

Development and Validation of Simultaneous Estimation of Ferulic Acid and Doxycycline Concentrations Based on Two Different UV Spectrophotometric Methods

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Abstract: According to the literature search, a method for simultaneously determining ferulic acid and doxycycline in combination by ultraviolet-visible spectroscopy was not included in any of the high-ranking pharmacopeias, monographs, or journals. Therefore, the development of a reliable system became inevitable, leading to the development and validation of two novel spectrophotometric techniques: simultaneous equation and absorption ratio spectroscopy methods. These methods were specifically developed to simultaneously determine ferulic acid and doxycycline monohydrate contents. These methods are characterized by simplicity, sensitivity, precision, and accuracy. The first method solved a simultaneous equation based on the absorbance measurements at two wavelengths, 216 nm and 274 nm, λ_{\max} for ferulic acid and doxycycline monohydrate, respectively. The second method was the absorption ratio method, which involves the formation of a Q-absorption equation at 276 nm (isoabsorptive point) and at 274 nm (λ_{\max} doxycycline monohydrate). Following the guidelines of ICH, the developed technique was validated against several specifications, including specificity, linearity and range, precision, accuracy, limit of detection, and limit of quantification. Both techniques produced linear responses at concentrations ranging from 1–10 $\mu\text{g/ml}$ and 2–20 $\mu\text{g/ml}$, respectively. The method was validated by the acceptable limits specified in the ICH guidelines. The technique proved to be simple, rapid, highly accurate, and cost-effective and can, therefore, be used for the simultaneous determination of drugs, including ferulic acid and doxycycline, in combination formulations for standard quality testing.

Keywords: ferulic acid; doxycycline monohydrate; simultaneous equation; absorbance ratio; spectrophotometric method.

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1. Introduction

The molecular formula for ferulic acid (FA) is $\text{C}_{10}\text{H}_{10}\text{O}_4$, and its IUPAC name is 3-methoxy-4-hydroxycinnamic acid [1,2]. Cairong *et al.* have provided the solubility data of FA in several pure solvents, including "water, ethanol, methanol, chloroform, dichloromethane (DCM), methyl acetate (MA), ethyl acetate (EA), and butyl acetate (BA)," both at atmospheric pressure and at temperatures ranging from 273.15 K to 333.15 K [3]. Polyphenols (FAs) are present in various plants and foods, including grains, nuts, fruits, and vegetables. FA has natural antioxidant, anti-inflammatory, and antibacterial properties [4]. It has been scientifically

documented that FA inhibits the growth of *Salmonella*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, and other bacteria killed [5-8]. In addition, FA has neuroprotective, radioprotective, antidiabetic, and anticancer effects [9].

The molecular formula for doxycycline monohydrate is $C_{22}H_{24}N_2O_8 \cdot H_2O$ and the IUPAC name is 4-[(2R)-2-amino-1,3-dimethyl-4-oxobutyl]-2,6-dihydroxy-3,5-dimethylpyridine-mono-hydrate; this compound is a yellowish, hygroscopic powder that dissolves readily in methanol but very slightly in water [10,11]. The sample had a molecular weight of 512.94 g/mol. 3.6–4.2 is the pH range. DOX is a derivative of oxytetracycline and is a potent semisynthetic drug [12]. It is widely used in the pharmaceutical production of drugs [13]. It is mostly available in the form of tablets and capsules but is also available as an oral suspension [14]. It shows a broad spectrum of activity against both gram-positive and gram-negative bacteria, including *Streptococcus pyogenes*, *Bartonella*, *Ehrlichia*, *Enterococci*, *Chlamydia elis*, *Hemoplasma*, *Actinomyces*, *Toxoplasma*, *Nocardia*, *Anaplama*, *Protozoa*, *Mycoplasma*, *Plasmodium species*, and some anaerobic species [15,16]. DOX blocks bacterial cell wall proteins by binding to the 30 ribosomal subunits and inhibiting mRNA translation, resulting in cell death. It also inhibits matrix metalloproteinase-9 (MMP-9), which is involved in various inflammatory and immunological responses. The drug is prescribed to treat bacterial infections such as skin infections, respiratory tract infections, sexually transmitted diseases, and urinary tract infections, among others; common side effects include nausea, vomiting, and diarrhea, but rare but severe liver damage should be monitored [17-21]. Limitations in the simultaneous determination of FA and DOX concentrations include absorption interference, spectral overlap, solubility problems, matrix interference, and method sensitivity. These problems can affect the accuracy and precision of the estimation procedure [22]—an illustration depicting (Figure 1) the molecular structures of ferulic acid and Doxycycline monohydrate.

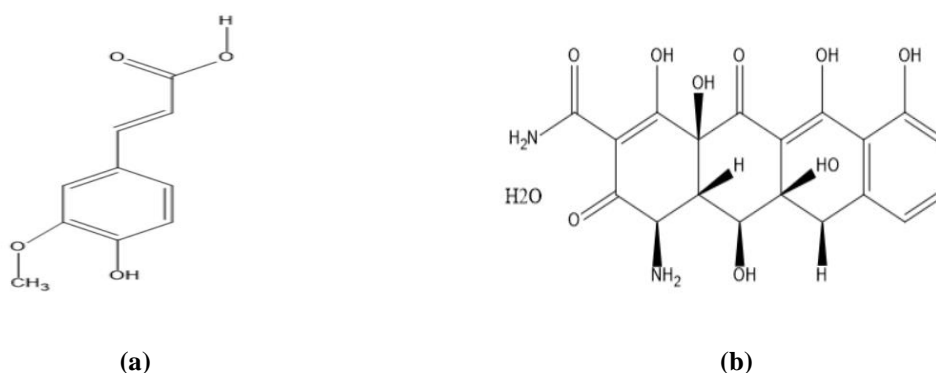


Figure 1. (a) Structure of ferulic acid; (b) Doxycycline monohydrate.

The novelty of developing and validating a UV spectrophotometer method for FA and DOX is that this technique determines their concentration in a sample. This requires optimizing conditions such as the choice of wavelength, linearity range, solvent system, and pH. Method validation ensures reliability, accuracy, and precision by evaluating parameters such as linearity, accuracy, precision, limit of detection, and limit of quantification. The specific application of UV spectrophotometry for these compounds addresses solubility, stability, and interference [23]. The study's novelty is determined by its particular objectives, approaches, and results. Among the simultaneous estimation methods for other drugs mentioned above, alternative techniques for the accurate and efficient determination of multiple drugs

simultaneously are important. This method contributes to the advancement of analytical chemistry by expanding the range of drugs that can be analyzed simultaneously and offering potential advantages in terms of time, cost, and resource utilization. Albayrak *et al.* developed a low-cost, rapid, and simple spectrophotometric method for determining etodolac and thiocolchicoside simultaneously. These methods can be reliably applied in quality control laboratories and provide alternatives to existing methods [24]. Sarkis and Sawan developed a UV spectrophotometric method for determining nicotinamide and tretinoin simultaneously. This method is suitable for routine quality control testing and allows direct determination of both compounds individually or simultaneously in a binary mixture [25]. Rathinam and Santhana developed a UV spectrophotometric chemometric method for simultaneously determining paracetamol, aceclofenac, and eperisone hydrochloride in pharmaceutical formulations. These methods suit routine quality control laboratories and provide an environmentally friendly, rapid, cost-effective analysis [26]. Attimarad *et al.* developed a UV spectrophotometric method for simultaneously determining metformin and remogliflozin in a formulation. The method was simple, accurate, sensitive, economical, and environmentally friendly, making it suitable for routine quality control [27]. Jadhav *et al.* developed a spectrophotometric method for simultaneously determining valsartan and hydrochlorothiazide in a single tablet formulation. It can be successfully used in the quality control of pharmaceutical formulations and routine laboratory analysis [28]. Ali and Elsaman developed a UV spectrophotometric method for simultaneously determining paracetamol and pseudoephedrine in bulk and combination pharmaceutical forms. The method was successfully applied to the analysis of commercial tablets and gave satisfactory results [29]. Pandey and Mishra developed a Q-absorption ratio method for the simultaneous determination of lamivudine and isoniazid; this method is characterized by simplicity and precision and has the potential for future applications [30]. Sen *et al.* developed three UV spectrophotometric methods for simultaneously determining teneligliptin hydrobromide and metformin hydrochloride, characterized by simplicity and cost-effectiveness for quality control [31]. Gholve *et al.* developed a UV spectrophotometric method for the simultaneous detection of doxycycline and levofloxacin suitable for routine quality analysis [32].

UV spectroscopy may be preferred over HPLC for its cost-effectiveness, simplicity, and speed, making it suitable for routine analysis when complex separation is not required. It provides accurate quantitative results for compounds with distinct UV absorption features. Additionally, UV spectroscopy is advantageous when reference standards for HPLC are limited or when purity determination is not the primary objective. In contrast, HPLC is a more versatile and robust technique for complex separations and the accurate quantitation of multiple compounds in a sample. However, the high cost of HPLC instruments and reagents, coupled with the need for intensive training and technical skill, limits its availability in many clinical laboratories and hinders its use for rapid, real-time quantification. Therefore, alternative approaches are needed for the analysis of FA and DOX. UV-Vis spectrophotometry offers a simpler and more practical solution, effectively detecting FA and DOX in pharmaceutical samples.

A thorough literature search revealed that no simultaneous method for assessing FA and DOX concentrations using UV/visible spectroscopy is available in any of the most commonly used pharmacopeias or publications. This study aimed to develop such a technique and, after validating it based on the International Conference on Harmonization (ICH) recommendations and acceptable laboratory practices, apply it to evaluate drug content. In the

present work, the simultaneous equation and absorbance ratio methods are two spectrophotometric techniques used for the joint estimation of FA and DOX.

2. Materials and Methods

Analytically pure samples of ferulic acid were obtained from SRL Chemical Mumbai, India. Doxycycline was obtained as a gift from Lupin Pharmaceutical Pvt. Ltd. Nagpur, India. Water was obtained from a Synergy water purification system for ultrapure water (Merck KGaA, Germany).

2.1. Instruments.

The experiment used a Lambda 25 dual-beam UV/VIS spectrometer (Perkin Elmer, Singapore). The cuvettes used were 1 cm wide quartz cuvettes with UV VinLab (Scan-Lambda 25) software, and the weighing device used an analytical balance (Wenar, model, 100291, India), a digital pH meter (Deluxe pH meter model, 101, India), volumetric dark flasks and glass pipettes of various sizes.

2.2. Solvent systems.

After extensive studies of the solubility of the two drugs FA and DOX in each solvent, phosphate buffer (pH 7.4) was selected as the final solvent system for method development.

2.3. pH 7.4 phosphate buffer preparation.

In preparing PBS, 8 g of sodium chloride, 2.38 g of sodium hydrogen phosphate, and 0.19 g of potassium dihydrogen phosphate were dissolved in an appropriate volume of 1000 ml of distilled water [33].

2.4. The FA standard stock solution.

To prepare a working standard solution with a 100 µg/ml concentration, FA (10 mg) was added to a 100 ml volumetric flask, dissolved, and diluted with phosphate buffer adjusted to a pH of 7.4. To obtain a working dilution in the concentration range of 1–10 µg/ml, an appropriate amount of this solution was pipetted and diluted with phosphate buffer (pH 7.4).

2.5. DOX standard stock solution.

A weighed amount of DOX (10 mg) was placed in a 100 ml volumetric flask, dissolved, and diluted with phosphate buffer (pH 7.4) to prepare a working standard solution with a 100 µg/ml concentration. A suitable volume of this solution was pipetted off and diluted with phosphate buffer (pH 7.4) to obtain a working dilution with a concentration ranging from 2-20 µg/ml.

2.6. Determination of isoabsorptive points and absorption maxima.

The absorbance maxima were determined, and overlapping spectra were obtained to study the standard solution of 10 µg/ml in range mode, covering the wavelength range of 200-400 nm.

2.7. Development of calibration curves.

Working dilutions of FA and DOX were prepared with phosphate buffer (pH 7.4) at 1–10 µg/ml and 2–20 µg/ml, respectively. A calibration curve was prepared after the absorbance of FA and DOX was measured at 216 nm, 274 nm, and 276 nm.

2.8. Absorptivity determination.

The absorbance was estimated at 216, 274, and 276 nm wavelengths. The average of three different absorbance measurements was used to determine the absorption maxima of FA and DOX, as well as the absorbance (A 1%, 1 cm) of FA and DOX at maxima of 216, 274, and 276 nm, respectively [34].

$$\text{Absorbance/concentration(g/100)} = A(1\%, 1 \text{ cm}) \quad (1)$$

The mean absorptivity values were determined, resulting in an a_x value. Additionally, a_y values were obtained at distinct λ_{max} values, specifically at 216, 274 nm, and at the isobestic point 276 nm.

2.9. Methods using simultaneous equations and absorbance ratios.

Appropriate dilution of a standard stock solution containing 1000 µg/ml FA or DOX in phosphate buffer (pH 7.4) resulted in drug solutions with concentrations of 7 µg/ml and 10 µg/ml, respectively. The spectra of the two solutions were recorded after scanning in the UV range of 200–400 nm. The first method used a solution of a simultaneous equation (SE) based on the absorbance at two wavelengths, 216 nm and 274 nm, λ_{max} for FA and DOX, respectively. The second method was the absorption ratio method (AR), which involves the formation of a Q absorption equation at 276 nm (isoabsorptive point) and 274 nm (λ_{max} DOX). To prepare both drugs for analysis, their respective stock solutions were diluted to different concentration ranges: 1–10 µg/ml for the first drug and 2–20 µg/ml for the second drug. Subsequently, all the prepared solutions were analyzed using a UV spectrophotometer. The absorbance measurements for the SE method were based on measuring the absorbance at two wavelengths, 216 nm and 274 nm, λ_{max} for FA and DOX, respectively. The AR signals were detected at 276 nm (isoabsorption point) and 274 nm (λ_{max} DOX) [35, 36].

2.10. A sample solution analysis method of simultaneous equations.

This technique absorbs two separate absorbing drugs at their respective λ_{max} . The two drugs can be estimated using a simultaneous equation. The absorption maxima of the two selected wavelengths, λ_1 -216 nm and λ_2 -274 nm, of FA and DOX were measured. The concentration at pH 7.4 in phosphate buffer was estimated using the following equation:

Considering the concentrations of FA and DOX and C_x and C_y , the two equations below were considered to be λ_1 (216 nm) and λ_2 (274 nm), respectively.

$$\text{At } \lambda_1 (216 \text{ nm}), A_1 = a_{x1} b C_x + a_{y1} b C_y \quad (2)$$

$$\text{At } \lambda_2 (274 \text{ nm}), A_2 = a_{x2} b C_x + a_{y2} b C_y \quad (3)$$

Where:

C_x and C_y = FA and DOX concentrations, respectively.

A_1 and A_2 = FA and DOX absorbance at 216 and 274 nm, respectively.

A_{x1} and a_{x2} = 216 nm and 274 nm FA absorptivity values, respectively.

A_{y1} and a_{y2} = 216 nm and 274 nm, respectively, for DOX absorptivity.

b=1, for a cell measurement of 1 cm

Rearranging Eq. 3

$$C_y = A_2 - a_{x_2} C_x / a_{y_2} \quad (4)$$

In Eq. 2, this value of C_y is replaced, and after rearranging, we obtain

$$C_x = A_1 a_{y_2} - A_2 a_{y_1} / a_{y_2} a_{x_1} - a_{x_2} a_{y_1} \quad (5)$$

$$C_y = A_1 a_{x_2} - A_2 a_{x_1} / a_{x_2} a_{y_1} - a_{x_1} a_{y_2} \quad (6)$$

2.11. Absorbance ratio method.

The ratio of the absorbance at any wavelength is a constant value in the absorbance ratio technique, which is equivalent to the Beer-Lambert equation at all wavelengths, regardless of the concentration or path length. The two drugs had similar absorbance at 276 nm at some concentrations, so there was no difference. The terms "isosbestic" or "isoabsorptive point" refer to wavelengths with equal absorptivity between two species [37].

The AR technique was used to estimate the amount of two drugs present in the sample solution at 276 nm and 274 nm by applying the following formula:

$$C_x = \frac{QM - QY}{QX - QY} \times \frac{A1}{a_{x1}} \quad C_y = \frac{QM - QX}{QY - QX} \times \frac{A2}{a_{y1}} \quad (7)$$

Where A_1 and A_2 are the absorbances of the mixture at 276 nm and 274 nm, respectively.

a_{x1} = FA absorptivity of 1%, 1 cm, at 276 nm

a_{y1} = A (276 nm, 1%, 1 cm) of FA

a_{x2} = A (274 nm, 1%, 1 cm) of FA

a_{y2} = A (274 nm, 1%, 1 cm) of Dox

FA and DOX were present at unknown concentrations (C_x and C_y).

2.12. Validation protocol.

The linearity, range, repeatability, precision, specificity, accuracy, and LOD and LOQ studies were performed to validate the proposed technique according to the ICH guidelines Q2 (R1) [38,39].

2.12.1. Linearity and range.

Different concentrations (1-10 $\mu\text{g/ml}$ of FA and 2-20 $\mu\text{g/ml}$ of DOX) of the standard solution were studied to determine the linearity and range of the reaction.

2.12.2. Repeatability.

When a series of experiments were performed in rapid succession and under identical conditions, they were considered compatible if they yielded consistent measurements of the same quantity. The proposed methodology involved developing and studying a standard solution consisting of FA and DOX at a concentration of 8+6 $\mu\text{g/ml}$. This solution was studied in 10 replicates of the experiment.

2.12.3. Precision.

The suggested approach's repeatability, intraday, and interday precision were assessed by utilizing a solution containing a concentration of FA (8 $\mu\text{g/ml}$) and DOX (6 $\mu\text{g/ml}$). The

absorbance was measured multiple times during the day to assess intraday precision. The sample was examined on different days to assess the interday variability. According to the research, the percentage relative standard deviation (% RSD) should ideally be less than 2% [40].

2.12.4. Specificity.

The spectra of the standard solution and the drug sample solution prepared for the experiment were measured to verify the specificity of the proposed analytical procedure.

2.12.5. Accuracy.

Additional amounts (80%, 100%, and 120%) of pure FA and DOX drugs were added to the prequantified FA and DOX sample solutions (2, 4, and 6 µg/ml, respectively) to evaluate accuracy via the standard addition procedure. The concentrations of the two drugs were determined by measuring the absorbance at 216 nm and 274 nm. Using the proposed method, the % RSD values were estimated. Calculations were performed to determine the % recovery of the sample and the % RSD [41,42].

2.12.6. Limit of detection (LOD)

The least detectable dose (LOD) is the lowest sample concentration that can be determined but is not always a precise measurement.

The single-to-noise ratio can be calculated according to the ICH guideline Q2 (R1) using the equation:

$$\text{LOD} = 3.3 \times \sigma/s \quad (8)$$

Where:

σ = Standard deviation of regression lines' y-intercepts

S = The slope of the calibration curve

2.12.7. Limit of quantification (LOQ).

The LOQ is the lowest concentration of a sample at which a quantitative measurement may be made with sufficient accuracy and precision.

$$\text{LOQ} = 10 \times \sigma/s \quad (9)$$

Where

σ = Standard deviation of regression lines' y-intercepts

S= The calibration curve's slope

3. Results and Discussion

The overlapping spectra of the two drugs present significant challenges for their estimation. However, the simultaneous estimation (SE) and absorbance ratio (AR) techniques were successfully applied to estimate both drugs in their mixture (Figure 2c). These UV spectroscopic methods were developed and validated following the ICH guideline [44], demonstrating simplicity, sensitivity, ease of use, precision, and accuracy. The data indicated acceptable accuracy, precision, and specificity over the specified linearity ranges. The overlapping spectra of FA and DOX detected two peaks at 216 nm and 274 nm, representing

their maximum absorption points. In addition, an isoabsorption band was observed at 276 nm (Figures 2, a, b, and c).

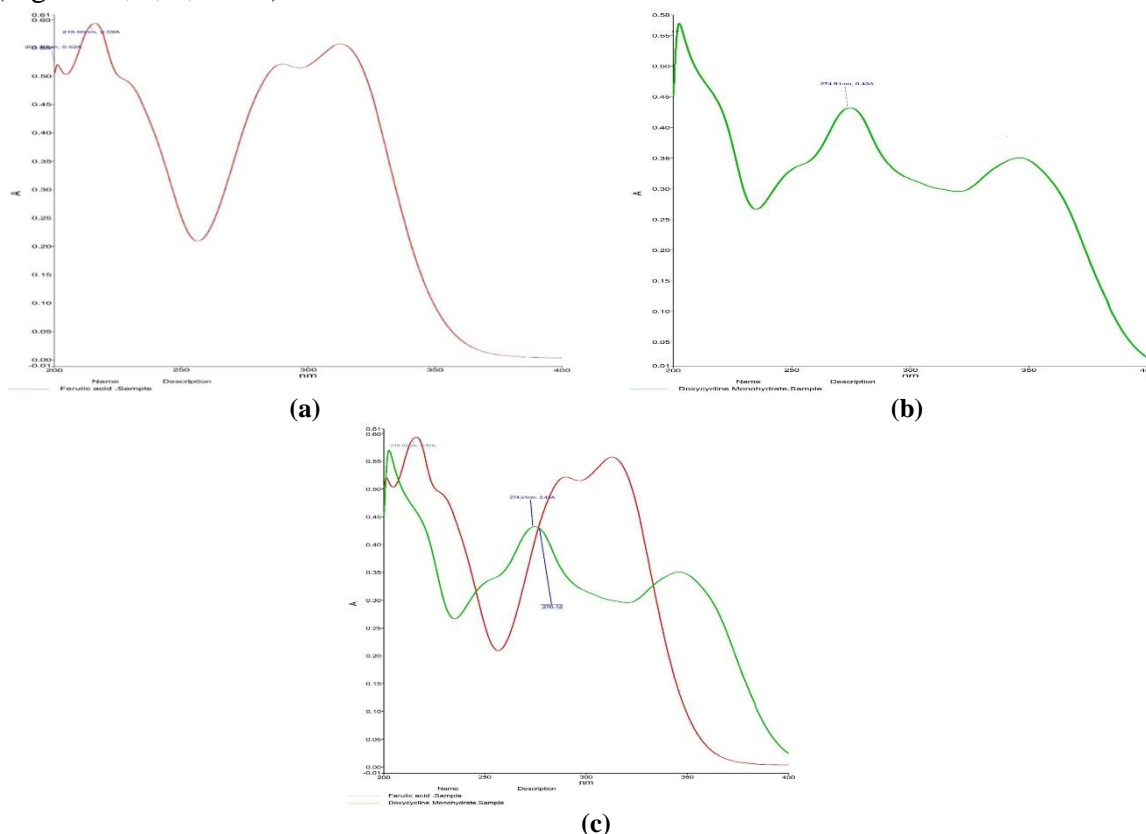


Figure 2. (a) Spectra of ferulic acid (λ_{max} 216 nm); (b) doxycycline monohydrate (λ_{max} 274 nm); (c) overall spectra of ferulic acid (λ_{max} 216 nm), and doxycycline monohydrate (λ_{max} 274 nm), displaying the isoabsorption point at 276 nm.

In the SE method, absorption at 216 and 274 nm was chosen for both drugs. In the AR method, 276 and 274 nm were chosen for detecting and quantifying FA and DOX, respectively.

A linear correlation was observed between the concentrations of FA and DOX within the respective ranges of 1–10 $\mu\text{g/ml}$ for FA and 2–20 $\mu\text{g/ml}$ for DOX. Calibration plots were prepared for the SE and AR methods, where the absorbance values of the drug standard solutions were plotted against their corresponding concentrations. The results of the correlation study indicate a strong linear relationship between the variables, as evidenced by the high correlation coefficients (R^2) of 0.9926, 0.9987, 0.9918, 0.9952, 0.9949, and 0.9977. These correlations were observed at different λ_{max} values, particularly at 216 nm, 274 nm, FA, and DOX, as well as at 216 nm, 274 nm, DOX, and FA and 276 nm, FA, and DOX. The linear regression equations for FA and DOX were $y = 0.0822x + 0.08$ and $y = 0.0383x + 0.046$, respectively. Similarly, the linear regression equations for FA and DOX were $y = 0.0211x + 0.088$ and $y = 0.0804x + 0.08$, respectively. These results are summarized in (Table 1), and shown in (Figures 3 a, b, c, d), and (Figures 4 a, b, c, d, e, and f).

Table 1. Absorptivity of FA and DOX as measured by the calibration curve.

| Drug | Concentration (ppm) | Absorbance | | | Absorptivity (abs/conc) | | |
|------|---------------------|-----------------|-----------------|-----------------|-------------------------|------------------|-----------------|
| | | $\lambda_1=216$ | $\lambda_2=274$ | $\lambda_2=276$ | $\lambda_1= 216$ | $\lambda_2= 274$ | $\lambda_2=276$ |
| | | nm | nm | nm | nm | nm | nm |
| FA | 1 | 0.12 | 0.15 | 0.16 | 0.12 | 0.15 | 0.16 |
| | 2 | 0.26 | 0.22 | 0.28 | 0.13 | 0.11 | 0.14 |
| | 3 | 0.35 | 0.33 | 0.38 | 0.117 | 0.11 | 0.127 |
| | 4 | 0.43 | 0.41 | 0.45 | 0.108 | 0.103 | 0.113 |
| | 5 | 0.5 | 0.48 | 0.54 | 0.100 | 0.096 | 0.108 |

| | | | | | | | | | | |
|-----|-------------|-------------|--------------|------|------------|-------------------|------------|-------------------|------------|-----------------|
| | 6 | 0.57 | 0.6 | 0.63 | 0.095 | 0.100 | 0.105 | | | |
| | 7 | 0.65 | 0.65 | 0.72 | 0.093 | 0.093 | 0.103 | | | |
| | 8 | 0.72 | 0.72 | 0.78 | 0.090 | 0.090 | 0.098 | | | |
| | 9 | 0.8 | 0.79 | 0.85 | 0.089 | 0.088 | 0.094 | | | |
| | 10 | 0.92 | 0.87 | 0.93 | 0.092 | 0.087 | 0.093 | | | |
| | Mean | | | | ax1 | 0.1032 | ax2 | 0.1026 | ax3 | 0.1139 |
| DOX | 2 | 0.14 | 0.12 | 0.09 | 0.07 | 0.06 | 0.045 | | | |
| | 4 | 0.18 | 0.21 | 0.15 | 0.045 | 0.053 | 0.038 | | | |
| | 6 | 0.22 | 0.28 | 0.19 | 0.037 | 0.047 | 0.032 | | | |
| | 8 | 0.25 | 0.35 | 0.27 | 0.031 | 0.044 | 0.034 | | | |
| | 10 | 0.29 | 0.43 | 0.33 | 0.029 | 0.043 | 0.033 | | | |
| | 12 | 0.32 | 0.49 | 0.39 | 0.027 | 0.041 | 0.033 | | | |
| | 14 | 0.38 | 0.57 | 0.46 | 0.027 | 0.041 | 0.033 | | | |
| | 16 | 0.42 | 0.66 | 0.54 | 0.026 | 0.041 | 0.034 | | | |
| | 18 | 0.47 | 0.74 | 0.6 | 0.026 | 0.041 | 0.033 | | | |
| | 20 | 0.53 | 0.82 | 0.66 | 0.027 | 0.041 | 0.033 | | | |
| | Mean | | | | ay1 | 0.03445873 | ay2 | 0.04508254 | ay3 | 0.034636 |
| | | A1= | A2= | | | | | | | |
| | | 0.32 | 0.467 | | | | | | | |

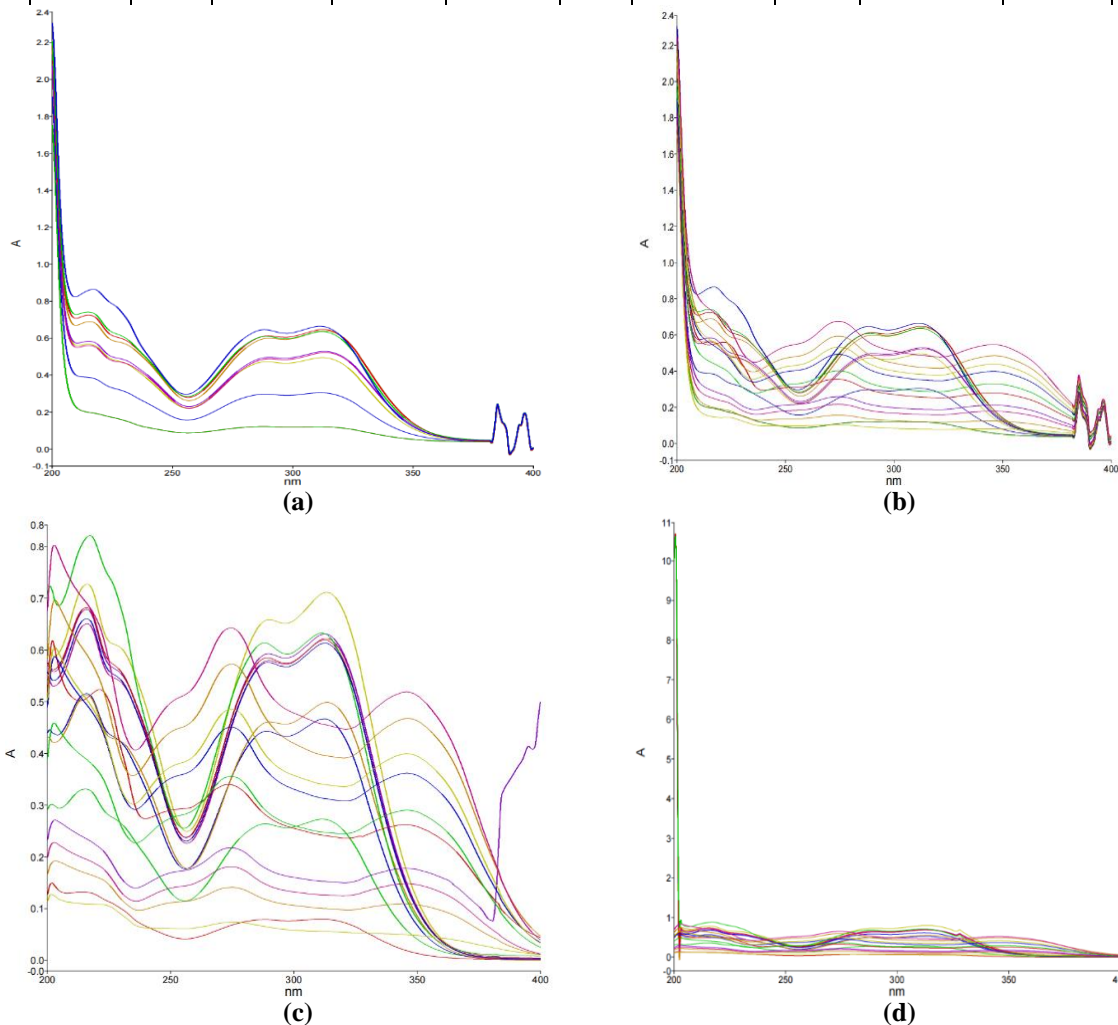


Figure 3. (a) Overlay spectra of ferulic acid at 216 nm; (b) Overlay spectra of FA and Dox at 216 nm; (c) Overlay spectra of doxycycline and ferulic acid at 274 nm; (d) Overlay spectra of FA and Dox at 276 nm.

The linearity was evaluated by the least-square method, indicating an acceptable accuracy in accordance with ICH guidelines [44].

The repeatability of FA and DOX concentrations was determined using the standard error method. For the SE method, the % RSD was 1.861% for FA and 1.786% for DOX.

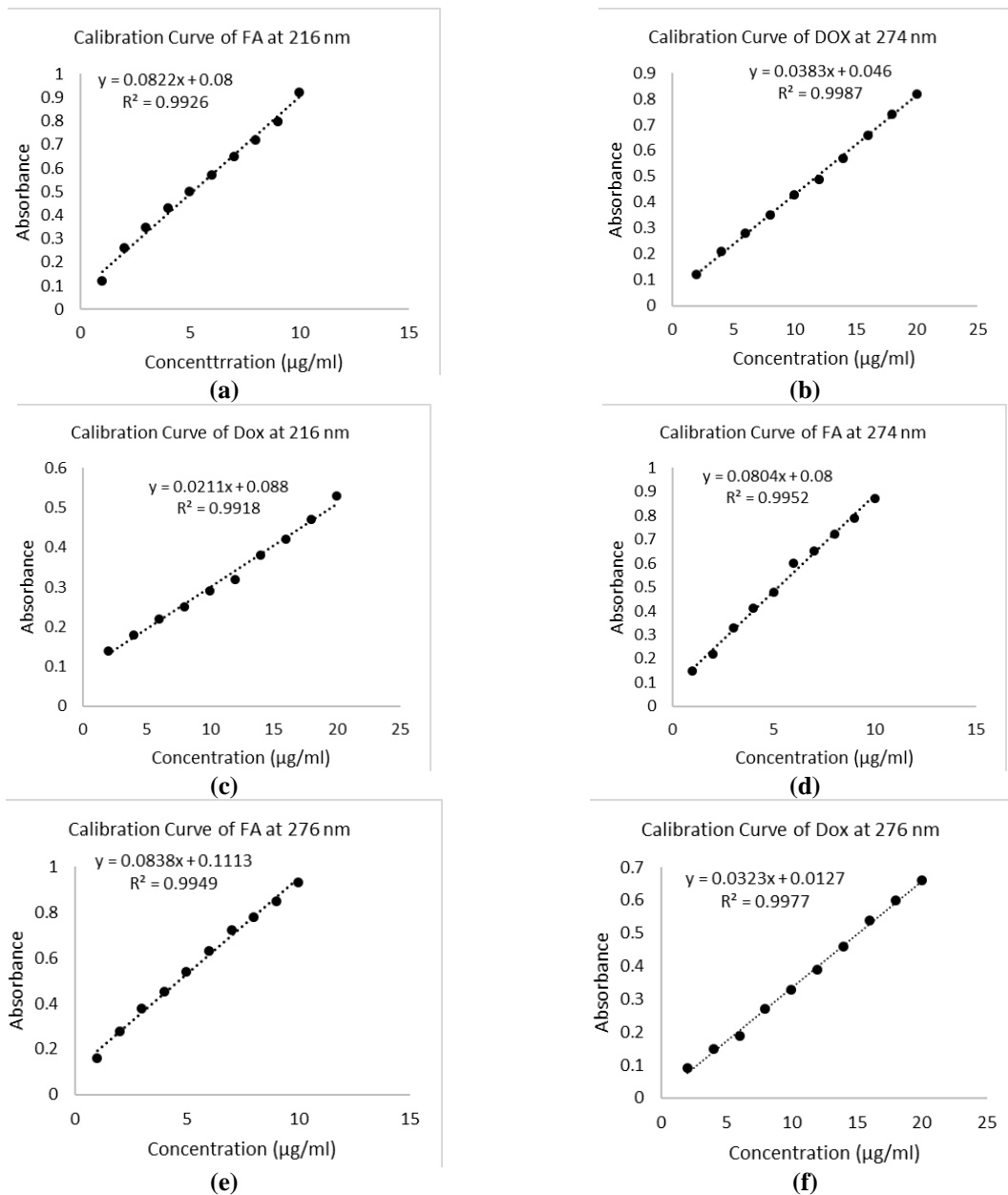


Figure 4. (a) FA at 216 nm calibration curve; (b) DOX at 274 nm calibration curve; (c) DOX at 216 nm calibration curve; (d) FA at 274 nm calibration curve; (e) FA at 276 nm calibration curve; (f) DOX at 276 nm calibration curve.

Table 2. Repeatability data of FA and DOX by SE and AR methods.

| SE Method | | AR Method | |
|-------------------------------|--------------------------------|-------------------------------|--------------------------------|
| Concentration of FA at 216 nm | Concentration of DOX at 274 nm | Concentration of FA at 276 nm | Concentration of DOX at 276 nm |
| 8.02919708 | 6.109660574 | 7.502386635 | 8.275541796 |
| 8.150851582 | 6.109660574 | 7.621718377 | 8.275541796 |
| 8.02919708 | 6.109660574 | 7.502386635 | 8.275541796 |
| 8.02919708 | 6.109660574 | 7.502386635 | 8.275541796 |
| 8.515815085 | 6.109660574 | 7.979713604 | 8.275541796 |
| 8.150851582 | 6.37075718 | 7.621718377 | 8.585139319 |
| 8.150851582 | 6.109660574 | 7.621718377 | 8.275541796 |
| 8.272506083 | 6.109660574 | 7.741050119 | 8.275541796 |
| 8.150851582 | 6.109660574 | 7.621718377 | 8.275541796 |
| 8.02919708 | 6.37075718 | 7.502386635 | 8.585139319 |
| Mean | 8.1508 | 6.161 | 8.337461 |
| ±SD | 0.151 | 0.110 | 0.130 |
| %RSD | 1.861 | 1.786 | 1.565 |

Similarly, the AR technique yielded % RSD values of 1.952% for FA and 1.565% for DOX, as shown in (Table 2).

These results indicate that the methods demonstrate acceptable repeatability, with % RSD values conforming to the ICH guidelines [44]. The low % RSD values suggest high precision and reliability of the SE and AR methods for the simultaneous estimation of FA and DOX.

To estimate accuracy, the results of the proposed SE and AR methods were compared to those obtained from previously reported methods, following ICH guidelines [44]. This comparison utilized three replicate measurements for the three concentrations within the linear range. The accuracy of the SE method was evaluated using recovery experiments at different levels: 80%, 100%, and 120%. The recoveries of FA were 100%, 101%, and 98.7%, while the recoveries of DOX were 102%, 104%, and 103%, as shown in (Table 3).

Table 3. Accuracy data of the proposed SE methods.

| SE method | | | | | | | | |
|-----------|---------|---------------|---------------------------------------|---------------------------------|-------------|----------|----------|----------|
| Drug | Level % | Conc. (µg/ml) | Quantity of standard additive (µg/ml) | Conc found after adding (µg/ml) | Recovery % | Mean | ±SD | %RSD |
| FA | 80% | 6 | 2 | 7.960199005 | 100.5389718 | 8.043118 | 0.143619 | 1.785619 |
| | | 6 | 2 | 7.960199005 | | | | |
| | | 6 | 2 | 8.208955224 | | | | |
| | 100% | 6 | 4 | 10.07462687 | 101.1608624 | 10.11609 | 0.07181 | 0.709857 |
| | | 6 | 4 | 10.07462687 | | | | |
| | | 6 | 4 | 10.19900498 | | | | |
| | 120% | 6 | 6 | 11.31840796 | 98.70830068 | 11.845 | 0.912077 | 1.700107 |
| | | 6 | 6 | 11.31840796 | | | | |
| | | 6 | 6 | 12.89817232 | | | | |
| DOX | 80% | 6 | 2 | 7.974276527 | 102.5809909 | 8.206479 | 0.201093 | 2.450423 |
| | | 6 | 2 | 8.322580645 | | | | |
| | | 6 | 2 | 8.322580645 | | | | |
| | 100% | 6 | 4 | 10.25806452 | 104.7311828 | 10.47312 | 0.186242 | 1.778286 |
| | | 6 | 4 | 10.58064516 | | | | |
| | | 6 | 4 | 10.58064516 | | | | |
| | 120% | 6 | 6 | 12.19354839 | 103.4050179 | 12.4086 | 0.186242 | 1.500911 |
| | | 6 | 6 | 12.51612903 | | | | |
| | | 6 | 6 | 12.51612903 | | | | |

Table 4. Accuracy data of the proposed AR methods.

| AR Method | | | | | | | | |
|-----------|---------|-----------------------|---------------------------------------|--|-------------|----------|----------|----------|
| Drug | Level % | Concentration (µg/ml) | Quantity of standard additive (µg/ml) | Concentration found after adding (µg/ml) | Recovery % | Mean | ±SD | %RSD |
| FA | 80% | 6 | 2 | 7.979713604 | 100.2436356 | 8.019491 | 0.068896 | 0.85911 |
| | | 6 | 2 | 7.979713604 | | | | |
| | | 6 | 2 | 8.099045346 | | | | |
| | 100% | 6 | 4 | 9.868735084 | 99.08512331 | 9.908512 | 0.068896 | 0.695323 |
| | | 6 | 4 | 9.988066826 | | | | |
| | | 6 | 4 | 9.868735084 | | | | |
| | 120% | 6 | 6 | 12.39498807 | 103.6230443 | 12.43477 | 0.068896 | 0.554061 |
| | | 6 | 6 | 12.51431981 | | | | |
| | | 6 | 6 | 12.39498807 | | | | |
| DOX | 80% | 6 | 2 | 7.656346749 | 97.22402543 | 7.777922 | 0.192614 | 2.476414 |
| | | 6 | 2 | 7.677419355 | | | | |
| | | 6 | 2 | 8 | | | | |
| | 100% | 6 | 4 | 9.513931889 | 97.20330237 | 9.72033 | 0.178746 | 1.83889 |
| | | 6 | 4 | 9.823529412 | | | | |
| | | 6 | 4 | 9.823529412 | | | | |
| | 120% | 6 | 6 | 11.99071207 | 100.7825937 | 12.09391 | 0.178746 | 1.477985 |
| | | 6 | 6 | 11.99071207 | | | | |
| | | 6 | 6 | 12.3003096 | | | | |

Similarly, the accuracy of the AR technique was evaluated using recovery experiments at wavelengths of 274 nm and 276 nm. The results showed recoveries of 100%, 99%, and 103% for FA at concentrations of 80%, 100%, and 120%, respectively. For DOX, the recoveries were 97%, 97%, and 100% at the same concentration levels, as shown in (Table 4).

These results indicate that the SE and AR methods accurately estimate FA and DOX, with recovery percentages well within the acceptable range according to ICH guidelines. The consistent recoveries across different concentrations further validate the reliability and robustness of these methods for simultaneous drug estimation.

Intraday and interday analyses demonstrated the precision of the proposed SE and AR methods. For the SE method, intraday precision in the analysis of FA showed %RSD values of 0.822%, 1.385%, and 1.272% during the first, second, and third hours, respectively. The intraday %RSD values for DOX were 1.894%, 1.97%, and 2.51% during the same time intervals. The interday precision for FA had %RSD values of 0.675%, 1.245%, and 1.209% for the first, second, and third hours, respectively. DOX's interday %RSD values were 1.927%, 2.30%, and 2.47% for the corresponding time points. Using the AR method, the interday precision for FA determination yielded %RSD values of 0.667%, 0.824%, and 1.715%, while the interday precision for DOX had %RSD values of 1.660%, 1.724%, and 2.179%. The intraday precision for FA using the AR method showed %RSD values of 0.709%, 1.305%, and 1.266%, and for DOX, the values were 1.685%, 2.01%, and 2.162% (Table 5 and Table 6).

Table 5. Precision data of the proposed SE methods.

| | | SE Method | | | | | | | |
|----------|---------|---------------|---------------|--------|-------|----------|-------|-------|-------|
| Days | Hour | Concentration | Concentration | Mean | Mean | ±SD | ±SD | %RSD | %RSD |
| | | FA | DOX | FA | DOX | FA | DOX | FA | DOX |
| Intraday | 1st | 8.0292 | 6.110 | 8.1022 | 6.162 | 0.067 | 0.117 | 0.822 | 1.894 |
| | | 8.0292 | 6.110 | | | | | | |
| | | 8.1509 | 6.110 | | | | | | |
| | | 8.1509 | 6.110 | | | | | | |
| | 2nd | 8.1509 | 6.371 | 7.8589 | 5.901 | 0.108811 | 0.117 | 1.385 | 1.978 |
| | | 7.7859 | 5.849 | | | | | | |
| | | 7.9075 | 5.849 | | | | | | |
| | | 8.0292 | 5.849 | | | | | | |
| | | 7.7859 | 5.849 | | | | | | |
| | 3rd | 7.7859 | 6.110 | 8.0049 | 5.692 | 0.101783 | 0.143 | 1.272 | 2.512 |
| | | 7.9075 | 5.849 | | | | | | |
| | | 8.0292 | 5.587 | | | | | | |
| 8.1509 | | 5.587 | | | | | | | |
| 8.0292 | | 5.849 | | | | | | | |
| Interday | Day-I | 7.9075 | 5.587 | 8.0535 | 6.057 | 0.054406 | 0.116 | 0.675 | 1.927 |
| | | 8.0292 | 6.110 | | | | | | |
| | | 8.0292 | 5.849 | | | | | | |
| | | 8.0292 | 6.110 | | | | | | |
| | | 8.1509 | 6.110 | | | | | | |
| | Day-II | 8.0292 | 6.110 | 8.1752 | 6.214 | 0.101783 | 0.143 | 1.245 | 2.301 |
| | | 8.1509 | 6.110 | | | | | | |
| | | 8.2725 | 6.110 | | | | | | |
| | | 8.2725 | 6.371 | | | | | | |
| | Day-III | 8.0292 | 6.371 | 8.4185 | 6.089 | 0.101783 | 0.150 | 1.209 | 2.470 |
| | | 8.2725 | 6.110 | | | | | | |
| | | 8.3942 | 5.849 | | | | | | |
| | | 8.5158 | 6.110 | | | | | | |
| | | 8.5158 | 6.110 | | | | | | |
| | | | 8.3942 | 6.266 | | | | | |

Table 6. Precision data of the proposed AR methods.

| DAY | Hours | AR Method | | | | | | | |
|----------|----------|-------------------------|-------------------------|--------|-------|----------|-------|-------|-------|
| | | Concentration at 276 nm | Concentration at 276 nm | Mean | Mean | ±SD | ±SD | %RSD | %RSD |
| | | FA | DOX | FA | DOX | FA | DOX | FA | DOX |
| Intraday | 1st | 7.9797 | 8.276 | 8.0036 | 8.337 | 0.053 | 0.138 | 0.667 | 1.660 |
| | | 7.9797 | 8.276 | | | | | | |
| | | 7.9797 | 8.276 | | | | | | |
| | | 7.9797 | 8.276 | | | | | | |
| | | 8.0990 | 8.585 | | | | | | |
| | 2nd | 7.9797 | 7.966 | 7.9320 | 8.028 | 0.065361 | 0.138 | 0.824 | 1.724 |
| | | 7.8604 | 7.966 | | | | | | |
| | | 7.8604 | 7.966 | | | | | | |
| | | 7.9797 | 7.966 | | | | | | |
| | | 7.9797 | 8.276 | | | | | | |
| | 3rd | 7.9797 | 7.966 | 7.9320 | 7.780 | 0.136059 | 0.170 | 1.715 | 2.179 |
| | | 7.7411 | 7.656 | | | | | | |
| | | 7.8604 | 7.656 | | | | | | |
| | | 7.9797 | 7.966 | | | | | | |
| | | 8.0990 | 7.656 | | | | | | |
| Interday | Day-I | 7.5024 | 8.276 | 7.5263 | 8.214 | 0.053367 | 0.138 | 0.709 | 1.685 |
| | | 7.5024 | 7.966 | | | | | | |
| | | 7.5024 | 8.276 | | | | | | |
| | | 7.6217 | 8.276 | | | | | | |
| | | 7.5024 | 8.276 | | | | | | |
| | Day - II | 7.6217 | 8.276 | 7.6456 | 8.399 | 0.09984 | 0.169 | 1.305 | 2.018 |
| | | 7.7411 | 8.276 | | | | | | |
| | | 7.6217 | 8.276 | | | | | | |
| | | 7.7411 | 8.585 | | | | | | |
| | | 7.5024 | 8.585 | | | | | | |
| | Day-III | 7.7411 | 8.276 | 7.8842 | 8.251 | 0.09984 | 0.178 | 1.266 | 2.162 |
| | | 7.8604 | 7.966 | | | | | | |
| | | 7.9797 | 8.276 | | | | | | |
| | | 7.9797 | 8.276 | | | | | | |
| | | 7.8604 | 8.461 | | | | | | |

In intraday and interday precision studies, the %RSD values were not more than 2.0%, indicating good repeatability and intermediate precision [45].

The limit of detection (LOD) and limit of quantification (LOQ) for each method were mathematically computed following ICH guidelines [44]. Using the SE method, the LOD for FA and DOX were 3.21 µg/ml and 3.96 µg/ml, respectively. The AR method yielded LOD values of 3.963 µg/ml for FA and 1.29 µg/ml for DOX. The SE method determined the LOQ for FA and DOX to be 9.73 µg/ml and 9.95 µg/ml, respectively. The AR method detected LOQ values of 13.26 µg/ml for FA and 3.93 µg/ml for DOX, as summarized in (Table 7).

Table 7. Summary of the data used for validating the proposed technique's method via linear regression.

| Parameters | SE | | | | AR | | | |
|---------------------------------|----------|--------|---------|--------|----------|--------|--------|--------|
| | FA | | DOX | | FA | | DOX | |
| Wavelength (nm) | 216 | 274 | 216 | 274 | 274 | 276 | 274 | 276 |
| Linearity ranges (µg/ml) | 1-10 | | 2-20 | | 1-10 | | 2-20 | |
| Specificity | Specific | | | | Specific | | | |
| Correlation coefficient | 0.9926 | 0.9952 | 0.9918 | 0.9987 | 0.9952 | 0.9949 | 0.9987 | 0.9977 |
| Regression equation slop | 0.0822 | 0.0804 | 0.0211 | 0.0383 | 0.0804 | 0.0838 | 0.0383 | 0.0323 |
| Intercept | 0.08 | 0.08 | 0.088 | 0.046 | 0.08 | 0.1113 | 0.046 | 0.0127 |
| Precision (% RSD) repeatability | 1.86 | | 1.78 | | 1.95 | | 1.56 | |
| Intra day | 0.822 | 1.385 | 1.894 | 1.978 | 0.667 | 0.824 | 1.660 | 1.724 |
| Inter day | 0.675 | 1.245 | 1.927 | 2.301 | 1.305 | 1.266 | 2.018 | 2.162 |
| Accuracy (% recovery) | 100-101 | | 100-103 | | 100-103 | | 97-100 | |
| LOD (µg/ml) | 3.21 | 3.28 | 4.382 | 3.28 | 3.963 | 4.382 | 3.96 | 1.29 |
| LOQ (µg/ml) | 9.73 | 9.95 | 13.26 | 9.95 | 12.0 | 13.26 | 12.01 | 3.931 |

These results indicate that the proposed SE and AR methods are precise, with acceptable LOD and LOQ values. The methods conform to ICH guidelines and demonstrate robust performance for the simultaneous estimation of FA and DOX.4.

4. Conclusion

This study aimed to implement two spectrophotometric methods for assessing the synthetic mixtures of a combination of FA and DOX. The applied methods, including the Simultaneous equation and Absorption ratio technique, revealed acceptable accuracy, good linearity, reproducibility, and precision and can be applied for the routine analysis and quality control of the combined mixture. Further, the methods also have the additional advantages of speed, simplicity, and environmental safety as they don't require hazardous solvents or sophisticated instruments. Statistical analysis has been accomplished, illustrating non-significant differences from the previously reported data. The proposed study poses simple, rapid, and reliable methods for analyzing the combination mixture.

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Conflicts of Interest

The author declares no competing financial interest.

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