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Development and Validation of Uv Spectrophotometric and Rp-Hplc Methods for Simultaneous Quantification of Doxorubicin Hydrochloride and Chloroquine

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ABSTRACT:
The combination of Doxorubicin hydrochloride and Chloroquine has been established as an efficacious and safe therapeutic regimen for cancer treatment. This study aimed to develop and validate simple, precise, accurate, and stability-indicating RP-HPLC and UV spectrophotometric methods for the simultaneous estimation of Doxorubicin hydrochloride and Chloroquine in synthetic mixture, adhering to ICH guideline Q2 (R2). Chromatographic separation was achieved under isocratic conditions using a C_{18} column. An optimized mobile phase consisting of 0.2% Acetic acid in Water: Acetonitrile: Methanol (50:25:25 %v /v /v) at 232 nm was employed to ensure well-resolved peaks for both analytes. The flow rate was maintained at 1.0 mL/min with detection at a predetermined optimal wavelength. For the UV spectrophotometric method, a simultaneous equation technique was applied using wavelengths corresponding to the respective of Doxorubicin hydrochloride and Chloroquine, with distilled water as the solvent. The linearity for Doxorubicin hydrochloride and Chloroquine was established across concentration ranges of 0.25-1.25 µg/mL and 1.5-7.5 µg/mL, respectively for both methods. The proposed methods were validated for specificity, linearity, range, precision, accuracy, LOD, LOQ, assay and system suitability. Results remained strictly within the limits defined by ICH guideline Q2 (R2). The data indicated that Doxorubicin hydrochloride and Chloroquine are amenable to highly precise and reproducible analysis, supporting their utility in anticancer pharmaceutical applications. The integrated analytical techniques proved highly sensitive, precise, and robust, making them suitable for reliable routine quantification.

1. INTRODUCTION:

Cancer represents a significant global health burden, characterized by aberrant cellular proliferation and metastatic dissemination to adjacent tissues. Notwithstanding the availability of diverse antineoplastic agents, therapeutic efficacy is frequently constrained by systemic toxicity and the emergence of multidrug resistance. To mitigate these challenges, combination therapy has emerged as a pivotal strategy in oncology, as it synergistically enhances therapeutic potency and forestalls resistance. Combination chemotherapy facilitates the concurrent administration of two or more agents with distinct mechanisms of action to target malignant cells[1]. This paradigm amplifies anticancer activity and optimizes clinical outcomes. Given the escalating clinical adoption of polypharmacy, the development of streamlined and robust analytical methodologies for the simultaneous quantification of drugs in co-formulated preparations has become imperative [2]. Doxorubicin hydrochloride is the chemical compound named (8S,10S)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl) oxy]-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy 7,8,9,10-tetrahydrotetracene-5,12-dione hydrochloride (Figure 1) is represents a cornerstone anthracycline antineoplastic

agent. It exerts its therapeutic efficacy by intercalating within DNA base pairs, inhibiting topoisomerase II, and generating reactive oxygen species, which collectively trigger programmed cell death. It is extensively utilized in the management of breast carcinoma, leukemia, lymphoma, and various solid malignancies; however, its clinical utility is frequently compromised by the development of chemoresistance [3].

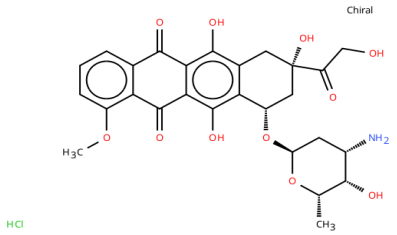

Figure 1: Structure of Doxorubicin hydrochloride

Chloroquine is the chemical compound 7-chloro-4-(4-diethylamino-1-methylbutylamino) quinoline (Figure 2). It is a lysosomotropic antimalarial agent that has garnered significant interest for its repurposed role in oncology. It functions by inhibiting macroautophagy, a highly conserved catabolic pathway that facilitates malignant cell survival under metabolic stress. By suppressing autophagic flux, chloroquine sensitizes neoplastic cells to chemotherapeutic regimens and potentates their inherent cytotoxic properties[4].

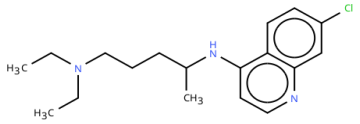

Figure 2: Structure of Chloroquine

Several Clinical studies have reported that the co-administration of Doxorubicin hydrochloride and Chloroquine exhibits synergistic antineoplastic activity. While doxorubicin induces genotoxicity within malignant cells, chloroquine compromises cellular repair and homeostatic survival pathways. This pharmacological synergy optimizes therapeutic response and serves to circumvent established mechanisms of chemoresistance [5]. The combination of Doxorubicin hydrochloride and Chloroquine has been investigated in clinical trial related studies and demonstrated synergistic anticancer activity in the chorioallantoic membrane (CAM) assay, which is attributed, at least in part, to the inhibitory effect of chloroquine on autophagy within the CAM model [6]. To develop a multifunctional drug delivery system, FP-MoS_2 nanosheets were co-loaded with DOX and CQ molecules. The resulting DOX/CQ@FP-MoS_2 nanocomposite demonstrated potential suitability for photochemotherapy applications [7]. Chloroquine and doxorubicin have also been successfully co-loaded into hypoxia-responsive liposomes, enabling synergistic therapeutic effects in the treatment of solid tumors [8]. The combination of two model drugs, doxorubicin (DOX) and chloroquine (CQ), was selected to enhance the therapeutic efficacy of small-molecule chemotherapeutic drugs (SMCDs) while simultaneously reducing their toxicity and associated side effects [9]. Combination therapy involving doxorubicin (DOX) and chloroquine (CQ) nanoparticles has been reported to exhibit potent anticancer activity against MCF-7/Adr, a multidrug-resistant breast cancer cell line [10]. The combination of two model drugs, doxorubicin (DOX) and chloroquine (CQ), was selected to enhance the therapeutic efficacy of small-molecule chemotherapeutic drugs (SMCDs) while simultaneously reducing their toxicity and associated side effects [11].

Various analytical techniques, including UV spectrophotometry [12-14], RP-HPLC [15-17], UPLC-DAD [18], Stability indicating RP-HPLC [19] and LC–MS [20], have been reported for the individual estimation of doxorubicin hydrochloride. Likewise, Chloroquine has been quantified using UV spectrophotometric and RP-HPLC techniques [21, 22], thin-layer chromatography [23], and LC–MS/MS [24]. However, an extensive literature survey indicates that, despite the availability of several analytical methods for the individual estimation of these drugs, no method has yet been reported for their simultaneous estimation in a synthetic mixture. Therefore, the present study is aimed at the development and validation of simple, accurate, precise, and reproducible UV spectrophotometric and RP-HPLC methods for the simultaneous estimation of Doxorubicin hydrochloride and Chloroquine in a synthetic mixture, in

accordance with ICH Q2(R2) guideline [23], with applicability to routine quality control analysis.

2. MATERIALS AND METHODS:

2.1 Chemicals and Components:

Doxorubicin hydrochloride and Chloroquine were generously provided as gift samples by Cadila Pharmaceuticals and Intas Pharmaceuticals, Ahmedabad. HPLC-grade methanol, acetonitrile, and water were procured from Finar Chemicals, Ahmedabad. Ortho-phosphoric acid (85%, AR Grade) and potassium dihydrogen phosphate were sourced from Astron Chemicals Ltd., India. Freshly prepared solutions were generated daily for all experimental procedures.

2.2 Scientific Conditions with Instrumentation:

The RP-HPLC analysis was performed using a Systronics LC-20-AD system integrated with Clarify® software, an SPD-20A UV detector, and a Rheodyne injector featuring a 20 µL sample loop. Chromatographic separation was executed using a reversed-phase strategy. Isocratic elution of both analytes was achieved utilizing a mobile phase consisting of 0.2% Acetic acid in Water: Acetonitrile: Methanol (50:25:25 %v /v /v) at a flow rate of 1.0 mL/min. Detection was maintained at an optimal wavelength of 232 nm. Mobile phases were prepared daily, filtered through 0.45 µm Millipore membrane filters, and degassed via an Equitron (India) sonicator prior to use. A Kromstar® C_{18} (250 x 4.6 mm, 5 µm) column was employed. The LC system was operated at ambient temperature. For the UV spectrophotometric method, a Shimadzu 1900 double-beam spectrophotometer with UV Probe 2.7 software and 1.0 cm quartz cells was utilized. Analytical weighing was conducted on a Scale-tec electronic precision balance.

2.3 Preparation of stock solution:

Accurately weighed portions of Doxorubicin Hydrochloride (0.25 mg) and Chloroquine (1.5 mg) were transferred into separate 100 ml volumetric flasks. The volume was adjusted to the mark using distilled water to obtain standard stock solutions with a final concentration of 2.5 µg/ml for Doxorubicin hydrochloride and 15 µg/ml for Chloroquine for the study.

2.4 Preparation of standard working solution:

The concentration levels of 0.25-1.25 µg/ml for Doxorubicin hydrochloride and 1.5-7.5 µg/ml for Chloroquine, aliquots from respective stocks (1, 2, 3, 4 and 5 ml and 1, 2, 3, 4 and 5 ml) were transferred into ten separate 10 ml volumetric flasks and diluted with distilled water to yield final values of 0.25, 0.5, 0.75, 1.0, 1.25 µg/ml and 1.5, 3.0, 4.5, 6.0, 7.5 µg/ml. Under refined spectrophotometric parameters, the preparations were examined via a 1 cm quartz cuvette. Similarly, employing the optimized chromatographic setup, 20 µL volumes of each working standard were introduced into the RP-HPLC system.

3. METHODOLOGY:

3.1 Development of UV Spectroscopy Method:

A zero-order UV spectrophotometric methodology was implemented for the simultaneous quantification of Doxorubicin hydrochloride and Chloroquine within a synthetic mixture. Discrete working standard solutions for each analyte were scanned across the 200–400 nm range to generate individual absorption profiles and identify respective absorption maxima (λmax) for subsequent quantitative evaluation. Primary stock solutions of Doxorubicin hydrochloride and Chloroquine were prepared in distilled water. Precise aliquots 2 ml of the mixture solution of Doxorubicin hydrochloride (2.5 µg/ml) and Chloroquine (15 µg/ml) was pipetted out into 10 ml volumetric flask and the volume was adjusted up to the mark with distilled water. Final concentration of Doxorubicin hydrochloride was 0.5 µg/ml and Chloroquine 3 µg/ml. These solutions were scanned within the UV region (200–400 nm) utilizing distilled water as a reagent blank.

The zero-order absorption spectra were recorded, and overlay spectra were examined to identify optimal analytical wavelengths for simultaneous estimation. Quantitative assessments were executed at wavelengths corresponding to the λmax of each compound, ensuring negligible spectral interference. The overlay UV spectra for Doxorubicin hydrochloride and Chloroquine are illustrated in Figure 3.

3.1.1 Procedure for the Selection of Wavelength:
A working standard solution of Doxorubicin hydrochloride (0.5 µg/mL) was prepared by transferring 2 mL of a 2.5 µg/mL stock solution into a 10 mL volumetric flask and diluting to volume with distilled water. Similarly, a working standard solution of Chloroquine (3 µg/mL) was obtained by pipetting 2 mL of a 15 µg/mL stock solution into a separate 10 mL volumetric flask and making up to the mark with distilled water.

The prepared solutions were scanned in the UV region (200–400 nm) against a distilled water blank using medium scan speed. The absorption maxima (λmax) were observed at 233 nm for Doxorubicin hydrochloride and 257 nm for Chloroquine which showed in figure 4 and 5, respectively. Quantitative analysis was carried out using the simultaneous equation method, and the concentrations of both drugs in the sample solution were calculated using the corresponding absorptivity coefficients at the selected wavelengths. This procedure applies the Simultaneous Equation technique based on Vierodt's principle, where the precise concentration of each drug within the sample is calculated according to the following mathematical expressions:
For Doxorubicin hydrochloride (x),

$$Cx = \frac{A2\ ay1 - A1\ ay2}{a\,x2\ aY1 - a\,x1ay2}$$

For Chloroquine (y),

$$Cy = \frac{A1ax2 - A2\ a\,x1}{ax2\ aY1 - a\,x1ay2}$$

Where Cx and Cy are the concentration of Doxorubicin hydrochloride and Chloroquine, respectively, A1 and A2 are absorbance at 233 nm and 257 nm respectively, ax1 and ax2 are absorptivity of Doxorubicin hydrochloride at 233 nm and 257 nm respectively; ay1 and ay2 are absorptivity of Chloroquine at 257 nm and 233 nm respectively. By solving the two simultaneous equations, the concentrations of Doxorubicin hydrochloride and Chloroquine in sample solutions were obtained.

3.2 Development of RP-HPLC Method:
Chromatographic analysis of Doxorubicin hydrochloride and Chloroquine was conducted utilizing a High-Performance Liquid Chromatography (HPLC) system in reversed-phase mode, a technique preferred for resolving moderately polar to non-polar and ionic analytes. Separation was facilitated by a Kromstar C_{18} column (250 mm × 4.6 mm i.d., 5 µm particle size) as the stationary phase. The mobile phase comprised 0.2% (v/v) aqueous acetic acid in water: acetonitrile: methanol (50:25:25 % v/v/v). This system was operated at a flow rate of 1 mL/min under isocratic conditions. Prior to analysis, the mobile phase was filtered through a 0.45 µm membrane filter and degassed via sonication for 30 minutes to eliminate dissolved gases and particulate contaminants. Detection was performed using a UV detector at 232 nm, ensuring optimal sensitivity and resolution for both Doxorubicin hydrochloride and Chloroquine. The methodology yielded robust separation of the two analytes, characterized by excellent peak symmetry and chromatographic resolution.

3.2.1 Selection detection wavelength:
The sensitivity of RP-HPLC method that uses UV detection depends upon proper selection of detection wavelength. At 232 nm both drugs give good peak height and shape. So, 232 nm was selected for simultaneous estimation of Doxorubicin hydrochloride and Chloroquine in synthetic mixture. Overlain UV spectra of Doxorubicin hydrochloride (0.5 µg/ml) and Chloroquine (3 µg/ml) in distilled water showed in figure 3.

3.3 Method Validation:
In accordance with ICH guideline Q2(R2) [23], the developed analytical methods were validated for the required performance characteristics [24–26]. The methods were systematically evaluated for system suitability, specificity, linearity, and range. Accuracy, precision, and sensitivity were also assessed, including the determination of the limit of detection (LOD) and limit of quantification (LOQ).

3.3.1 Specificity:
Specificity denotes the capacity to unequivocally assess the target analyte despite the potential presence of

concomitant components. Typically, these encompass impurities, degradation products, and matrix constituents. Analytical specificity was substantiated by contrasting the chromatograms of the mobile phase and test preparations; this comparison demonstrated a total absence of interference from the mobile phase or excipient peaks at the retention times of Doxorubicin hydrochloride and Chloroquine.

3.3.2 Linearity and Range:

The linearity for Doxorubicin hydrochloride and Chloroquine was established across concentration ranges of 0.25-1.25 µg/mL and 1.5-7.5 µg/mL, respectively (n=6). Regarding the UV spectrophotometric methodology, calibration curves were generated by plotting absorbance as a function of concentration (µg/mL). In the HPLC protocol, calibration curves were constructed by plotting peak area against the respective concentrations of both analytes. Linear regression equations were subsequently derived, and the linearity for both drugs was appraised via slope, y-intercept, and correlation coefficient (R^2), confirming a robust linear correlation within the investigated ranges.

3.3.3 Precision:

Intraday and interday precision, corresponding to repeatability and intermediate precision, respectively, were evaluated to assess the robustness of Methods I and II. Assessments were conducted within a single day and across three consecutive days, analyzing freshly prepared solutions at concentrations of 0.25, 0.5, and 0.75 µg/mL for Doxorubicin hydrochloride and 1.5, 3, and 4.5 µg/mL for Chloroquine (n=3). To determine intermediate precision (n=3), mean absorbance (UV) and peak area (HPLC) were recorded for each experimental set. For repeatability (n=6) studies, specific concentrations of 0.5 µg/mL and 3 µg/mL were utilized. Results were expressed as percentage Relative Standard Deviation (%RSD), where values below 2 % were deemed acceptable. This rigorous validation ensures a comprehensive appraisal of analytical precision, confirming the reliability and reproducibility of the quantified concentrations for both Doxorubicin hydrochloride and Chloroquine within the investigated matrices.

3.3.4 Limit of Detection (LOD):

Limit of detection can be calculated using following equation as per ICH Q2 (R2) guideline.

$$LOD = 3.3 * \frac{\sigma}{S}$$

where, σ = Standard deviation of the Y intercept of calibration curve
S = Mean slope of the corresponding calibration curve.

3.3.5 Limit of Quantification (LOQ):

Limit of quantification can be calculated using following equation using the standard deviation of the Y-intercept (σ) and the mean slope (S) of the calibration curve according to ICH Q2 (R2) guideline.

$$LOQ = 10 * \frac{\sigma}{S}$$

3.3.6 Accuracy (Recovery study):

The accuracy of an analytical procedure denotes the degree of concordance between an accepted conventional true value or reference standard and the experimentally determined value. The accuracy of the developed methodology was substantiated by performing recovery studies in accordance with ICH guidelines at three distinct concentration levels 50%, 100%, and 150% relative to target concentrations of Doxorubicin hydrochloride (0.5 µg/ml) and Chloroquine (3 µg/ml). This assessment was executed in triplicate (n=3) to ensure statistical reliability

3.3.7 Assay as an analysis of a Synthetic Mixture:

A synthetic mixture of doxorubicin hydrochloride and chloroquine was prepared in a 0.25:1.5 ratio. Common excipients, namely lactose (22 mg), talc (26 mg), magnesium stearate (16 mg), microcrystalline cellulose (MCC, 24 mg), and croscarmellose sodium (10.5 mg), were blended with doxorubicin hydrochloride (0.25 mg) and chloroquine (1.5 mg) using a mortar and pestle to achieve uniform mixing. An accurately weighed portion of the resultant powder blend, equivalent to 0.25 mg of doxorubicin hydrochloride and 1.5 mg of chloroquine, was transferred into a 100 mL volumetric flask. The flask was filled to approximately half its volume with deionized water and sonicated to ensure complete dissolution of the analytes, after which the volume was made up to the mark with the same solvent. The solution was then filtered through Whatman filter paper, yielding a stock solution containing 2.5 µg/mL of doxorubicin hydrochloride and 15 µg/mL of chloroquine. For preparation of the sample

solution, 2 mL of the above mixture solution containing 2.5 µg/mL doxorubicin hydrochloride and 15 µg/mL chloroquine was transferred into a 10 mL volumetric flask, and the volume was adjusted to the mark with distilled water. The final concentrations obtained were 0.5 µg/mL of doxorubicin hydrochloride and 3 µg/mL of chloroquine (n = 3). This sample solution was used for assay by UV spectrophotometric and RP-HPLC methods.

3.3.8 Robustness:

The robustness of the developed analytical methodologies was appraised to ascertain their capacity to remain unaffected by subtle, deliberate fluctuations in experimental parameters. For the RP-HPLC method, sample solutions of Doxorubicin hydrochloride and Chloroquine were analyzed under systematically altered conditions, including modifications to the flow rate (±0.1 mL/min), detection wavelength (±2 nm), and mobile phase composition (±2%). The influence of these perturbations on critical system suitability parameters specifically retention time, peak area, and chromatographic resolution was rigorously monitored.

3.3.9 System Suitability Tests:

System suitability testing represents an integral facet of liquid chromatography, conducted to verify that the chromatographic assembly functions optimally and is capable of generating reliable, reproducible data. The appraised system suitability parameters encompassed chromatographic resolution, column efficiency (theoretical plate count), and the asymmetry (tailing) factor. The outcomes of these evaluations are detailed in Table 1, confirming that the optimized RP-HPLC methodology is proficient for the simultaneous quantification of Doxorubicin hydrochloride and Chloroquine.

4. RESULTS AND DISCUSSION:

4.1 Method I: UV Method:

The simultaneous equation method is a simple and reliable UV spectrophotometric technique for the quantitative analysis of two components present in a mixture without prior separation. The method involves measuring absorbance at two selected wavelengths, generally the respective λmax values of each analyte, where Beer-Lambert's law is obeyed. Two linear equations are constructed using absorptivity coefficients, and the concentrations of both components are obtained by solving these equations simultaneously. Compared to other UV methods, this approach does not require an isoabsorptive point or derivative spectral processing, thereby reducing computational complexity and instrumental noise. Its simplicity, reproducibility, and cost-effectiveness make it particularly suitable for routine quality control analysis.

4.1.1 Selection of wavelength for Doxorubicin hydrochloride and Chloroquine:

The sensitivity of the RP-HPLC method, coupled with UV detection, is fundamentally dependent upon the strategic selection of an optimal detection wavelength. The prominent absorbance profile of Doxorubicin hydrochloride exhibited an absorption maximum at 233 nm (Figure 4), while Chloroquine demonstrated an absorption maximum at 257 nm (Figure 5). The zero-order UV absorption spectra for Doxorubicin hydrochloride (0.5 µg/mL) and Chloroquine (3 µg/mL) in distilled water are illustrated in Figures 3.

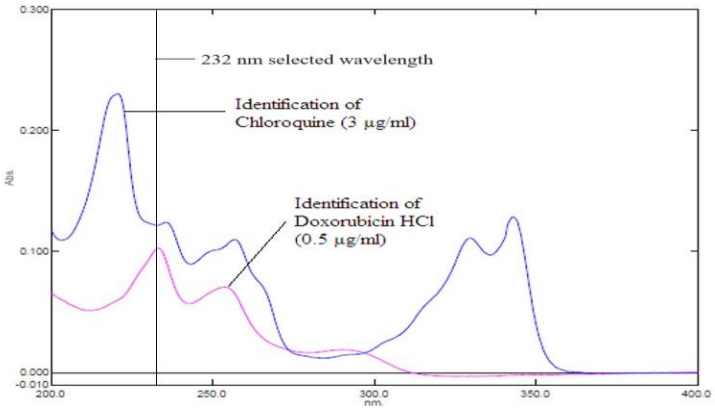

Figure 3: Overlain UV spectra of Doxorubicin hydrochloride (0.5 µg/ml) and Chloroquine (3 µg/ml) in distilled water

4.1.2 Vierordt's Method Development:

The zero-order UV spectra of Doxorubicin hydrochloride and Chloroquine exhibit spectral overlap within the 200–400 nm range, complicating direct quantification via conventional UV spectrophotometry without addressing mutual interference. Under the zero-order methodology, the total absorbance of a binary mixture at a specific wavelength is treated as the sum of the individual absorbances of each constituent. Consequently, the concentrations of Doxorubicin hydrochloride and Chloroquine within the formulation can be resolved directly from their zero-order profiles by implementing the simultaneous equation (Vierordt's) method, utilizing respective absorptivities at optimized analytical wavelengths. Linearity of UV spectra for Doxorubicin hydrochloride and Chloroquine are illustrated in Figures 4 and 5, respectively.

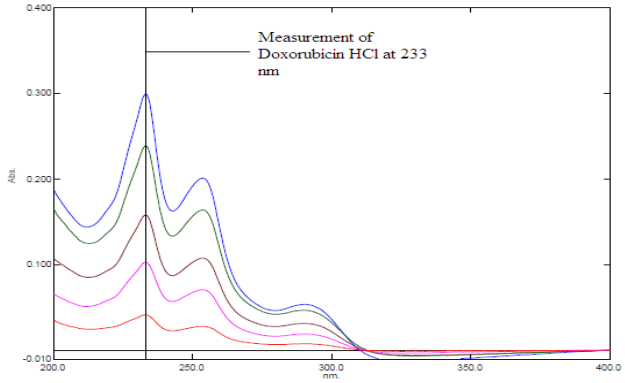

Figure 4: Overlain UV Spectra of Doxorubicin hydrochloride (0.25-1.25 µg/ml) at 233 nm

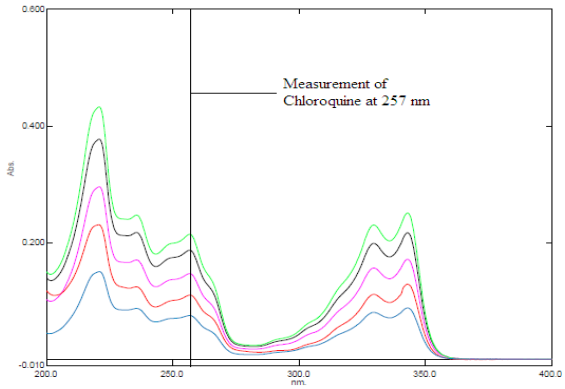

Figure 5: Overlain UV Spectra of Chloroquine (1.5-7.5 µg/ml) at 257 nm

4.2 Method II: RP-HPLC Method:

In pharmaceutical analysis, simultaneous estimation via RP-HPLC is extensively utilized for the determination of multi-component analytes within a single matrix. Numerous validated methodologies have been developed for the concurrent quantification of active pharmaceutical ingredients and their related substances in dosage forms. The RP-HPLC technique employs an optimized configuration of stationary phase, mobile phase, and detection systems to achieve robust resolution and accurate quantification of target analytes. Reversed-phase high-performance liquid chromatography (RP-HPLC) was selected for the simultaneous estimation of Doxorubicin hydrochloride and Chloroquine due to its suitability for ionic and moderately polar to non-polar molecules. RP-HPLC is preferred for its procedural simplicity, reproducibility, high theoretical efficiency, and stability. A C_{18} column was utilized owing to its increased hydrophobicity relative to C_4 and C_8 variants, which facilitates the accelerated elution of polar compounds compared to non-polar constituents. Furthermore, a UV detector was integrated, enabling streamlined and sensitive detection of the analytes in UV-transparent mobile phases. Consequently, a Kromstar C_{18} column (250 × 4.6 mm, 5 µm particle size) was employed for the efficacious separation and simultaneous estimation of Doxorubicin hydrochloride and Chloroquine.

4.2.1 RP-HPLC Method Development:

Liquid chromatography coupled with UV detection was implemented to establish a methodology for the simultaneous quantification of Doxorubicin hydrochloride and Chloroquine. The protocol was optimized to achieve superior peak symmetry and high theoretical plate counts within an efficient analytical timeframe. Chromatographic parameters were refined through the systematic evaluation of various stationary and mobile phase configurations. The selected mobile phase, comprising 0.2% acetic acid in water: acetonitrile: methanol (50:25:25 %v /v /v) at 232 nm (Figure 3), provided optimal resolution of the analytes, yielding retention times of approximately 2.1 min for Doxorubicin hydrochloride and 4.5 min for Chloroquine. Separation was executed on a Kromstar C_{18} column (250 × 4.6 mm, 5 µm) at a constant flow rate of 1.0 mL/min.

4.3 *Validation of the Proposed Methods*:

4.3.1 Specificity:

Specificity denotes the capacity to unequivocally assess the target analyte despite the potential presence of concomitant components. Typically, these encompass impurities, degradation products, and matrix constituents. Analytical specificity was substantiated by contrasting the chromatograms of the mobile phase and test preparations; this comparison demonstrated a total absence of interference from the mobile phase or excipient peaks at the retention times of Doxorubicin hydrochloride and Chloroquine (table 1).

Table 1: System suitability parameter

Parameters	Retention Time	Tailing Factor	Number of Theoretical plates	Resolution
Doxorubicin Hydrochloride	2.1 min	0.8	7514	3.0
Chloroquine	4.5 min	0.9	8987	

4.3.2 Linearity and range

4.3.2.1 Vierordt's Method

Doxorubicin hydrochloride was demonstrated a proportional increase in absorbance with increasing concentration (linear) over the range of 0.25-1.25 µg/mL at both 233 nm and 257 nm. Correlation Coefficient for Doxorubicin hydrochloride 0.9976 at 233 nm and 0.9963 at 257 nm were obtained (figure 6 and 7). Chloroquine in the concentration range of 1.5-7.5 µg/mL at 257 nm and 233 nm with Correlation Coefficient 0.9986 and nm and 0.9985, respectively (figure 8 and 9). The linearity data of Doxorubicin hydrochloride for UV method is shown in Table 2 and 3.

Table 2: Linearity data of Doxorubicin hydrochloride for Vierordt's UV method

Doxorubicin hydrochloride						
Conc. (µg/ml)	Mean Absorbance ± SD (n=6)		Absorptivity		% RSD	
	233 nm	257 nm	233 nm	257 nm	233 nm	257 nm
0.25	0.042 ± 0.0018	0.025 ± 0.0014	0.168	0.100	1.80	1.20
0.50	0.102 ± 0.0015	0.063 ± 0.0017	0.204	0.126	1.40	1.50
0.75	0.158 ± 0.0013	0.093 ± 0.0015	0.210	0.124	0.82	1.6
1.0	0.239 ± 0.0012	0.139 ± 0.0012	0.239	0.139	0.50	0.86
1.25	0.301 ± 0.0010	0.178 ± 0.0010	0.240	0.142	0.33	0.56

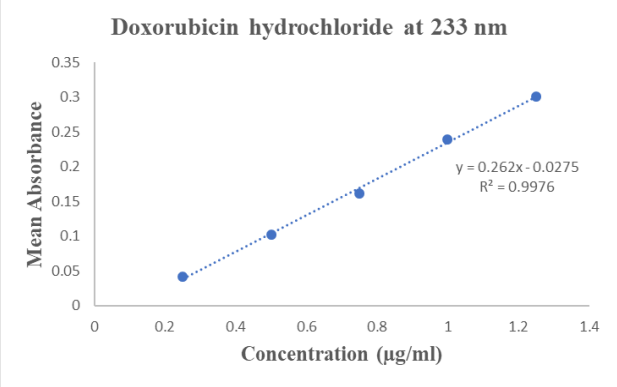

Figure 6: Calibration curve of Doxorubicin hydrochloride at 233 nm

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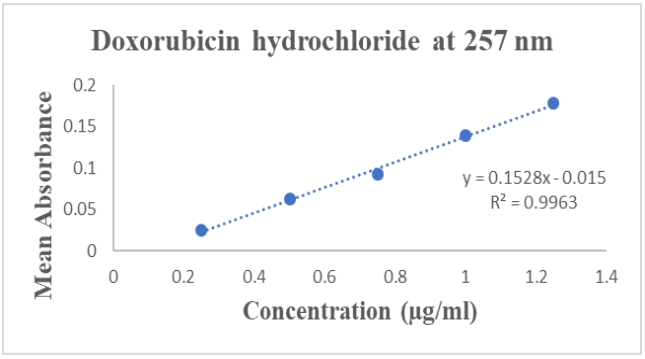

Figure 7: Calibration curve of Doxorubicin hydrochloride at 257 nm

Table 3: Linearity data of Chloroquine for Vierordt's UV method

Chloroquine						
Conc. (µg/ml)	**Mean Absorbance ± SD (n=6)**		**Absorptivity**		**% RSD**	
	257 nm	**233 nm**	**257 nm**	**233 nm**	**257 nm**	**233 nm**
1.5	0.073 ± 0.0011	0.086 ± 0.0013	0.048	0.057	1.50	1.60
3.0	0.109 ± 0.0014	0.122 ± 0.004	0.036	0.040	1.20	1.70
4.5	0.146 ± 0.0016	0.166 ± 0.0016	0.032	0.036	1.09	0.96
6.0	0.184 ± 0.0018	0.210 ± 0.0024	0.030	0.035	0.97	1.10
7.5	0.214 ± 0.0019	0.246 ± 0.0019	0.028	0.032	0.88	0.77

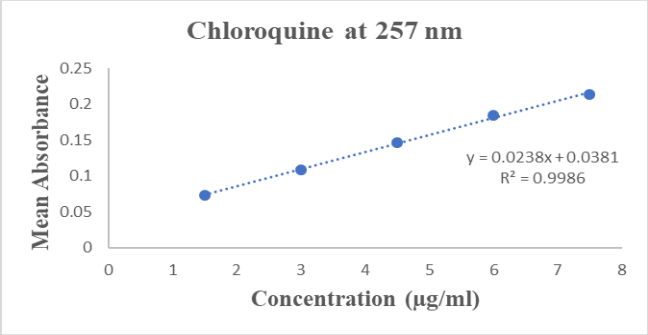

Figure 8: Calibration curve of Chloroquine at 257 nm

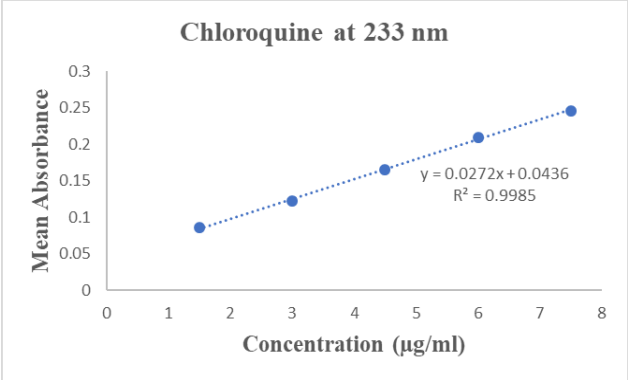

Figure 9: Calibration curve of Chloroquine at 233 nm

4.3.2.1.1 Calculation for Simultaneous Equation (Vierordt's) Method for Doxorubicin hydrochloride and Chloroquine in Synthetic Mixture.

Doxorubicin hydrochloride (0.50 µg/ml) and Chloroquine (3 µg/ml) in distilled water, both the solutions were scanned over range of 200-400 nm against distilled water as blank, using medium scan speed. The sampling wavelength for analysis includes 233 nm for Doxorubicin hydrochloride and 257 nm for Chloroquine. The method

employs Simultaneous Equation as per Vierodt's method and the concentrations of drugs in sample solution were determined by using the following formula:
For Doxorubicin hydrochloride,

$$Cx = \frac{A2\ ay1 - A1\ ay2}{a\ x2\ aY1 - a\ x1ay2}$$

Where ax_1 and ax_2 represented the absorptivity of Doxorubicin hydrochloride at 233 nm and 257 nm, respectively; ay_1 and ay_2 denoted the absorptivity of Chloroquine at 257 nm and 233 nm, respectively; and A_1 and A_2 corresponded to the absorbance of the sample measured at 233 nm and 257 nm, respectively.

C (Doxorubicin hydrochloride) = (0.063) (0.0348) – (0.102) (0.04)/ (0.1262) (0.0348) – (0.2122) (0.04)
=0.0021924 -0.00408/0.00439176-0.008488
= -0.0018876/-0.00409624
=0.46 µg/mL

The concentration of Doxorubicin hydrochloride (Cx), calculated using Vierordt's simultaneous equation method, was found to be 0.46 µg/mL.
For Chloroquine,

$$Cy = \frac{A1ax2 - A2\ a\ x1}{ax2\ aY1 - a\ x1ay2}$$

where a_{x1} and a_{x2} are the absorptivity values of Doxorubicin hydrochloride at 257 nm and 233 nm, respectively; a_{y1} and a_{y2} represent the absorptivity of Chloroquine at 257 nm and 233 nm, respectively; and A_1 and A_2 are the absorbance values of the sample measured at 257 nm and 233 nm, respectively.

C(Chloroquine) = (0.109) (0.1262) - (0.122) (0.2122)/ (0.1262) (0.0348) – (0.2122) (0.04)
= 0.0137558 - 0.0258884/ 0.00439176 - 0.008488
= - 0.0121326/ - 0.00409624
= 2.96 µg/mL

The concentration of Chloroquine (Cy), calculated using Vierordt's simultaneous equation method, was found to be 2.96 µg/mL.

4.3.2.2 RP-HPLC Method:

The RP-HPLC chromatogram of Doxorubicin hydrochloride (0.25-1.25 µg/ml) and Chloroquine (1.5-7.5 µg/mL) at 232 nm. The Peak Area was found. Calibration curves were plotted between concentrations and peak areas. The regression equation of calibration curve was generated and the correlation coefficient (R^2) values were observed to be 0.9990 and 0.9992 (table 4).

Table 4: Linearity and precision data for HPLC method

Sr. No.	Parameters	Doxorubicin hydrochloride	Chloroquine
1	Detection wavelength (nm)	232 nm	
2	Linearity Range (µg/ml)	0.25-1.5	1.5-7.5
3	Regression equation (y = mx +c)	y = 698.52x + 108.36	y = 91.84x + 69.25
4	Correlation Coefficient (r^2)	0.9990	0.9992
5	Intraday Precision (%RSD, n=3)	0.75-1.31	0.52-1.26
6	Interday Precision (% RSD, n=3)	0.95-1.37	0.59-1.28
7	Repeatability (% RSD, n=6)	0.89	0.73

4.3.3 Precision:

The precision of the developed analytical methodologies was appraised through intraday, interday, and repeatability assessments. Analyses were executed at three distinct concentrations 0.25, 0.50, and 0.75 µg/mL for doxorubicin hydrochloride and 1.5, 3, and 4.5 µg/mL for chloroquine with each concentration evaluated in triplicate within a single diurnal cycle (intraday) and across three consecutive days (interday). The absorbance values (UV method) and chromatographic peak areas (HPLC method) for these analytes were recorded. For repeatability verification, a solitary concentration of 0.5 µg/mL for doxorubicin hydrochloride and 3 µg/mL for chloroquine was analyzed. The resultant percentage relative standard deviation (%RSD) values for the intraday, interday, and repeatability studies

for RP-HPLC are summarized in Tables 4, confirming that the developed protocols are highly precise and reproducible.

4.3.4 Accuracy:

The accuracy of the developed methodology was appraised utilizing the standard addition (recovery) technique. Predetermined quantities of Doxorubicin hydrochloride and Chloroquine were spiked into pre-analyzed samples at three distinct levels: 50%, 100%, and 150% of the nominal concentrations. These recovery investigations were executed in triplicate, with accuracy expressed as a percentage recovery. UV method findings demonstrated superior accuracy, with recovery values ranging from 99.33% to 99.68% for doxorubicin hydrochloride and 99.75% to 100.06% for chloroquine the accuracy of the RP-HPLC methodology was confirmed via standard addition at 50%, 100%, and 150% levels in triplicate, yielding excellent recoveries of 99.73-100.08% for doxorubicin hydrochloride and 99.88-100.38% for chloroquine (Table 5), proving the protocol's reliability for quantitative analysis.

Table 5: Recovery study data for Vierordt's Method and RP-HPLC Method

Vierordt's Method						
Name of Drug	**% Level of recovery**	**Test Amount (µg/ml)**	**Amount of drug taken (µg/ml)**	**Total Std Amt (µg/ml)**	**Total amount recovered (µg/ml)**	**% Mean Recovery ± SD(n=3)**
Doxorubicin hydrochloride	50	0.5	0.25	0.75	0.745	99.33±0.9325
	100	0.5	0.5	1.0	0.999	99.90±0.010
	150	0.5	0.75	1.25	1.246	99.68±0.0059
Chloroquine	50	3	1.5	4.5	4.489	99.75±0.8652
	100	3	3	6	5.997	99.95±1.2148
	150	3	4.5	7.5	7.505	100.06±1.3433
HPLC METHOD						
Name of Drug	**%Level of Recovery**	**Test Amount (µg/ml)**	**Amount of drug taken (µg/ml)**	**Spiked Std Amt. (µg/ml)**	**Total amt. Recovered (µg/ml)**	**% Recovery ±S. D (n=3)**
Doxorubicin hydrochloride	50	0.5	0.25	0.75	0.748	99.73±0.0027
	100	0.5	0.5	1.0	0.998	99.80±1.3143
	150	0.5	0.75	1.25	1.251	100.08±1.1361
Chloroquine	50	3	1.5	4.5	4.495	99.88±0.0055
	100	3	3	6	6.023	100.38±0.067
	150	3	4.5	7.5	7.493	99.90±0.015

4.3.5 Assay as Analysis of Synthetic Mixture

From the assay procedure, final concentrations of 1 µg/mL doxorubicin hydrochloride and 20 µg/mL chloroquine were subjected to UV spectroscopic analysis. The assay percentages for Doxorubicin hydrochloride and Chloroquine were determined to be 99.60 % and 99.93%, respectively. Furthermore, the RP-HPLC assay results for these analytes were found to be between 99.80% to 99.96%. The comprehensive outcomes of these determinations are documented within Table 6.

Table 6: Analysis of synthetic mixture for UV and HPLC method

Vierordt's UV Method			
Drug Name	**Amount in synthetic mixture (µg/ml)**	**Amount found (µg/ml)**	**% Assay± S.D. (n=3)**
Doxorubicin hydrochloride	0.5	0.498	99.60±0.91
Chloroquine	3.0	2.998	99.93±1.01
HPLC METHOD			
Doxorubicin hydrochloride	0.5	0.499	99.80±0.94
Chloroquine	2.999	2.999	99.96±1.12

4.3.6 LOD and LOQ:

The limits of detection (LOD) and quantification (LOQ) are calculated using the standard deviation responses and slopes obtained from the calibration curves of each drug at their specific wavelengths. The results showed in table 7.

Table 7: LOD and LOQ data for UV and HPLC method

Sr. No.	**Parameters**	**Doxorubicin hydrochloride**	**Chloroquine**

UV Method					
1	Wavelength (nm)	233 nm	257 nm	257 nm	233 nm
2	LOD (µg/ml)	0.023	0.031	0.16	0.16
3	LOQ (µg/ml)	0.069	0.092	0.47	0.48
HPLC Method					
1	Detection wavelength (nm)	232 nm			
2	LOD (µg/ml)	0.016		0.093	
3	LOQ (µg/ml)	0.051		0.281	

4.3.7 Robustness:

The robustness of the developed RP-HPLC methodology was appraised by investigating the impact of subtle deliberate perturbations, encompassing inter-analyst variability, minor fluctuations in flow rate, chromatographic run time, and detection wavelength. The %RSD values consistently remained within established acceptance criteria, signifying that the protocol is robust and reproducible. Its results were observed in the analytical outcomes, substantiated the method's reliability across diverse experimental conditions.

5. CONCLUSION:

A robust, rapid, and stability-indicating analytical platform was successfully established for the simultaneous quantification of doxorubicin hydrochloride and chloroquine using RP-HPLC and complementary UV spectrophotometric techniques. Systematic optimization of chromatographic conditions resulted in the selection of 0.2% acetic acid in Water: Acetonitrile: Methanol (50:25:25, %v/v/v) as the optimized mobile phase at a flow rate of 1.0 mL/min with detection at 232 nm, providing excellent chromatographic performance in terms of adequate retention, resolution, peak symmetry, and recovery. The short retention times (~2.1 min for doxorubicin hydrochloride and ~4.5 min for chloroquine) enable rapid, high-throughput analysis suitable for routine quality control. The UV spectrophotometric method was performed using distilled water as the solvent of choice, selected for its simplicity, cost-effectiveness, and absence of spectral interference, thereby ensuring reliable and reproducible absorbance measurements. Specificity of both analytical approaches was confirmed by the absence of interference from reagent blanks and formulation excipients at the respective analytical wavelengths and retention times. Comprehensive validation demonstrated that the developed RP-HPLC and UV methods are sensitive, accurate, precise, and reproducible across the studied concentration ranges. All validation parameters complied with the acceptance criteria outlined in International Council for Harmonisation guideline Q2 (R2). In conclusion, the validated analytical procedures offer streamlined, reliable, and regulatory-compliant tools for the concurrent estimation of Doxorubicin hydrochloride and Chloroquine. Their simplicity, robustness, and high analytical performance support their suitability for routine quality surveillance, and batch release testing of combined pharmaceutical formulations.

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CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

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