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Validated Spectrophotometric Methods Based on Charge Transfer Complexation Reactions for the Quantification of Antimalarial Drug: Chloroquine Phosphate in Pure and Dosage Forms

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ABSTRACT: **Chloroquine phosphate (CQP), an antimalarial medication, has been determined using two straightforward, sensitive, quick, and verified spectrophotometric techniques. The procedures were based on the generation of charge transfer complexes in methanol utilizing alizarin red S (ARS) and quinalizarin (Quinz) as chromogenic reagents, respectively, with absorption maxima at 560 and 531 nm for each. Investigated was the optimization of the reaction conditions, including the kind of solvent, reagent concentration, and reaction time. Beer's law is effectively observed using Quinz and ARS, respectively, in the concentration ranges of 1.0-20 and 1.0-16 µg mL-1, with strong correlation coefficients (r2 ≥0.9995) and low relative standard deviations (RSD%≤ 1.07). Calculations were also made for the molar absorptivity, Sandell sensitivity, detection, and quantification limitations. The approaches were successfully used to identify CQP in pharmaceutical formulations, and the standard addition technique. was used to evaluate the methods' validity. Results from the suggested procedures for pure CQP and** dosage forms were in good agreement with those from the previously described approach.

Keywords: **Chloroquine phosphate; Spectrophotometry; Charge transfer reaction; Dosage forms; Method validation.**

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I. INTRODUCTION

Chloroquine phosphate (CQP), is a 4-aminoquinoline compound and chemically is 7-chloro-4-[[4-(diethylamino)-1-methylbutyl]amino] quinoline phosphate (Fig. 1). As the first and most effective synthetic antimalarial drug, it is used to treat all forms of malaria [1, 2]. The FDA's approval of hydroxychloroquine and chloroquine as an emergency treatment for COVID-19 will expire on June 15, 2020 [3]. CQP has been officially acknowledged by the US Pharmacopoeia [2].

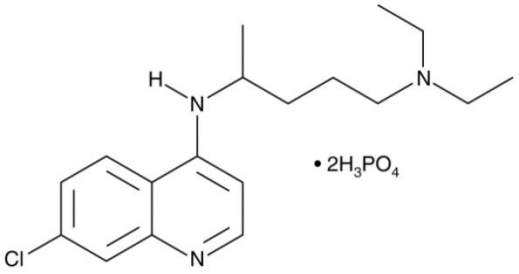


Fig. 1. The chemical structure of Chloroquine phosphate (CQP).

High-performance liquid chromatography [4,5], electrochemical [6,7], conductometric and indirect AAS [8], and spectrofluorimetry [9,10] were just some of the methods described in the literature for the determination of CQP in pure, dosage forms, and biological fluids. These stated approaches typically involve extensive sample pre-treatment, cleanup processes before analysis, and the use of costly instruments that are beyond the reach of most quality control labs**.**

Since it is simple, cheap, quick, sensitive, selective, accurate, precise, widely available, and applicable for pharmaceutical analysis, visible spectrophotometry is widely used in quality control and clinical laboratories, hospitals, and pharmaceutical industries for the assay of various classes of drugs in pure form, pharmaceutical formulations, and biological samples To the best of our knowledge, only a handful of spectrophotometric approaches have been described for quantifying CQP in pharmaceutical dosage forms [10-27] (Table 1).

Table 1. Comparison between the reported spectrophotometric methods for determination of CQP.

Method	**Wavelength (nm)**	**Beer's law ($\mu g\ mL^{-1}$)**	**Molar absorptivity ($L\ mol^{-1}cm^{-1}$)**	**Reference**
DDQ	462	5-53	NA	[11]
I_2	293	1-15	NA	
Chloranilic acid	520	8.0-80	NA	[12]
BCG	420	1.0-20	1.79×10^4	[13]
BCP	420	0.5-12	3.09×10^4	
BCP	420	1.25-8.75	4.09×10^4	[14]
Rose bengal	420		NA	[15]
BCG	420	50-250	NA	[16]
Wool fast blue	590	50-250	NA	
$[Co(SCN)_4]^{2-}$	625	2.0-60	NA	[17]
$[Mo(SCN)_6]^-$	467	2.0-42	6.82×10^3	[18]
$KBrO_3$-KBr	350	40-200	NA	[19]
Fe^{3+}/ 1, 10-phenanthroline	510	20-320	666.6	[20]
$KBrO_3$/ H_2SO_4	343	0.5-50	NA	[21]
2,4-dinitrophenol	430	3-70	1.47×10^3	[22]
picric acid	420	1.2-30	1.1×10^3	
UV/(0.06 M monosodium phosphate)	343	7.2 - 19.2	NA	[23]
Calmagite	665	2-26	1.67×10^4	[24]
7, 7, 8, 8-tetracyanoquino-dimethane (TCNQ)		0.4-4.0	NA	[25]
Tetracyanoethylene (TCNE)	415	1-8	8.3×10^4	[26]
Quinz	560	1.0-20	1.2370×10^4	This work
ARS	531	1.0-16	1.6742×10^4	

NA: not available.

Few of the methods require a strict pH control and a tedious and time-consuming liquid-liquid extraction step; measurements are done at shorter wavelengths; some other methods have a relatively narrow dynamic linear range, involve a heating step, and/or use expensive reagents; however, all of these drawbacks are present in the previously reported methods. As a result, it was important to create a new, straightforward, low-cost, and selective spectrophotometric approach for the analysis of CQP in their pharmaceutical dosage forms.

The purpose of this study is to create spectrophotometric methods for the pure and dose measurement of CQP that are easy to use, sensitive, accurate, precise, cost-effective, and verified. The proposed techniques make use of the chromogenic reagents quinalizarin (Quinz) and alizarin red S (ARS), both of which may form stable charge transfer complexes with CQP. CQP assays using typical excipients at levels observed in dosage forms showed no interference. Statistical evidence supports the accuracy of these procedures.

II. Materials and Methods

2.1. Instrumentation:

All the absorption spectral measurements were made using Shimadzu UV-1601 UV/Visible double beam spectrophotometer (Sweden) with a fixed slit width (2 nm) and equipped with 10 mm matched quartz cells.

2.2. Chemicals and reagents

All employed chemicals and solvents (dimethyl sulfoxide, methanol, acetonitrile, acetone and ethanol) were of analytical-reagent grade and used throughout the study.

Pharmaceutical grade CQP was kindly supplied by Alexandria Co. for Pharmaceuticals, Alexandria, Egypt. The commercial pharmaceutical formulations: Alexoquine tablets labeled to contain 250 mg CQP per tablet (Alexandria Co. for Pharmaceuticals, Alexandria, Egypt).

A standard stock solutions of CQP containing 100 µg mL^{-1} and 1.0×10^{-3} mol L^{-1} were prepared by dissolving 10 and 51.6 mg of pure medication in the smallest volume of DMSO and then diluting to 100 mL with methanol to produce the working concentration. When stored at a cool (< 25 ºC) and dark location, the standard solution was shown to be stable for at least a week.

Both the alizarin red S, 3,4-dihydroxy-9, 10-dioxo-2-anthracene sulfonic acid (ARS) and quinolizarin 1,2,5,8-tetrahydroxy-anthraquinone (Quinz) were purchased from Sigma-Aldrich and utilized as-is. To make a stock solution of 1.0×10^{-3} mol L^{-1}, the required amount of the reagent was dissolved in around 25 mL of methanol, and the volume of the 100 mL volumetric flask was filled to the mark with more of the solvent. After a week, this answer held steady.

2.3. General procedure

A series of 10 mL volumetric flasks were filled with aliquots of the CQP standard working solution in the concentration ranges (1.0-20 µg mL^{-1}) and (1.0-16 µg mL^{-1}) using Quinz and ARS, respectively. Two milliliters of either a Quinz or ARS solution (1.0 x 10^{-3} mol L^{-1}) were poured into each flask. After giving the mixture a good shake to speed up the reaction, methanol was added until the volume was correct. Quinz and ARS were used to test the absorbance of the resultant solutions at 560 and 531 nm, respectively, against a reagent blank that was made at the same time. The calibration graph was created by plotting the absorbance *versus* the CQP concentration. The corresponding regression equation was derived.

2.4. Assay procedure for dosage forms

Using an agate mortar, the 250 mg CQP in each of 10 tablets was finely ground and precisely weighed. The powder was transferred to a 100 mL calibrated flask and dissolved in 10 mL DMSO after being precisely weighed. After the contents of the flask had been sonicated and agitated for about 10 minutes to ensure thorough mixing, they were filtered through Whatman No.42 filter paper. A stock solution of 100 µg mL^{-1} was prepared by discarding the initial portion of the filtrate and bringing the solution to volume with methanol. The required concentration ranges were obtained by further diluting this solution using the same solvent. A series of 10 mL volumetric flasks were used to apply the suggested procedures to aliquots spanning the working concentration ranges for each approach. Using the associated regression equations or the calibration graphs, the nominal content of the tablets was calculated.

2.5. Stoichiometric relationship

At the optimum wavelengths, we used the continuous variation approach originally developed by Job [28] and modified by Vosburgh and Coober [29] to calculate the stoichiometric ratios of the charge transfer complexes generated between CQP and reagents. Both the CQP standard solution and the reagent solution 1.0×10^{-3} mol L^{-1} were prepared using Job's method of continuous variation. Each solution in a series was made with a total of 2.0 mL of medication and reagent. Following the foregoing steps, a 10-mL calibrated flask was filled with methanol and the reagents were mixed with the medication in varied amounts

III. RESLUTS AND DISCUSSIONS

3.1. Absorption spectra

Under ideal circumstances, the radical anion, which serves as the absorbing species, was promptly generated in the medium following the combination of the reagents. It exhibited its highest level of absorption at wavelengths of 560 nm and 531 nm when employing Quinz and ARS, respectively, inside a methanol medium (as depicted in Fig. 2a and 2b). Therefore, these wavelengths were selected for all subsequent observations in order to achieve the highest sensitivity for the proposed methodologies. It is of significance to note that the Quinz and ARS, when present in a methanol medium, demonstrate peak absorption wavelengths at 491 nm and 420 nm, respectively. The substantial disparity in the peak values of the absorption bands for the reagent and the product, specifically 69 nm for Quinz and 111 nm for ARS, facilitated the accurate assessment of the products while minimizing the influence of the excess reagents present in the medium.

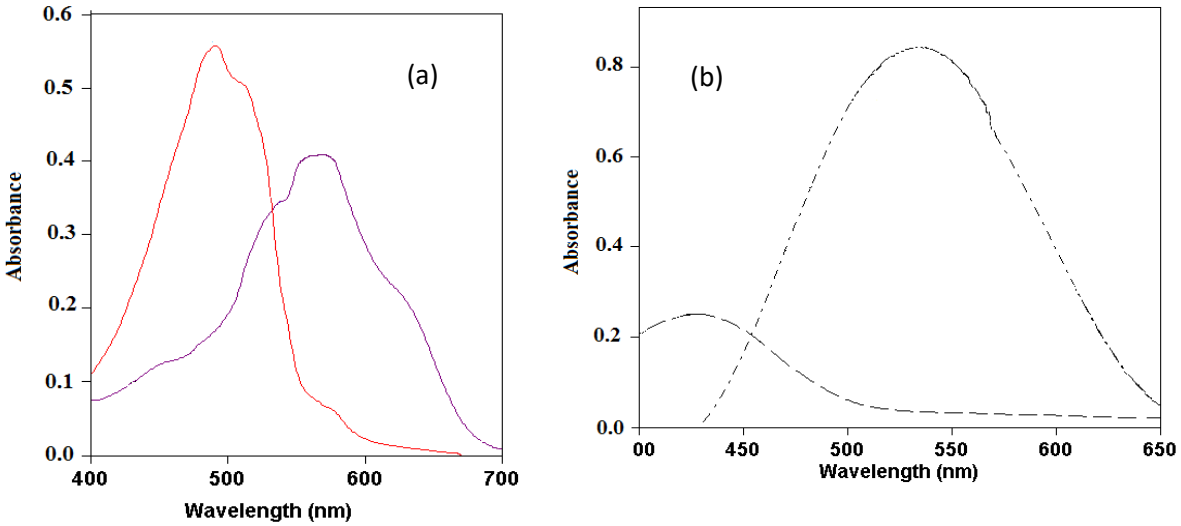


Figure 2. Absorption spectra of charge transfer complexes of 15 µg mL^{-1} CQP with (1.0 x 10^{-3} mol L^{-1}) (a) Quinz and (b) ARS in methanol solvent obtained against reagent blank solution prepared in the same solvent.

3.2. Optimization of the experimental conditions

3.2.1. Effect of the solvent

The charge transfer reaction was investigated in various solvents, namely DMSO, methanol, acetonitrile, acetone, and ethanol. While DMSO and acetonitrile exhibit the highest dielectric constants, it is noteworthy that methanol yielded the most optimal sensitivity. This outcome can likely be attributed to methanol's ability to establish stable hydrogen bonds with the radical anion. Subsequently, methanol was selected as the substance for subsequent experimental investigations (Figure 3).

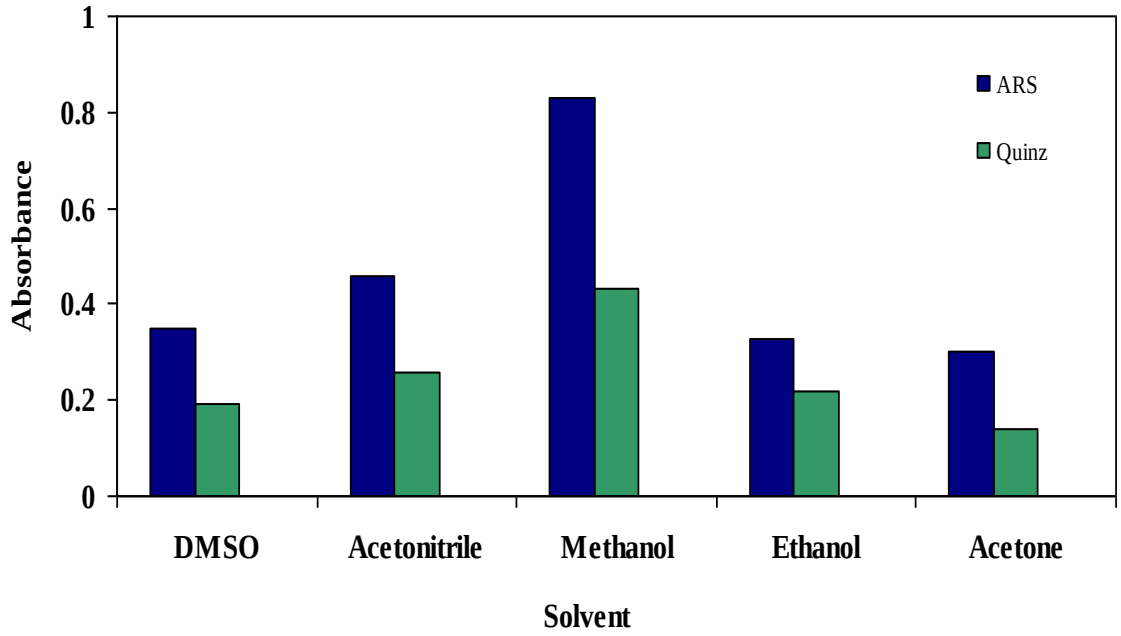


Figure 3. Effect of different solvents on the charge transfer complex of Quinz-CQP and ARS-CQP solution obtained against (1.0 × 10^{-3} mol L^{-1}) Quinz and ARS solutions, respectively also prepared in each solvent. CQP concentration = 15 µg mL^{-1}.

3.2.2. Effect of the reagent concentration

To accomplish the stated goal, an experimental procedure was conducted wherein different quantities of reagent solutions (with a concentration of 1.0×10^{-3} mol L^{-1}) ranging from 0.2 mL to 3.0 mL were introduced into a fixed concentration of CQP (15 µg mL^{-1}) (refer to Figure 4). The experimental findings indicate that a volume of 2.0 mL of a Quinz or ARS reagent solution with a concentration of 1.0×10^{-3} mol L^{-1} was sufficient to achieve the maximum color intensity and consistently produced the greatest absorbance values.

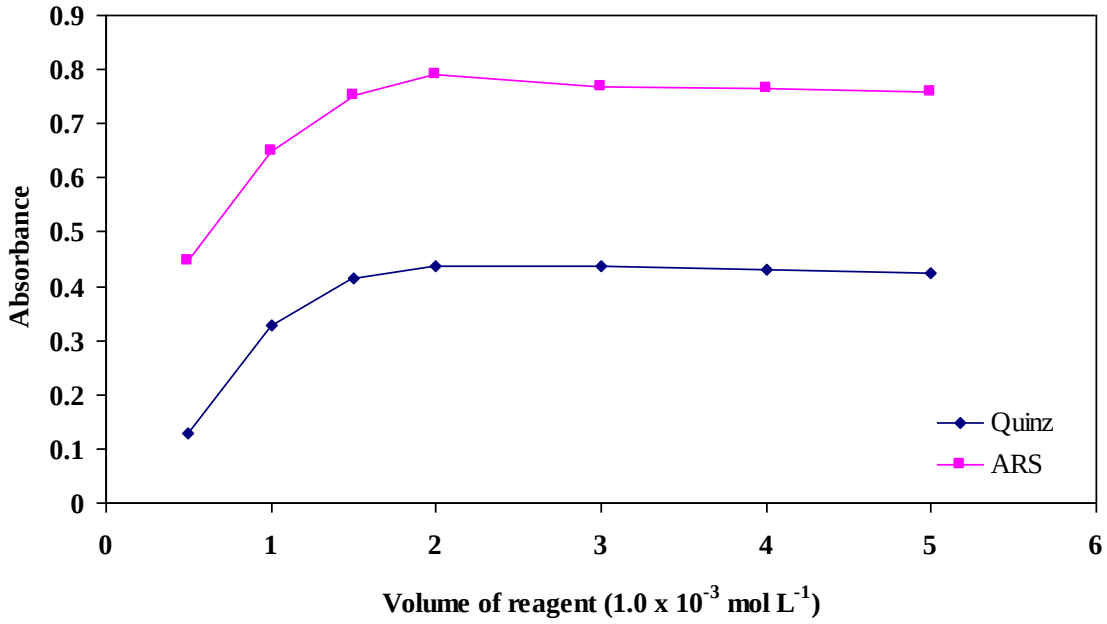


Figure 4. Effect of (1.0×10^{-3} mol L^{-1}) Quinz or ARS concentration on the absorbance of CQP-Quinz and CQP-ARS solutions. CQP concentration = 15 µg mL^{-1}.

3.2.3. Effect of the reaction time and temperature

The optimal reaction time was found by monitoring the progression of color development under laboratory ambient conditions at a temperature of 25±2°C. Full color development was achieved after a duration of 2.0 minutes for CQP when both reagents were used. When the temperature was increased, the absorbance of the charge transfer complex exhibited a reduction accompanied by a hypochromic shift, ultimately decaying at a temperature of 50 °C.

3.2.4. Sequence of additions

The optimal order of addition for achieving complete color development, maximal absorbance, and stability at the specified wavelength is "CQP-reagent-solvent." In addition to exhibiting lesser stability, certain sequences necessitated a longer duration. The complexes exhibiting this particular sequence demonstrate stability for a minimum duration of 8.0 hours.

3.2.5. Stoichiometric ratio

The determination of the molar ratio between the charge transfer complex's CQP and reagent (Quinz or ARS) was carried out using Job's approach [28], which involves continual modifications while maintaining a constant sum of the molar concentrations of the CQP and reagent under investigation. According to the data presented in Figure 5, it was determined that the molar ratio resulting in the highest absorbance was (1:1) (CQP: reagent).

The literature review indicates that molecular charge-transfer complexes tend to develop in non-polar solvents, whereas polar solvents seem to favor the prevalence of radical anion species [30-

35]. Moreover, it is widely accepted that the introduction of basic compounds possessing a lone pair of electrons, such as CQP, leads to the generation of charge-transfer complexes characterized as n-π type. These types of complexes can be regarded as an intermediate molecular-association compound that gives rise to the formation of a corresponding radical anion in polar liquids. In the present scenario, radical anions are formed as a consequence of complete charge transfer (as depicted in Scheme 1).

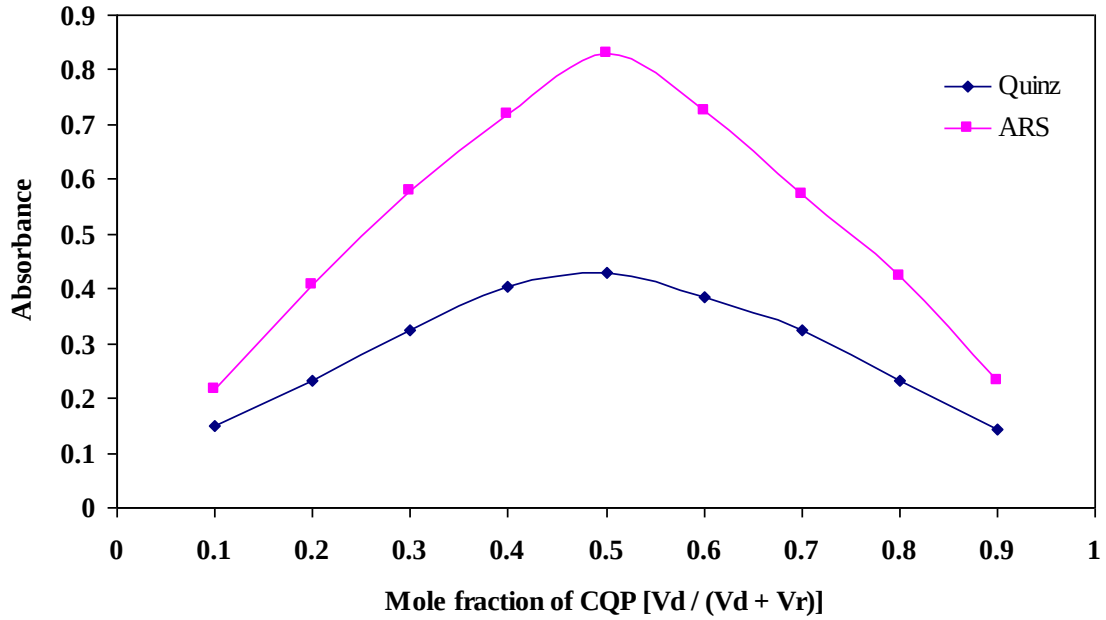
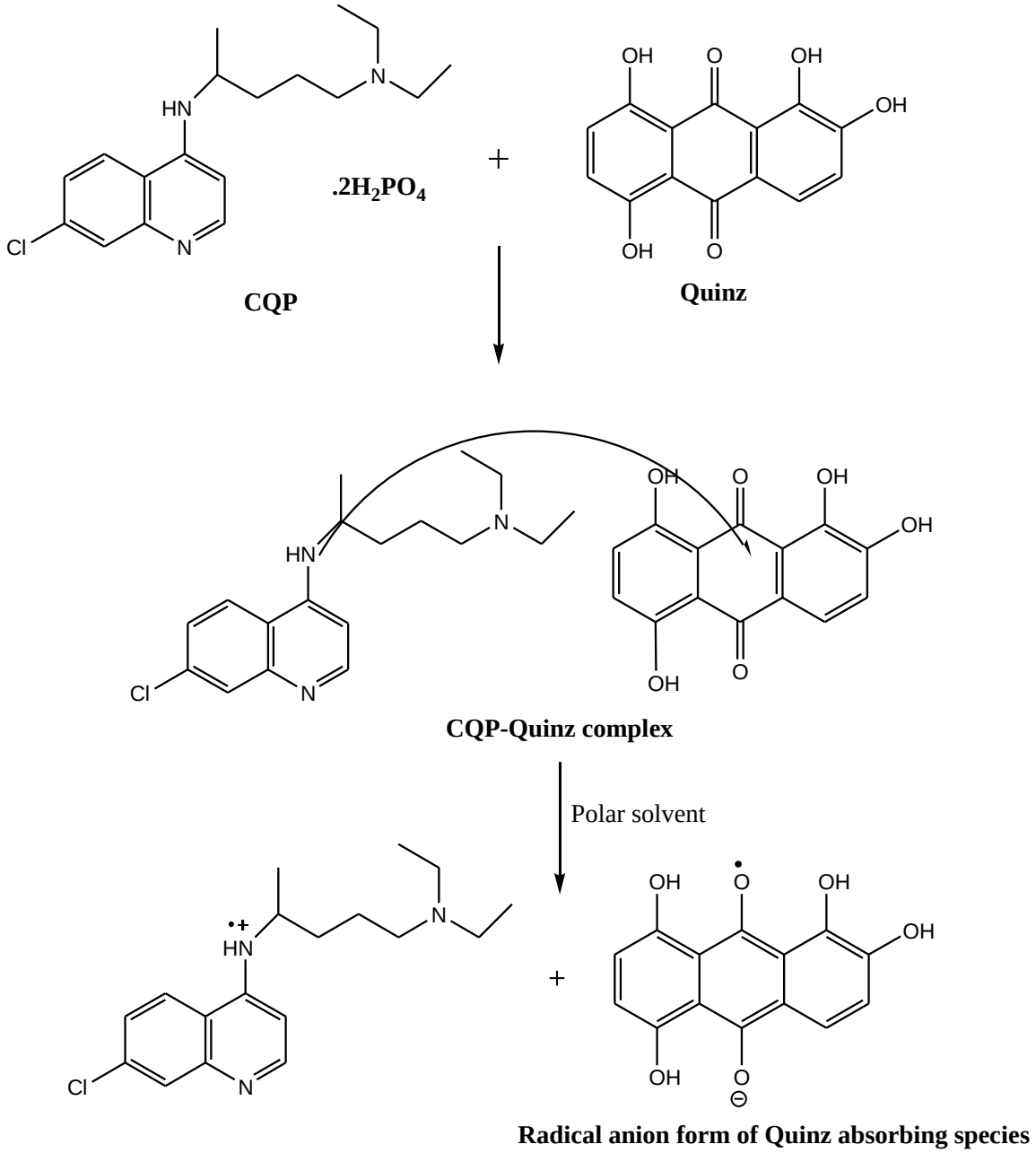


Figure 5. Application of Job's method to the reaction between Quinz or ARS and CQP.



Scheme 1. Possible mechanism of radical anion formation from CQP and Quinz reaction.

3.3. Validation of the proposed methods

The methods' validity was assessed in terms of linearity, specificity, accuracy, repeatability, and precision, in accordance with the principles set forth by the International Conference on Harmonization (ICH) [36].

3.3.1. Linearity, detection, and quantification limits

The linear regression equations were derived by the utilization of the aforementioned approaches. The regression plots demonstrated a clear linear relationship between the analytical response in the two procedures and the concentration of CQP within the specified ranges as indicated in Table 2. The data was subjected to linear regression analysis, resulting in the derivation of the following equations. In the context of Quinz, the relationship between the absorbance (A) and the

concentration of CQP (C) can be represented by the equations A = -0.0053 + 0.0218C (with a correlation coefficient r^2 of 0.9995) and A = 0.0114 + 0.0512C (with a correlation coefficient r^2 of 0.9998), as determined using the ARS method. Here, A represents the absorbance values, C represents the concentration of CQP in micrograms per milliliter, and r^2 denotes the correlation coefficient. The determination of the limit of quantification (LOQ) was achieved by defining the minimum concentration that can be measured in accordance with the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines [36]. The findings are presented in Table 2. The determination of the limits of detection (LOD) involved establishing the minimal threshold at which the analyte may be identified with a high degree of reliability. The findings pertaining to the LOD are also presented in a concise manner in Table 2. The equations used to calculate the Limit of Quantification (LOQ) and Limit of Detection (LOD) were as follows:

$$LOQ=10s/b$$

$$LOD=3.3s/b$$

Where s is the standard deviation of replicate determination values under the same conditions as for the sample analysis in the absence of the analyte. b: is the slope of the calibration curve.

Table 2: Analytical parameters for the determination of CQP by the proposed methods.

Parameters	Quinz	ARS
Beer's law limits, µg mL^{-1}	1.0-20	1.0-16
Ringboom limits, µg mL^{-1}	3.0-18	3.0-14
Molar absorptivity, x 10^4 L mol^{-1} cm^{-1}	1.2370	1.6742
Sandell sensitivity, ng cm^{-2}	41.71	30.81
Regression equation [a]		
Intercept (a)	-0.0053	0.0114
Standard deviation of intercept (S_a)	0.04	0.03
Slope (b)	0.0218	0.0512
Standard deviation of slope (S_b)	0.06	0.07
Correlation coefficient, (r)	0.9995	0.9998
Mean ± SD[b]	100.20±1.07	99.20±0.69
RSD%	1.07	0.70
RE%	1.12	0.73
Limit of detection, µg mL^{-1}	0.29	0.30
Limit of quantification, µg mL^{-1}	0.97	1.0
Calculated *t*-value [b]	0.67	1.27
Calculated *F*-value [b]	1.79	1.34

[a] *A=a + bC, where C is the concentration in (µg mL^{-1}), A is the absorbance, a is the intercept and b is the slope.*

[b] *Mean of six determinations.*

[c] *Theoretical values of t (2.57) and F (5.05) for five degrees of freedom and 95 % confidence level at p = 0.05.*

3.3.2. Accuracy and precision

To evaluate the validity and reliability of the proposed methodologies, the determination of CQP was conducted intraday and inter-day at three distinct concentrations for each approach. The intraday investigations were conducted inside a single day, while the inter-day studies were conducted over a period of five days, with each level (n = 6) being analyzed. The recorded values in Table 3 include the percent relative error (RE%) and relative standard deviation (RSD%) as measures of accuracy and precision, respectively. Additionally, the findings of both intraday and inter-day analyses were included. The data demonstrated high levels of accuracy and precision for the methodologies that were created.

Table 3: Intra-day and inter-day precision and accuracy for CQP obtained by the proposed methods.

Method	Taken (µg mL^{-1})	Recovery %	Precision RSD % [a]	Accuracy RE %	Confidence Limit [b]
			Intra-day		
Quinz	6.0	99.10	0.67	-0.90	5.946 ± 0.04
	12	101.0	1.10	1.0	12.12 ± 0.14
	18	99.50	1.68	-0.50	17.91 ± 0.32
ARS	5.0	99.80	0.47	-0.20	4.99 ± 0.02
	10	99.20	0.72	-0.80	9.92 ± 0.07
	15	100.70	1.25	0.70	15.105 ± 0.20
			Inter-day		
Quinz	6.0	99.40	0.60	-0.60	5.964 ± 0.04
	12	100.50	1.10	0.50	12.06 ±0.14
	18	100.90	1.50	0.90	18.162 ± 0.29
ARS	5.0	100.30	0.76	-0.30	5.015 ± 0.04
	10	98.70	0.98	-1.30	9.87 ± 0.10
	15	99.80	1.70	0.20	14.97 ± 0.27

[a] *Mean of six determination, RSD%, percentage relative standard deviation; R.E%, percentage relative error.*
[b] *Confidence limit at 95% confidence level and five degrees of freedom (t = 2.571). Mean ± standard error.*

3.3.3. Ruggedness and robustness

The robustness of the suggested methodology was evaluated by implementing the processes using two distinct instruments in two separate laboratories at different time intervals, and with the involvement of two different analysts. The reproducibility of results obtained from variations between laboratories and analysts was confirmed, as the relative standard deviation (RSD) did not surpass 2.0% .

The robustness of the proposed approach was evaluated by examining the impact of slight variations in experimental variables, specifically the concentrations of the reagent and the reaction time, on the analytical performance of the method. In the conducted tests, a single experimental parameter was manipulated while all other factors remained constant. Subsequently, the recovery % was determined for each instance. The minor fluctuations in any of the variables had no meaningful impact on the outcomes. The recorded values for recovery, expressed as the mean ± %RSD, may be found in Table 4. The aforementioned observation demonstrates the dependability of the proposed methodology when regularly employed for the examination of CQP.

Table 4: Results of method robustness and ruggedness (all values in RSD%) studies (n=3).

Methods	Nominal amount concentration (µg mL^{-1})	RSD%			
		Robustness		Ruggedness	
		Variable alerted [a]			
		Reagent volume	Reaction time	Different analysts	Different instruments
	6.0	0.53	0.60	0.63	0.35
Quinz	12	0.91	1.0	1.20	0.92
	18	1.37	2.10	1.90	1.42
	5.0	0.70	0.53	0.74	0.82
ARS	10	0.98	0.80	1.13	1.30
	15	1.60	1.40	1.85	2.20

[a] *Volume of reagent is (2.0±0.2 mL) and reaction time is (5.0±2.0 min) were used.*

3.3.4. Specificity and effect of excipients

The investigation focused on evaluating the extent to which the proposed approach was affected by potential interference arising from the excipients commonly included in tablets. The experimental procedure involved the utilization of the standard addition method, wherein predetermined quantities of pure CQP were added to a tablet solution that had been previously analyzed. The determination of the recovery of the additional compound quantification procedure (CQP) was achieved through a comparative analysis of the concentration levels of the artificially introduced mixtures with the previously established reference value. Table 5 demonstrates that the acquired findings were satisfactory and superior to the spectrophotometric methods published in the literature. The proposed approaches demonstrated good recovery values, suggesting that the presence of excipients did not cause interference. This observation further supports the notion that the proposed methods exhibit a high level of selectivity.

3.4. Analysis of the pharmaceutical preparations

The approach presented in this study was utilized for the quantification of CQP in pharmaceutical formulations. The method underwent testing to evaluate its linearity, specificity, accuracy, repeatability, and precision in accordance with the criteria provided by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). The statistical comparison was conducted between the findings obtained from the suggested approaches and those obtained from the reference method [13]. The recovery values, along with their corresponding standard deviations (SD), were collected. The statistical analysis of the data was conducted using Student's t-test and the variance ratio F-test at a 95% confidence level. The results indicated that there was no significant difference between the performance of the proposed and reference techniques in terms of accuracy and precision. This information can be found in Table 5 of the referenced study [37]. The results presented in this study demonstrate the applicability of the proposed methodologies for analyzing CQP in its various dose forms, with equivalent analytical performance.

Table 5: **Application of the standard addition technique for the determination of CQP in pharmaceutical preparations using the proposed methods.**

Sample	Taken ($\mu g\ mL^{-1}$)	Quinz		ARS		Reported method *(Nagib Qarah et al. 2017)*
		Added ($\mu g\ mL^{-1}$)	Recovery [a] (%)	Added ($\mu g\ mL^{-1}$)	Recovery [a] (%)	
Alexoquine tablets	4.0	-	99.20	-	99.70	
		4.0	99.60	4.0	99.20	
		8.0	100.80	8.0	98.60	
		12	99.70	12	100.70	
Mean ± SD [b]			99.83±0.68		99.55±0.89	99.80±0.81
RSD% [b]			0.68		0.89	0.81
V [b]			0.47		0.79	0.66
S.E [b]			0.34		0.44	0.33
t-value [c]			0.06		0.46	
F-value [c]			1.42		1.21	

[a] *The average of at least three determinations.*

[b] *V= variance; RSD%= percentage relative standard deviation; SE= standard error.*

[c] *Theoretical values of t and F are 2.571 and 5.05, respectively at confidence limit at 95% confidence level and five degrees of freedom (p= 0.05).*

IV. Conclusion

The two approaches that have been developed exhibit characteristics such as simplicity, rapidity, sensitivity, accuracy, robustness, and cost-effectiveness. Extraction, heating, or pH correction are not necessary for its implementation. The resulting chromophore has a high degree of stability. The aforementioned attributes render the proposed methodologies highly appropriate for the regular analysis of CQP in quality control laboratories.

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