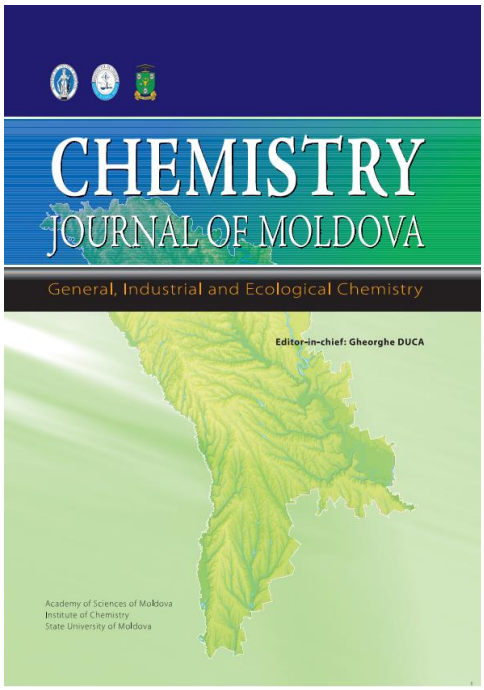

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COMPARATIVE ANALYSIS AND APPLICATION OF NOVEL SPECTROPHOTOMETRIC APPROACHES AND BIOASSAY FOR FAST MACROLIDE QUANTIFICATIONIN TABLETS

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Abstract. Novel spectrophotometric methods and bioassays have been developed and validated for clarithromycin quantitation in tablets. Spectrophotometric techniques were based on charge transfer complexation through naphthoquinone derivatives. Reactions were carried out in alkaline medium using 1,2-naphthoquinone-4-sulphonate and phylloquinone, which showed an absorption maximum at 452 and 455 nm, respectively. While bioassay was conducted dependent on the inhibitory effect upon the strain of *Bacillus subtilis* ATCC 9372, by applying cylinder-plate. Linear calibration curves with correlation coefficients of 0.9980-0.9998 were obtained. Molar absorptivity and *Sandell's* sensitivity were less than 10.73 L/mole/cm and 0.0099 µg/cm, respectively, with a detection limit down to 0.27 µg/mL and quantification limits of 0.68-0.78 µg/mL. The validation of the developed methods was performed for selectivity, precision, accuracy and robustness. Recoveries were found between 97.5–101.9% with % RSD being bellow to 3.5%. A comparative analysis was established and the methods were successfully applied for clarithromycin quantification in dosage forms.

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Introduction

Macrolide antibiotics, composed of a 12-, 14-, or 16-membered macrocyclic lactone, amino groups, and deoxy sugars, are derived from various Streptomyces species and/or obtained by semisynthetic processes. Erythromycin, roxithromycin, clarithromycin (CLA), and azithromycin are widely utilised macrolide antibiotics for treating infections caused by both Gram-positive and Gram-negative bacteria [1,2].

CLA is a semi-synthetic variant of the 14-membered macrolide known as erythromycin. Its chemical structure (Figure 1) closely resembles that of erythromycin, with the only difference being the replacement of the O-methyl group with a hydroxyl group at the sixth position of the lactone ring [3]. This alteration enhances CLA's performance compared to erythromycin, particularly regarding acid stability, pharmacokinetics, gastrointestinal side effects, and its antibacterial range. As a broad-spectrum antibiotic, CLA is commonly used to treat respiratory infections caused by bacteria such as *Pseudomonas aeruginosa*, *Chlamydophila pneumoniae*, *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, *Streptococcus pneumoniae*, and *Haemophilus influenza* [4–6]. It works usually by disrupting RNA-dependent protein synthesis in bacteria, leading to a bacteriostatic effect [3].

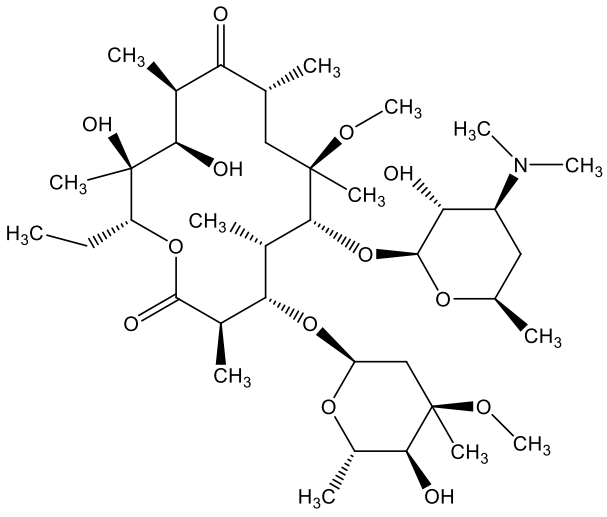

Figure 1. Clarithromycin chemical structure.

Literature survey reveals that clarithromycin determination in biological fluids (like plasma, blood and urine) and dosage forms were conducted by microbiological assay [7] and physico-chemical techniques, including ultraviolet (UV) spectrophotometry [8], spectrofluorimetry [9], thin layer chromatography [10], voltammetry [11], and capillary zone electrophoresis [12]. As well, several methods have been reported for CLA analysis using high-performance liquid chromatography (HPLC) with electrochemical detection [13], mass spectrometry [14], fluorescence detection after pre-column derivatisation [15] and UV detection [16]. But they usually require complex procedures and expensive equipment. Due to the weak chromophores of macrolides, indirect UV methods are generally more effective than direct absorbance methods. Moreover, different spectrophotometric methods were published for macrolides analysis using several derivative agents like: quinalizarin, 7,7,8,8-tetracyanoquinodimethane, alizarin, bromocresol green, eosin Y, rose Bengal, bromophenol blue, purpurin [17], 2,4-dinitrophenyl hydrazine [18], 1,2-naphthoquinone-4-sulphonate (NQS) [19] and 3-methylbenzothiazolin-2-one hydrazone/ferric chloride system [20]. Nevertheless, naphthoquinone reagents have not reported yet for the spectrophotometric determination of clarithromycin. A recent literature investigation has revealed that only a few *Bacillus subtilis bioassays are* available for the analysis of 14-membered macrolides, since it requires not only no specialized equipment but also no toxic solvents [21]. Concordance between spectrophotometric technique and bioassay for clarithromycin test has never been established until today. Hence, the present study was set out to provide such data and to overcome the existing disadvantages.

In the current work, simple, rapid, low-cost and robust bioassay and spectrophotometric methods were reported for CLA analysis in pure and dosage forms. In bioassay, the cylinder-plate was applied using the *Bacillus subtilis* ATCC 9372 as microorganisms. Beside, spectrophotometric techniques involved charge transfer reaction using two naphthoquinone reagents, 1,2-naphthoquinone-4-sulphonate and phylloquinone (NQ1), as chromogenic agents. It was encouraged to perform this study, since it has many advantages over the previously reported methods mentioned in literature, such as simpler procedure, good linearity range and higher sensitivity. Of note, an important feature of the present study is that naphthoquinone was never employed for such a task before.

Experimental

Materials

Standard of clarithromycin (CLA) was obtained from Sigma-Aldrich (Steinheim, Germany), even as CLA tablets were commercially purchased. Claridar® tablets (Dar Al Dawa, Algeria): labelled to contain CLA as 500 mg/tablet; Clarital® tablets (Sophal, Algeria): labelled to contain CLA as 500 mg/tablet. The 2-methyl-3-[(E)-3,7,11,15-tetramethylhexadec-2-enyl]naphthalene-1,4-dione (phylloquinone) and 1,2-naphthoquinone-4-sulphonate were provided from Sigma-Aldrich (Steinheim, Germany), as well as methanol, ethanol, isopropanol and acetone.

Instrumentation

Lambda 365 Spectrophotometer (Perkin Elmer, USA), with double beam and quartz cuvettes (l= 1 cm) were used for absorbance measurements. Vortex mixer (Genesis) and Toledo balance (Woluwe), were also used.

Preparation of stock and work solutions

CLA stock standard solutions were prepared by dissolving a precisely measured quantity of the compound in methanol to achieve a concentration of 50 µg/mL and kept in dark storage conditions. To prepare working standard solutions, the stock solution was diluted with methanol. These solutions were freshly made each day for use as working standards, with concentrations set at 1.0–18.0 µg/mL for spectrophotometric methods and 1.1–1.3 µg/mL for bioassay [7].

Preparation of tablet samples

The sample preparation was done based on an already published protocol [7,22,23]. Ten tablets were weighed and finely powdered. A weighed portion equivalent to the weight of one tablet was transferred to a 100 mL volumetric flask, sonicated for 20 min with about 10 mL of methanol. Then the solution was completed to volume with the same solvent. The mixture was mixed well and then filtered. A measured volume of the filtrate was diluted quantitatively with methanol to yield sample solutions having working concentrations.

For spectrophotometric methods

Naphthoquinones solutions

To prepare 0.2% w/v naphthoquinone (NQ) solution, 0.2 g of NQ was weighed and dissolved in distilled water (NQS) or in ethanol 70% (NQ1), transferred into a 100 mL standard volumetric flask, diluted to the mark with the same solvent and mixed well and protected from the light.

Derivatization procedure

An accurately measured 1.0 mL of CLA (15 μg/mL) was transferred into a 10mL standard volumetric flask. Then a volume of 0.2 M NaOH solution was added, followed by a volume of 0.2% (w/v) NQ solution, and the reaction mixture was diluted to the mark with d istilled water. The solutions were left at room temperature for a certain time and the absorbance was measured at 452 nm and 455 nm for CLA–NQS and CLA–NQ1, respectively, against reagent blank.

Composition of formed complex

To determine the stoichiometric ratio, Job's method of continuous variation (Job, 1964) [24] was applied using equimolar solutions of CLA and NQ (2.1×10^{-3} M). In this method, solution series of CLA and NQ were prepared as described under the general procedures and comprising different proportions (0:10, 1:9, ..., 9:1, 10:0). Then, the absorbance of each solution was plotted against the mole fraction of the drug.

For microbiological assay

Microorganism and inoculum standardization

The cultures of *Bacillus subtilis* ATCC 9372, after reconstitution, were cultivated and maintained on Grove-Randall's 1 agar (Merck). The microorganism standardisation was made according to the procedure described in The United States Pharmacopeia [25] for microbiological assay with antibiotics. Prior to use, the microorganism was grown in a test tube containing the same medium, which was incubated in a dry air oven during 24h at 34±3°C. The growth was suspended in tryptic soy broth (TSB) using a glass homogenizer and diluted to give a suspension of 25± 1% turbidity determined through the transmittance that was registered at 580nm, using a suitable spectrophotometer and 10 mm path length test tubes as absorption cells against TSB as blank. From this standardized suspension, aliquots of 1.0 mL were added to 50mL of Grove-Randall's number 1 agar at 46±1.5°C and used as the inoculated layer in the plate.

Cylinder-plate assay

To evaluate the standard samples, the bioassay utilised a 3×3 parallel line assay design, which included three doses of the standard and three doses of the sample on each plate, with a total of eight plates for each assay, in accordance with the guidelines of the European and US Pharmacopoeias [25,26]. The agar consisted of two distinct layers. Initially, 20 mL of Grove-Randall's number 1 agar solution was poured into a 100 mm × 20 mm Petri dish to form the base layer. Once this layer solidified, 5.0 mL of the inoculated layer was added on top. Each plate contained six stainless steel cylinders of the same dimensions (8mm × 6mm × 10mm) placed on the surface of the inoculated medium. Three of these cylinders were filled with 200 μL of reference solutions, while the remaining three contained the sample solutions. Both the standard solutions and samples were diluted in PBS (pH 4.0) [25] and tested at a concentration of 1μg/mL. The bioassay plates were incubated aerobically at 35 °C for 18 hours. The diameters of the growth inhibition zones were meticulously measured using a ruler. All procedures were conducted within a biological safety cabinet, and any contaminated materials were decontaminated prior to disposal.

Validation of the proposed methodology

Procedures were validated according to ICH recommendations [27] for linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), robustness and relative recovery.

Linearity, detection and quantification limits

The stock standard solutions of CLA were diluted with methanol to prepare working solutions at concentration ranges of 1.0–18.0 μg/mL for UV assays and 1.1–1.3 μg/mL for bioassay. The calibration graphs were obtained by plotting the absorbance average versus the corresponding concentrations, with confidence intervals of 95% at $p = 0.05$. LOD and LOQ were estimated using the successive dilution technique.

Accuracy, precision and robustness

Method accuracy [28] was estimated as recovery percentage for CLA solutions at three concentration levels (80%, 100% and 120%) in three replicates (n= 3) on the same day (intra-day) and on 3 consecutive days (inter-day).

The precision was evaluated in the same way of accuracy. Precision and accuracy were calculated in terms of relative standard deviation (RSD) and relative error (RE), respectively.

Robustness was carried out to evaluate the influence of small changes at the methods conditions. For UV methods, it was evaluated by changing the following parameters: NQ concentration, alkaline medium and reaction time. For the microbiological assay, it was checked by changing the parameters: inoculum concentration (2.0%), incubation temperature (35°C), and volume (thickness) of the inoculated layer (5.0 mL).

Results and discussion

Development and optimization of analytical methods

For spectrophotometric methods

CLA in methanol usually showed absorption peak at 210 nm and after its derivatization with naphthoquinones (NQS and NQ1), orange complexes were formed. Solutions of these reaction products were scanned in the 200–800 nm regions. The wavelengths maxima (λ_{max}) were observed at 452 nm for CLA-NQS and at 455 nm for CLA-NQS, while both values were adopted for absorbance measurement. These bands were probably formed by the dissociation of an original donor-acceptor complex [29]. Furthermore, UV spectra showed good selectivity and revealed no overlapping absorbance peaks from potential excipients.

Optimization studies

To select the optimum conditions of charge transfer reaction of CLA with NQ, the effect of various parameters such as the solvent nature, NQ reagent concentration, alkaline medium concentration, reaction time and temperature was examined in order to optimize the detection, by examining each reaction variable in a turn while keeping the others constant. In preliminary tests, pH parameter was investigated and no effect on the products stability was observed. The optimum conditions were fixed based on the measurement of absorbance values at the respective λ_{max} of each method.

Solvent nature and NQ volume effects

The role of the solvent's nature can facilitate the total transfer charge, the complex dissociation and the stabilization of the radical anion formed [29]. Consequently, different concentrations of the following solvents: water, methanol, ethanol, isopropanol, acetone and acetonitrile were tested as solvents for the considered reaction.

It was found that water was optimum for method A and method B and presented a maximum absorbance against the reagent blank of the orange products. It may be due to its favourable dielectric constant and the high solubility of the NQ(s) as well its capacity to form stable hydrogen bonds with the radical anion. It was chosen for all further measurements.

Hence, different concentrations of NQ reagent were reacted with CLA drug at a fixed concentration of 15 µg/mL in alkaline medium. This enables us to evaluate the concentration ranges over which CLA could be determined, by adding various volumes ranging between 0.25–2.5 mL of 0.2% (w/v) NQ and the results are shown in Figure 2.

The investigation of the results revealed that the reaction between CLA and NQ was completed with 1.75 mL for both reagent NQS and NQ1 and, it was fixed as the optimum concentration of NQ reagent for methods A and B. Therefore, the selected concentration of NQ offers high precise readings and after this maximum level the absorbance remained almost constant and a higher NQ volume had no beneficial effect on the absorption values.

Effect of the alkalinity

Macrolides have difficulty to react with NQ in acidic media, probably to lower electron density at the level of the amino group, in comparison with alkaline media [29]. From the preliminary investigations, alkaline medium was necessary for the derivatization reaction between CLA and NQS/NQ1. The reaction was tested in sodium hydroxide, disodium hydrogen phosphate, borax and sodium bicarbonate in a range of 0.1–0.6 M, and the orange colour product showed maximum absorbance with of sodium hydroxide (0.2 M) for both methods.

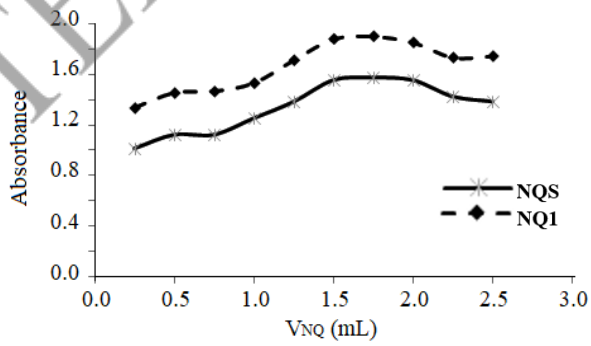

Figure 2. Influence of NQ volume (0.2% w/v) derivatization reactions of CLA (15µg/mL) with NQS and NQ2; NaOH 0.2 M: 1 mL; temperature: 25°C; reaction time: 20 min.

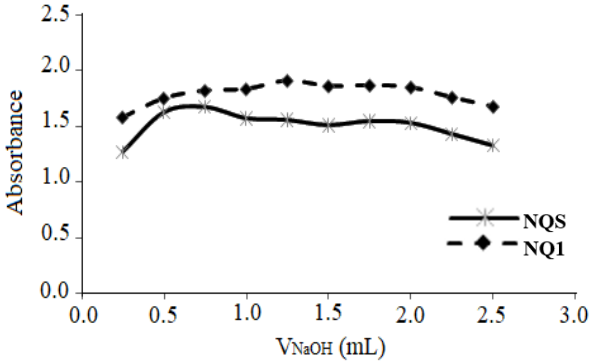

Figure 3. Effect of NaOH volume on the charge transfer reaction of CLA (15µg/mL) with NQS and NQ2; 0.2% w/v NQ: 1 mL; temperature: 25°C; reaction time: 20 min.

The effect of different volumes of NaOH (0.2 M) in the range of 0.25–2.5 mL was studied and absorbance of the formed product was measuring against the blank solution at 452 nm and 455 nm for CLA–NQS and CLA–NQ1, respectively (Figure 3). The results showed that the optimum values of NaOH solutions were 0.75 mL and 1.25 mL for CLA–NQS and CLA–NQ1, respectively, and hence they were selected as optimal condition. No appreciable changes were observed in increasing or decreasing NaOH concentrations around these values, indicating that the degree of derivatization was maximal for both reagents NQS and NQ1.

Optimization of reaction temperature and time

In order to select the optimum temperature of CLA-NQ reaction, various values from 25 to 80°C were examined under the other optimum factors. It was also found from the investigation that the ambient temperature in methods A and B yielded the highest sensitivity at their respective λ_{max}, which means that the reaction was achieved completion limits. Furthermore, the findings indicated that higher temperatures adversely affected the absorption values of the reaction solution, likely due to the instability of the obtained derivatives for both reagents. Therefore, a temperature of 25°C was determined to be ideal for achieving maximum orange complexes.

In this step of work, derivatization solutions of CLA with NQs were prepared and allowed the reaction to proceed for various time intervals between 0 and 60 minutes at room temperature (25±1°C). The optimal reaction time was estimated by measuring the absorbance of the resulting complexes for methods A and B. The obtained products were monitored, revealing that the ideal reaction time was 14 minutes for method A and 18 minutes for method B. Complete colour development occurred at these times, indicating that the complexes formed rapidly. Therefore, under these optimal conditions coloured products were more or less stable up to 1.5 h.

Molar ratio and reaction mechanism

Stoichiometric ratio between CLA and each NQ in alkaline medium was studied by Job's method of continuous variation [30]. Different ratios of equimolar solutions of the macrolide and the reagent were combined, and the absorbance of each mixture was measured. Job's plot in Figure 4 showed with the maximum shape at 0.5 ratio, which indicated that the stoichiometry of CLA : NQ is 1:1.The log(Kf) values were found to be 4.08 and 5.17 for methods A and B, respectively, which confirms the good stability of the formed complexes (Table 1).

The possible mechanism of the derivatization reaction can be proposed as found in Figures 4 and 5, where one mole of CLA interacted with one mole of NQ. So, one free electron of the nitrogen atom was transferred to the charge-deficient centre of one NQ molecule.

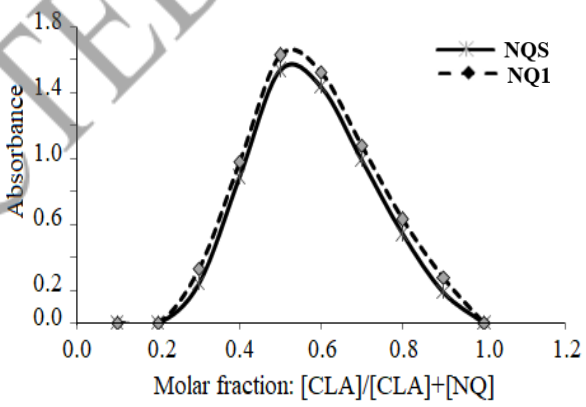

Figure 4. Job's method for naphthoquinones reaction with CLA

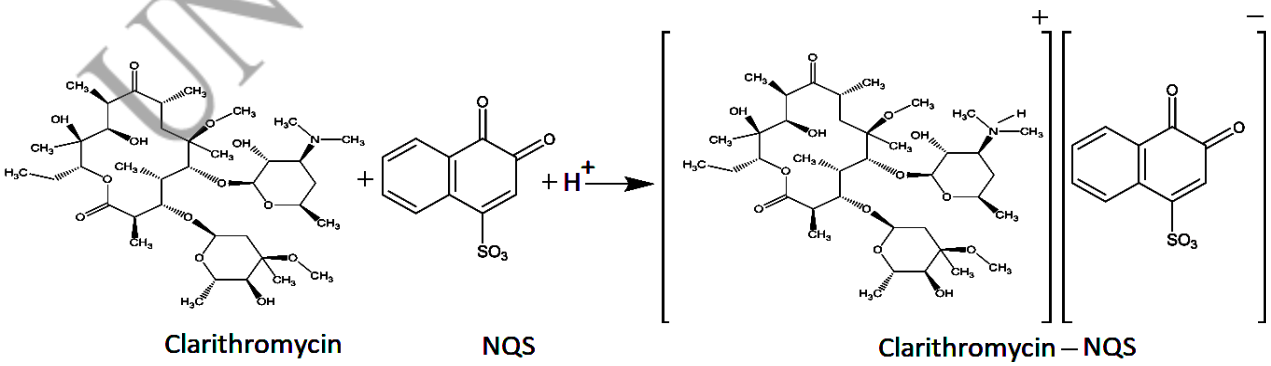

Figure 5. Scheme for the possible reaction mechanism pathway of CLA with NQS.

For Bioassay

Recently, there has been a growing emphasis on analytical methods for quantitatively determining substances, especially from regulatory agencies, due to their role in ensuring reliable medicine quality control. Choosing the right analytical methodology is essential and depends on factors like the drug's source, complexity, and purity. Biological methods are advantageous as they correlate measured parameters with the drug's therapeutic properties, minimising interference from impurities. Microbial assays are used to evaluate antibiotic potency by comparing their effects on sensitive microorganisms to reference standards. In this context, a 3×3 experimental design was utilised with three dose levels for both standard and sample solutions, following established Pharmacopoeia procedures.

Bioassay experimental conditions were carefully selected to ensure high test performance. Initial steps were conducted to establish an effective protocol, as cultures and sample preparation as well incubation conditions (like incubation time). In preliminary step, to mentioned potential influence of bacterial strain variability, many common strains were tested and compared. Due to its high sensitivity to CLA, *Bacillus subtilis* ATCC 9372 strain was chosen as a suitable test microorganism. Throughout the handling of microorganisms, all safety protocols were followed. All experiments were carried out in a laminar air flow cabinet, and any contaminated materials were decontaminated prior to disposal. The optimal conditions were cited in the experimental section.

Methods validation

The developed methods were validated according to the frequently recommended references and guidelines for the validation of analytical procedures [25,26].

Linearity, limits of detection and quantification

Data on CLA working range for the developed methods are shown in Table 1. For both UV methods, the calibration curves resulted in a coefficient of determination (R^2) greater than 0.9995, and was found to be 0.998 for the bioassay, thus satisfying the minimum acceptable criteria of ICH [27].

In UV methods, the detection (LOD) and quantification (LOQ) limits were 0.25–0.77 and 0.22–0.68 µg/ mL for CLA–NQS and CLA–NQ1, respectively, whereas in the Bioassay, LOD and LOQ values were much lower than 0.79 µg/ mL, these were indicating high sensitivity of the developed methods.

Table 1

Optimized characteristics and regression data of CLA analysis.

Parameters	*Value*		
	NQS	*NQ1*	*Bioassay*
Colour	Orange	Orange	-
λ_{max}(nm)	452	455	-
Reaction time (min)	14	18	-
Logformation constant ($\log K_f$)	4.08	5.17	-
Linear range (µg/mL)	1.0-18.0	1.0-18.0	1.1-1.3
Regression equation	y = 0.0654x + 0.0892	y = 0.0691x + 0.0812	y = 6.904 x + 5.492
Determination coefficient(R^2)	0.9996	0.9998	0.998
Limit of detection (µg/mL)	0.25	0.22	0.26
Limit of quantification (µg/mL)	0.77	0.68	0.78
Molar absorptivity ($L\times mole^{-1}cm^{-1}$)	9.83	10.72	-
Sandell's sensitivity ($\mu g\times cm^{-2}$)	0.0094	0.0098	-

Table 2

Data of intra-day and inter-day accuracy and precision for CLA analysis.

		Intra-day			*Inter-day(n=3)*		
NQ	*Taken/added* µg/mL*	*Found µg/mL*	*%RSD*	*%RE*	*Found µg/mL*	*%RSD*	*%RE*
NQS	12.0	12.1	0.9	0.9	12.2	1.8	2.0
	15.0	14.7	2.3	1.7	15.2	0.8	1.3
	18.0	18.4	1.6	2.1	18.5	2.1	2.8
NQ1	12.0	12.2	1.0	1.3	11.8	1.4	1.5
	15.0	15.3	2.1	2.0	15.3	0.9	2.2
	18.0	17.8	2.2	1.1	18.5	2.5	3.1
Bioassay*	1.0	1.01	1.0	1.4	1.02	0.7	1.7
	2.0	2.02	0.9	0.9	2.04	1.7	1.9
	3.0	3.06	2.5	1.9	3.10	2.2	3.2

Accuracy and precision

Accuracy of the proposed methods was evaluated by recovery studies. The average recovery was in the range 98.0%– 103.4% for concentration levels 80, 100 and 120% of CLA working concentration. It can be noted from Table 2 that the coefficients of % RSD were between 0.8% and 2.5% for UV methods and between 0.7% and 2.5% for Bioassay. These values were satisfactory, since they are lower than 5%, which is the maximum limit acceptable by ICH [27].

The coefficient of variation the studied methods was found in the range of 0.9– 2.1% for intra-day and 1.3– 3.2% for inter-day assay (Table 2), which are lower than 5%, according to the criteria of the recommended guidelines, indicates that the accuracy and precision of the proposed methods are acceptable.

Stability, selectivity and robustness

The selectivity of CLA was verified simultaneously to the determination of the accuracy. All methods were selective and specific to CLA without interference with other components such as: tablets matrix (potential excipients), impurities and degradation products. Else, the effect of daylight on the formed product stability was investigated and it was found that NQ derivatives were stable at least for 1.5 hours, after that, the absorbance value decreased and the derivative molecule will completely breakdown after five hours. Whereas, upon the storage in the refrigerator (4°C) for they showed a good stability for 15 days, with %RSD less than 3.2%.

The robustness was evaluated by examining the influence of NQ concentration, alkaline medium and time of the reaction. The % recovery was within 98.8–101.3% with % RSD of 0.8–2.1% for methods A and B (Table 3), whereas for the bioassay, this maximum value of such coefficient reached 1.5% (Table 3). These results revealed that minor deviations have no practical significance in pharmaceutical analysis of CLA. So, it is considered that the proposed methods are robust, since these coefficients were lower than 5.0%.

Application to the pharmaceutical dosage forms

After the validation of the proposed analytical methods, they were applied for the analysis of collected tablets containing CLA, as well as HPLC method [31] was used too, and the results were statistically compared with each other.

Using the suggested methodology, tablets of Claridar® and Clarital® showed an average percentage content of 99.0%–101.9% for spectrophotometric methods and bioassay which means a good accordance with the declared values (Table 4).

Table 3

Robustness results of CLA by spectrophotometric methods and bioassay.

Spectrophotometric methods					*Bioassay*			
Investigated condition		*Parameters*	*Recovery (%)*	*RSD (%)*	*Investigated condition*	*Parameters*	*Recovery (%)*	*RSD (%)*
t (min)	NQS	12	98.9	1.9	Inoculum	1.8	99.2	0.7
		16	100.8	1.1	Conc (%)	2.2	101.3	1.2
	NQ1	16	101.2	2.1				
		20	99.1	1.7				
NQ (mL)	NQS	1.65	100.7	0.8	Incubation	33	100.8	1.5
		1.85	99.2	1.7	T (°C)	37	98.9	0.8
	NQ1	1.65	99.4	1.2				
		1.85	101.1	1.8				
NaOH (mL)	NQS	0.65	99.3	1.5	Inoculated	4.5	101.0	1.3
		0.85	101.0	2.0	layer (mL)	5.5	100.9	0.9
	NQ1	1.15	98.8	1.6				
		1.35	100.9	1.4				

Table 4

CLA determination in tablets by the developed methods and HPLC.

Samples	*Parameters*	*Proposed methods*			*Reported*
		NQS	*NQ1*	*Bioassay*	*HPLC*
Claridar® tablets	% Content[a]	100.8	99.3	99.1	99.0
	%RSD	2.1	1.9	2.3	2.5
	t-value [b]	1.72	1.64	1.55	-
Clarital® tablets	% Content[a]	101.1	100.7	99.4	101.9
	%RSD	1.9	2.4	2.7	2.7
	t-value [b]	1.65	1.59	1.71	-

Besides, no interference from coexisting substances were found, indicating highly specific and selective of the newly methods. Moreover, with respect to *t*-tests, no significant differences were found between the proposed and the reported methods at 95% confidence level, and this demonstrated the feasibility and reliability of the present methods in the selected dosage forms.

Methods comparison

Spectrophotometric, bioassay and HPLC methods were applied to assay CLA in tablets. The mean contents were found to be (99.48– 99.56%), 100.52% and 99.54% using spectrophotometric techniques, bioassay and HPLC, respectively (Table 5).

Table 5

Determination of CLA in Clarital® tablets by different techniques.

Samples	*% Content*			
	Spectrophotometric methods		*Bioassay*	*HPLC*
	NQS	*NQ1*		
1	98.8	100.8	101.9	100.4
2	101.1	98.6	99.4	98.5
3	100.9	99.2	100.7	101.4
4	97.5	101.1	98.9	98.3
5	99.1	98.1	101.7	99.4
Average of determinations	99.48	99.56	100.52	99.54

According to the obtained data, a good correlation between the studied techniques was observed. Using the developed methods, results were comparable with previous studies [31] and within the acceptable limits of 95–105% [26]. Furthermore, novel methods are cost-effective than existing tools and are more suitable for CLA quantification.

CLA content was determined in Clarital® tablets by NQS method and bioassay. Obtained results indicate a satisfactory correlation, with linear curve equation of $y= 0.257x + 0.862$ ($n= 12$; $R^2= 0.993$). It is noteworthy to mention that cross-referencing the developed procedures with another effectual method creates a possibility to provide more useful data than some publish results with direct spectrophotometric method [21].

Considering their advantages, developed methods are inexpensive and simple compared to other methods like HPLC. HPLC can be used for the quantitative determination of antibiotics with high precision, but it cannot offer any accurate data about biological activity [21]. However, the microbiological tests have certain changeability, but the findings of this study confirmed the possibility to correlate the obtained spectrophotometric results with those of bioassay. To authenticate the quality of any drug, it is necessary to combine various performance and sensitive analytical methods, in order to match the quality and to predict the therapeutic efficacy as well as drug security [31]. Hence, the studied techniques are quite practical for CLA quantitative determination in its dosage forms, being an adequate alternative technique for CLA routine quality control.

Conclusions

In view of the significance of quantifying macrolide antibiotics in dosage forms, it was essential to develop and validate simple, cost-effective methods for analysing CLA in tablet formulations. The novel naphthoquinones UV methods and bioassay showed adequate linearity ($R^2> 0.998$) at the ranges of 1.0–18.0 µg/mL and 1.1–1.3 µg/mL, respectively, with LOD (0.22–0.26 µg/mL) and LOQ (0.68–0.78 µg/mL) values compatible with the targeted approaches. Precision (RSD< 2.6%), robustness (RSD< 2.2%) and recovery (> 97.5%) values confirm that these methods were precise, specific and robust. Moreover, tablets quantification showed good agreement with the label claim without any interference with the interacting components (excipients, impurities and degradation products).

Conversely, the developed procedures have several advantages such as simplicity, sensitivity, specificity, versatility and cost-effectiveness (chemicals and apparatus), which make them more suitable for routine quality control of CLA and can be interchangeable with the existing conventional techniques. However, they have their own limitations, which often depend on the specific goals of the analysis and the nature of the samples (*e.g.* dependence on chemicals quality, standardisation issues of bioassay and calibration challenges). The proposed methods showed a particular specificity towards formulation matrix, and can be broader their applicability to other macrolides.

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References

1. Kaneko, T.; Dougherty, T.J.; Magee, T.V. Macrolide Antibiotics. Triggle, D.J.; Taylor, J.B. Eds. Comprehensive Medicinal Chemistry II. Elsevier: Oxford, 2007, 7, pp. 519–566. DOI: https://doi.org/10.1016/B0-08-045044-X/00219-4
2. Bijay, L.P.; Lekhan, L.K.; Kishor, D.; Manasi, G. Analyzing atomic scale structural details and nuclear spin dynamics of four macrolide antibiotics: erythromycin, clarithromycin, azithromycin, and roxithromycin. RSC Advances, 2024, 14(25), pp. 17733–17770. DOI: https://doi.org/10.1039/D4RA00718B
3. Omura, S. Macrolide Antibiotics. Chemistry, Biology, and Practice. Academic Press: London, 2002, 635 p. DOI: https://doi.org/10.1016/B978-0-12-526451-8.X5000-0
4. Brunton, L.; Lazo, J.; Parker, K. Goodman and Gilman's the Pharmacological Basis of Therapeutics. McGraw Hill: New York, 2005, 1984 p.
5. Nakagawa, Y.; Itai, S.; Yoshida, T.; Nagai, T. Physicochemical properties and stability in the acidic solution of a new macrolide antibiotic, clarithromycin, in comparison with erythromycin. Chemical and Pharmaceutical Bulletin, 1992, 40(3), pp. 725–728. DOI: https://doi.org/10.1248/cpb.40.725
6. Rajinikanth, P.S.; Mishra, B. Floating *in situ* gelling system for stomach site-specific delivery of clarithromycin to eradicate *H. pylori*. Journal of Controlled Release, 2008, 125(1), pp. 33–41. DOI: https://doi.org/10.1016/j.jconrel.2007.07.011
7. Mahmoudi, A.; Fourar, R.E.-A.; Boukhechem, M.S.; Zarkout, S. Microbiological assay for the analysis of certain macrolides in pharmaceutical dosage forms. International Journal of Pharmaceutics, 2015, 491(1-2), pp. 285–291. DOI: https://doi.org/10.1016/j.ijpharm.2015.06.027
8. Hassan, W.; Zaman, B.; Ali, B. Derivative spectrophotometric method for the determination of clarithromycin in pharmaceutical formulation and application to *in-vitro* dissolution. Research and Reports in Immunology, 2018, 2, pp. 1–8. DOI: https://doi.org/10.35841/immunology.2.1.1-8
9. Khashaba, P.Y. Spectrofluorimetric analysis of certain macrolide antibiotics in bulk and pharmaceutical formulations. Journal of Pharmaceutical and Biomedical Analysis, 2002, 27(6), pp. 923–932. DOI: https://doi.org/10.1016/s0731-7085(01)00609-4
10. Kwiecień, A.; Krzek, J.; Gądek, M. Simultaneous identification and quantitative determination of azithromycin, clarithromycin, roxithromycin, spiramycin and troleandomycin by thin-layer chromatography and densitometry. Acta Chromatographica, 2013, 26(4), pp. 657–670. DOI: https://doi.org/10.1556/AChrom.26.2014.4.8
11. Vajdle, O.; Guzsvány, V.; Škorić, D.; Csanádi, J.; Petković, M.; Avramov-Ivić, M.; Kónya, Z.; Petrović, S.; Bobrowski, A. Voltammetric behavior and determination of the macrolide antibiotics azithromycin, clarithromycin and roxithromycin at a renewable silver–amalgam film electrode. Electrochimica Acta, 2017, 229, pp. 334–344. DOI: https://doi.org/10.1016/j.electacta.2017.01.146
12. Paul, P.; Duchateau, T.; Sänger-van de Griend, C.; Adams, E.; Van Schepdael, A. Capillary electrophoresis with capacitively coupled contactless conductivity detection method development and validation for the determination of azithromycin, clarithromycin and clindamycin. Journal of Separation Science, 2017, 40(17), pp. 3535–3544. DOI: https://doi.org/10.1002/jssc.201700560
13. Zaater, M.F.; Tahboub, Y.R.; Ghanem, E. Determination and stability assessment of clarithromycin in human plasma using RP-LC with electrochemical detection. Journal of Chromatographic Science, 2012, 50(9), pp. 763–768. DOI: https://doi.org/10.1093/chromsci/bms055
14. Alvi, S.N.; Dgither, S.A.; Hammami, M.M. Rapid determination of clarithromycin in human plasma by LCMS/MS assay. Pharmaceutical Analytical Chemistry, 2016, 2(1), pp. 110. DOI: https://doi.org/10.4172/2471-2698.1000110
15. Sastre Toraño, J.; Guchelaar, H.-J. 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. Journal of Chromatography B. 1998, 720(1-2), pp. 89–97. DOI: https://doi.org/10.1016/s0378-4347(98)00456-3
16. Li, W.; Jia, H.; Zhao, K. Determination of clarithromycin in rat plasma by HPLC–UV method with pre-column derivatization. Talanta, 2007, 71(1), pp. 385–390. DOI: https://doi.org/10.1016/j.talanta.2006.04.015
17. Keskar, M.R.; Jugade, R.M. Spectrophotometric investigations of macrolide antibiotics: A brief review. Analytical Chemistry Insights, 2015, 10, pp. 29–37. DOI: https://doi.org/10.4137/ACI.S31857
18. Mahmoudi, A.; Van Schepdael, A. Derivatization techniques based on charge transfer reactions for spectrophotometric determination of josamycin in various dosage forms. Chemistry Journal of Moldova, 2024, 1(19), pp. 37–46. DOI: https://doi.org/10.19261/cjm.2024.1165
19. Gouda, A.A.; Hashem, H.; Hassan, W. Spectophotometric methods for determination of cefdinir in pharmaceutical formulations *via* derivatization with 1,2-naphthoquinone-4-sulfonate and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole. Drug Testing and Analysis, 2012, 4(12), pp. 991–1000. DOI: https://doi.org/10.1002/dta.280

20. Al-Majed, A.A.; Belal, F.; Khalil, N.Y.; Ibrahim, K.E.E. Kinetic spectrophotometric determination of josamycin in formulations and spiked human plasma using 3-methylbenzo thiazolin-2-one hydrazone/Fe^{+3}system. Journal of AOAC International, 2004, 87(2), pp. 352–359. DOI: https://doi.org/10.1093/jaoac/87.2.352
21. Mahmoudi, A.; Tertiș, M.; Simon, L.M.; Van Schepdael, A.; De Francia, S.; Junie, L.M.; Săndulescu, R. Correlated quantification using microbiological and electrochemical assays for roxithromycin determination in biological and pharmaceutical samples. Talanta, 2020, 211, 120703, pp. 1–10. DOI: https://doi.org/10.1016/j.talanta.2019.120703
22. Mahmoudi, A. LC determination and stability assessment of macrolide antibiotics azithromycin and spiramycin in bulk and tablet samples. International Letters of Natural Sciences, 2015, 47, pp. 1–10. DOI: https://doi.org/10.56431/p-so7039
23. Mahmoudi, A.; Boukhechem, M.S. Novel liquid chromatographic method for the simultaneous evaluation of erythromycin and azithromycin in human urine. Journal of Materials and Environmental Science, 2017, 8(6), pp. 1953–1959. https://www.jmaterenvironsci.com/Document/vol8/vol8_N6/207-JMES-2050-Mahmoudi.pdf
24. Sawyer, D.T.; Heineman, W.R.; Beebe, J.M. Chemistry Experiments for Instrumental Methods. John Wiley & Sons: New York, 1984, 427 p.
25. The United States Pharmacopeia (USP) The United States Pharmacopeial Convention. Rockville, 2014. https://www.usp.org/
26. European Pharmacopoeia, E.D.Q.M. Council of Europe, Strasbourg France, 2023. https://www.edqm.eu/en/european-pharmacopoeia-ph.-eur.-11th-edition
27. ICH–Guidelines, Validation of analytical procedures Q2(R2). Switzerland: Geneva, 2022, 38 p. https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline
28. Karnes, T.H.; March, C. Precision, accuracy, and data acceptance criteria in biopharmaceutical analysis. Pharmaceutical Research, 1993, 10, pp. 1420–1426. DOI: https://doi.org/10.1023/a:1018958805795
29. Ashour, S.; Bayram, R. Novel spectrophotometric method for determination of some macrolide antibiotics in pharmaceutical formulations using 1,2-napthoquinone-4-sulphonate. Spectrochimica Acta Part A, 2012, 99, pp. 74–80. DOI: https://doi.org/10.1016/j.saa.2012.08.024
30. Mahmoudi, A.; De Francia, S.; Van Schepdael, A. Development and validation of new spectrophotometric procedures for the analysis of roxithromycin in bulk and dosage forms. Journal of Microbiology, Biotechnology and Food Sciences, 2024, 14(3), e10743. DOI: https://doi.org/10.55251/jmbfs.10743
31. Mahmoudi, A.; De Francia, S.; Paul, P. Development and validation of high performance liquid chromatography method for determination of clarithromycin in pharmaceutical tablets. Journal of Separation Science, 2023, 46(21), pp. 2300424. DOI: https://doi.org/10.1002/jssc.202300424
32. Naveed, S.; Qamar, F. Simple UV spectrophotometric assay of clarithromycin. International Journal of Pharma Sciences and Research, 2014, 5(9), pp. 582–585. https://www.ijpsr.info/docs/IJPSR14-05-09-015.pdf