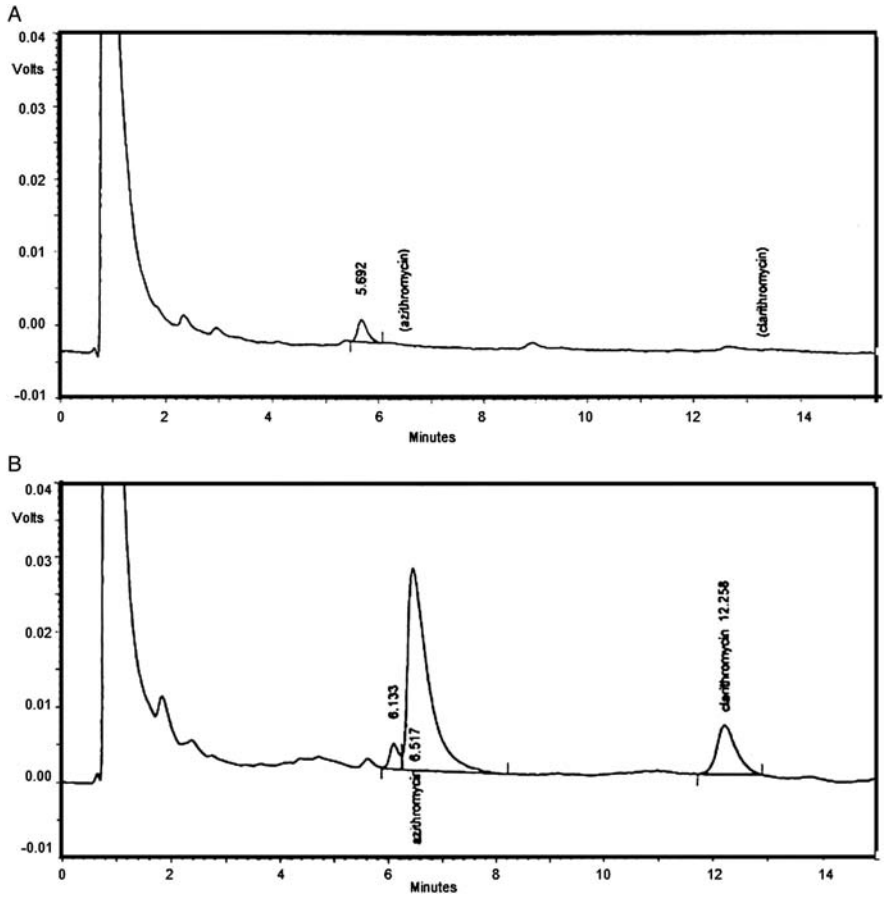

Figure 2. LC chromatograms: blank human plasma (A); human plasma spiked with CLA (0.30 μg/mL) and azithromycin (0.250 μg/mL) (B).

Table I
Calibration Curve Data of CLA in Human Plasma ($n = 6$)

Nominal concentration (μg/mL)	Mean peak area ratio	Backcalculated concentration (μg/mL)	Accuracy (%)	SD	CV%
0.05	0.03690	0.05	100.0	0.006	12.00
0.10	0.07254	0.098	98.0	0.008	8.16
0.20	0.14699	0.197	98.5	0.014	7.11
0.50	0.36848	0.499	99.4	0.008	1.61
1.00	0.74310	1.002	100.2	0.030	2.99
2.00	1.48833	2.010	100.5	0.071	3.53
3.00	2.22494	3.00	100.0	0.053	1.77
5.00	3.70984	4.997	99.94	0.014	0.28
Mean			99.59	0.096	3.66

Table II
Intra-Day and Inter-Day Accuracy and Precision Data of CLA in Human Plasma

	Within-day concentration ($n = 10$) (μg/mL)				Between-day concentration ($n = 20$) (μg/mL)			
Concentration added	Found	Accuracy (%)	SD	CV%	Found	Accuracy (%)	SD	CV%
0.05	0.05	100	0.00	0.00	0.051	102.0	0.003	3.88
0.30	0.299	99.67	0.011	3.68	0.298	99.33	0.009	3.02
2.5	2.455	98.2	0.079	3.22	2.474	98.96	0.109	4.41
4.0	3.964	99.1	0.138	3.48	4.024	100.10	0.151	3.75
Mean		99.24	0.159	2.60		100.02	0.186	3.76

Sensitivity

The lowest standard concentration on the calibration curves (0.05 μg/mL) was regarded as the lower limit of quantitation (LLOQ), because it meets the criteria of an identifiable response ≥5 times that of blank with an accuracy ≥80% and precision ≤20%. The sensitivity was tested by preparing and analyzing QC samples at LLOQ with a complete set of calibration curves. No measurements were made below the reported limit.

Recoveries

The percentage recoveries were determined by analyzing five replicates of three QC samples that were prepared to contain CLA at concentrations of 0.30, 2.5 and 4.0 μg/mL of plasma in presence of a fixed amount (0.2 μg) of the IS. Absolute analytical recoveries were estimated by comparing peak area ratios of the extracted measurements with that of an equivalent unextracted aqueous counterparts (Table III). However, relative analytical recoveries were deduced by comparing the measured and backcalculated concentrations of QC samples with their nominal concentrations and presented in Table IV.

Table III
Absolute Recovery Data of CLA Samples from Human Plasma ($n = 5$)

QC (μg/mL)	Peak area ratio (aqueous)	Peak area ratio (extracted-plasma)	Recovery (%)
0.3	122,575	120,315	98.16
2.5	943,528	936,859	99.29
4	1,579,143	1,417,528	89.77
(Mean recovery ± SD)			95.74 ± 5.20

Table IV
Relative Recovery Data of CLA from QC Samples ($n = 5$)

QC (μg/mL)	Found	Recovery (%)	SD	CV%
0.30	0.298	99.33	1.49	1.50
2.5	2.54	101.76	4.28	4.20
4.00	4.20	105.00	2.78	2.65
Mean recovery		102.03	5.32	2.78

Table V
Stability Data of CLA in Human Plasma for Two QC Levels under Various Conditions ($n = 5$)*

Stability	Accuracy (%) 0.30 μg/mL	(mean ± SD) 4.0 μg/mL
Short term (RT/4h)	0.288 ± 0.015	4.094 ± 0.177
(Mean Stability% ± CV)	96.01 ± 5.21	102.09 ± 4.32
Long term (−20°C/2w)	0.270 ± 0.007	4.054 ± 0.169
Long term (−20°C/5w)	0.274 ± 0.015	3.77 ± 0.170
Long term (−20°C/9w)	0.286 ± 0.019	3.624 ± 0.099
(Mean Stability % ± CV)	92.33 ± 6.86	96.52 ± 6.79
Freeze/thaw (Cycle I)	0.296 ± 0.022	3.912 ± 0. 264
Freeze/thaw (Cycle II)	0.294 ± 0.009	3.532 ± 0.267
Freeze/thaw (Cycle III)	0.300 ± 0.023	3.638 ± 0.438
(Mean stability % ± CV)	99.00 ± 11.14	92.35 ± 14.42
Post-preparative (RT/27h)	0.292 ± 0.020	3.723 ± 0.102
Post-preparative (−20?C/45h)	0.277 ± 0.005	4.056 ± 0.483
(Mean stability % ± CV)	95.83 ± 7.37	97.25 ± 12.96
Stock Solution (RT/6h)	0.254 ± 0.006	3.664 ± 0.064
(Mean stability % ± CV)	84.60 ± 2.66	91.61 ± 1.75
Stock Solution (−20°C/10D)	0.280 ± 0.007	3.944 ± 0.039
(Mean stability % ± CV)	93.24 ± 2.42	98.60 ± 1.01

*RT = room temperature, w = week, D = day, h = hour.

Stability

Drug stabilities of CLA during handling and/or storage in plasma were evaluated at low and high QC standards after short and long times of storage at room temperature for 4 and 6 h, at −20°C for two, five and nine weeks and over three freeze-and-thaw cycles. Stabilities were computed by comparing the pertinent detector response from tested solutions after storage with that of freshly prepared ones measured at zero time. Results in this context are shown in Table V.

Utility

The applicability and robustness of the method for analysis was evaluated by analyzing many samples taken from recruited healthy volunteers given a single dose of 500 mg tablet of CLA. A typical mean plasma concentration–time profile is presented in Figure 3.

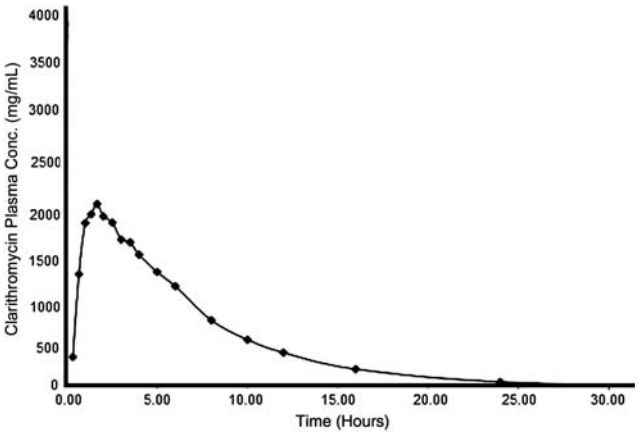

Figure 3. Concentration–time profile of CLA in human plasma following oral administration of 500 mg CLA tablet.

Results and Discussion

Targeting active substances in biological fluids is an important issue with regards to clinical, forensic and bioavailability studies. Analysis of CLA in human plasma is complicated because it exhibits a weak and non-specific UV absorption below 220 nm. Additionally, it provides low plasma concentration following its oral administration. Accordingly, its monitoring and quantitation needs to be specific and sensitive with high throughput.

Representative chromatograms of drug-free plasma (blank) and plasma sample spiked with CLA and the IS are shown in Figure 2, which shows a good resolution with no interference peaks from endogenous matter at the retention times of CLA and the IS at 12.26 and 6.52 min, respectively, indicating the selectivity of measurement.

Six calibration plots of eight point standards were constructed and studied separately by least-squares regression analysis. The plots were linear over the concentration range of 0.05–5.0 μg/mL with a high mean regression coefficient, $R^2 = 0.9999$, and a mathematical expression $Y = 0.7426X - 0.0005$. This equation was utilized to backcalculate calibration standards and QC concentrations.

The lowest standard of concentration (0.05 μg/mL) in the calibration plot was regarded as the LLOQ because it provided a distinguishable response from blank with an accuracy of 100% and precision of 12%.

To establish reliability of this assay in terms of accuracy and repeatability, QC samples of low, medium and high concentrations that contained 100 μL of the IS were analyzed in one day and in three consecutive days. The concentration of each analysis was backcalculated using the respective regression equations in each day and presented in Table II. The overall means of intra-day and inter-day accuracy coupled with CV% over the studied range were 99.24 ± 2.6 and 100.02 ± 3.76, respectively.

To verify the efficiency of the extraction procedure and to account for any potential variations during measurements, absolute recoveries were estimated on the basis of comparing peak area of the extracted QC sample with those of an equivalent unextracted sample in aqueous phase and found to vary from 89.8–98.2% with an average of 95.74 ± 5.20. Similarly, the

relative analytical recoveries were determined by comparing the extracted and measured concentrations with the added concentration and found to extend from 99.3–105% with an average close to 100%, as demonstrated in Tables III and IV.

Stability testing of CLA stock solution and QC standards at low and high concentrations in plasma was studied at room temperature and at −20°C after short and long periods of time, extended up to nine weeks, and found to be very stable, because no significant changes were observed in the concentration measured after storage in comparison with freshly prepared analogues measured at zero time. Additionally, CLA was found to be highly stable after three cycles of freeze and thaw, as shown in Table V.

Eventually, the utility of the method was conducted and proven, because a high number of real samples from volunteers given a CLA tablet were successfully analyzed and are presented in Figure 3.

Conclusions

In view of our findings, the method included linearity, high recovery, low LOQ and the absence of co-eluted interfering peaks, together with the use of liquid–liquid extraction and a fast response ECD instead of the expensive MS. These merits reflect the simplicity, sensitivity, selectivity and cost-effectiveness of this method over its reported counterparts; all are in agreement with the requirements for clinical, forensic toxicology and bioavailability studies.

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