

Spectroscopic Estimation of Doxylamine Succinate in Tablets and Human Plasma by Formation Ion-Pair Complex

Running title: Spectroscopic Estimation of Doxylamine

ABSTRACT

Aim: To develop a simple spectroscopic method for estimation of doxylamine (DOX) succinate in its tablet dosage form and human plasma with the aid of an ion-pair complex formation.

Methods: In this method, methyl orange (0.05 % w/v) dye was used to form an ion-pair complex in acetate buffer (1M; pH: 5.00) at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The ion-pair complex formed was extracted with chloroform. The maximum absorbance for the ion-pair complex was measured at 420 nm.

Results and discussion: The method conditions were obeyed Beer's law in the concentrations ranging from 5-25 $\mu\text{g/mL}$ of DOX succinate with a correlation coefficient (r^2) of 0.992. The ion-pair (drug-dye) complex was formed in a 1:1 ratio which was demonstrated by Jobs' method of continuous variation. The method was satisfied the validation criteria as per ICH (Q_2R_1) guidelines. Accuracy studies showed 99.06-100.9 % recovery of the analyte. The responses of the precision and robustness were found within acceptable limits (<2% RSD). The LOD and LOQ values were found as 0.31 and 0.939 $\mu\text{g/mL}$, respectively.

Conclusion: The developed method was simple, specific, and economical and requires a short analysis time. Therefore it could be considered for precise analysis of DOX succinate in biological matrices.

Keywords: Doxylamine succinate, spectroscopy, ion-pair complex, methyl orange, linearity, method validation, etc.

1. INTRODUCTION

An ion-pair formation strategy works with the concept that ionizable species of both analyte and pairing reagents are more readily extracted with the help of suitable organic solvents as ion-pairs. The separation could affect the type and concentration of the counter ion and the type of organic phase used. An ion-pair composes of oppositely charged ions held together *via* Columbic attraction that behaves as a single unit. In general, an ion-pair interaction has great importance in drug delivery to enhance the permeability of several agents such that they will be ionized throughout the physiological pH range (Libermann *et al.*, 2015). Besides its applications in drug delivery systems, the ion-pair complex formation has a tremendous advantage in quantifying the analyte drugs in different biological matrices with good sensitivity.

Doxylamine (DOX) succinate is a first-generation antihistamine. It is an ethanolamine derivative that reduces the effects of histamine in the body. Chemically, it is *N, N*-dimethyl-2-[1-phenyl-1-(2-pyridinyl)ethoxy]-butanedioate succinate. The drug is freely soluble in water and chloroform and slightly soluble in diethyl ether and benzene. DOX succinate tablets are commercially available under different brand names (Ex.: UNISOM sleep tablets).

An extensive literature survey revealed several spectrophotometric [1-3] and chromatographic [4-12] methods for estimation of DOX succinate in various dosage forms in individuals and combination with other drugs. Majority of reported methods employed with single drug as well as with combinations in different dosage forms, in which no ion-pair spectroscopic approach was developed for analysis in biological samples. The present work attempted to develop a simple, economical, and sensitive spectroscopic method that could be also useful for precise quantification of the drug in biological matrices. In this direction, the present work aimed to estimate the DOX succinate in commercial tablet dosage forms and spiked human plasma [13-17]. Further, the method was validated according to the International Council for Harmonization (ICH) guidelines. The present communication described the experimental protocols for the method development, optimization, and validation studies for estimation of DOX succinate in tablet dosage forms and blank human plasma.

2. MATERIALS AND METHODS

2.1 Apparatus: The analysis was performed in 10 mm quartz cells using a UV-Visible spectrophotometer (Lab India, UV-3000+) with a fixed 2 nm spectral bandwidth. UV-Win 5 software v5.1.1 was used for all absorbance measurements in the region of 200-800 nm [3]. The pH of the solutions was measured with the help of a digital pH meter (Elico, L1 120).

2.2 Materials and reagents: DOX succinate was procured as a gift sample from Granules India Limited, Hyderabad. Tablet formulation (UNISOM tablets) was obtained from the local market. The blank human plasma was kindly obtained as a gift sample from Needs Blood Bank, Guntur. Analytical grade solvents and chemicals were purchased from Merck India Limited, Mumbai, and SD Fine Chemicals, Mumbai. The pH of the acetate buffer (1 M; pH: 5.00) was adjusted with glacial acetic acid. 0.05% w/v of Methyl Orange (MO) dye solution was prepared using 50 mg of MO dye in distilled water (solvent diluent) [13].

2.3 Determination of absorption maxima: The analysis was performed by measuring all the absorbances in 10 mm quartz cells as the instrument enabled with high-resolution capacity and better reproducibility. The maximum absorbance wavelength was measured for the standard solution (10 µg/mL) and ion-pair (DOX-MO) complex by taking approximately 3.0 mL of each solution in cuvette under UV-visible region (200-800 nm).

2.4 Optimization of experimental variables: The method was optimized to achieve the degree of completion of the reaction, effective extraction, and sensitivity. The spectroscopic conditions were optimized by studying the effect of different variables such as pH range, extracting solvent, reaction time, the volume of dye used, and temperature conditions for the extraction of DOX-MO complex.

2.5 General method for DOX-MO complex formation [13, 15]: The aliquots (1-6 mL) of the standard stock solution (100 µg/ mL) was taken in each separating funnel (six 250 mL separating funnels) in order to get the final concentration of 5-30 µg/mL DOX succinate in diluent (made to 10 mL). About 2 mL of acetate buffer solution (1M; pH: 5.00) was added to each separating flask, followed by 1 mL of MO (0.05% w/v) dye, and the volume was made to 20 mL with diluent with thorough mixing. The contents of each funnel were allowed to stand for one minute after shaking with 10 mL of chloroform.

Two layers were separated and the absorbance was recorded for the organic layer at 420 nm against a reagent blank.

2.6 Preparation of standard solution of DOX succinate: The standard solution (1000 µg/ mL) was prepared by accurately weighing 10 mg of DOX succinate in a 10 mL volumetric flask containing 3 mL of diluent and sonicated (20 mins). The volume was made up to the mark with diluent. 1 mL of the above solution was transferred into a 10 mL volumetric flask and diluted to get 100 µg/ mL of the drug. From this 2 mL was transferred into a 10 mL volumetric flask and diluent to get the working standard solution (20 µg/ mL).

2.7 Preparation of sample solution: Accurately weighed twenty tablets (Unisom tablets) and triturated to get a fine powder. Tablet powder equivalent to 10 mg of DOX succinate was taken in a 100 mL calibrated flask containing 50 mL of diluent. The contents were shaken for 20 mins and the volume was made to the mark with diluent. The resulting solution was passed through a 0.45 µ membrane filter paper to remove insoluble matter if any. The sample solution was further prepared as described in the general method by taking 5 mL of aliquot and analyzed in five replicates.

2.8 Assay in the spiked plasma sample [18, 19]: To the about 1 mL of blank human plasma containing in polypropylene tubes spiked with 1 mL of standard stock solution (100 µg/mL). To the resulted solution added 2 mL of buffer solution (1 M; pH: 5.00) followed by 1mL MO solution (0.05% w/v) and the volume was adjusted to 10 mL by adding diluent and mixed thoroughly. A 5 mL portion of chloroform was added to the mixture for liquid-liquid extraction. A cloudy solution obtained was centrifuged for 5 minutes (3000 rpm). The absorbance of the chloroform layer was measured at 420 nm against the reagent blank in five replicates. The regression equation was applied to know the content of DOX succinate.

2.9. METHOD VALIDATION

The method was validated for various parameters according to the ICH (Q₂R₁) guidelines [20, 21].

2.9.1 Specificity: The specificity of the method was determined by using 20 µg/mL of working standard solution and sample solution as per the general method. Also, a blank extract was performed to know the selectivity of the method.

2.9.2 Linearity: Five concentrations of DOX succinate were freshly prepared for linearity studies and measured the absorbance at 420 nm. The calibration curve was plotted with absorbances against concentration. The standard solutions (1.0-5.0 mL) were transferred into respective volumetric flasks by using a grade bulb pipette and the remaining procedure was followed to get DOX-MO complex in the concentration of 5-25 µg/ mL. The solutions were then filtered through a 0.45 µ membrane filter. Each solution was scanned at 420 nm in triplicate and linearity was evaluated by linear regression analysis.

2.9.3 Accuracy: A known amount of the standard was added to the sample at three levels (50, 100, and 150 % - of the labeled claim). Three determinations were considered for each level at the detection wavelength (420 nm) and were expressed in percent analyte recovered with %RSD.

Accuracy level 1 (50%): About 2.5 mL of DOX standard solution (100 µg/mL) was added to the sample in a 20 mL volumetric flask and followed the general procedure. The resulted mixture was extracted with chloroform and measured for absorbance at 420 nm.

Accuracy level 2 (100%): About 5 mL of DOX standard solution (100 µg/mL) was added to the sample in a 20 mL volumetric flask and followed the general procedure. The resulted mixture was extracted with chloroform and measured for absorbance at 420 nm.

Accuracy level 3 (150%): About 7.5 mL of DOX standard solution (100 µg/mL) was added to sample a 20 mL volumetric flask and followed the general procedure. The resulted mixture was extracted with chloroform and measured for absorbance at 420 nm.

2.9.4 Precision: Precision was determined as repeatability, in which system precision and method precision was established by following ICH guidelines. The system precision was established by taking the standard solution at optimized conditions. Six replicates of sample solutions established the method's precision.

2.9.5 Robustness: Deliberate changes in the optimized spectroscopic conditions like buffer volume, dye content were made to know the robustness of the method.

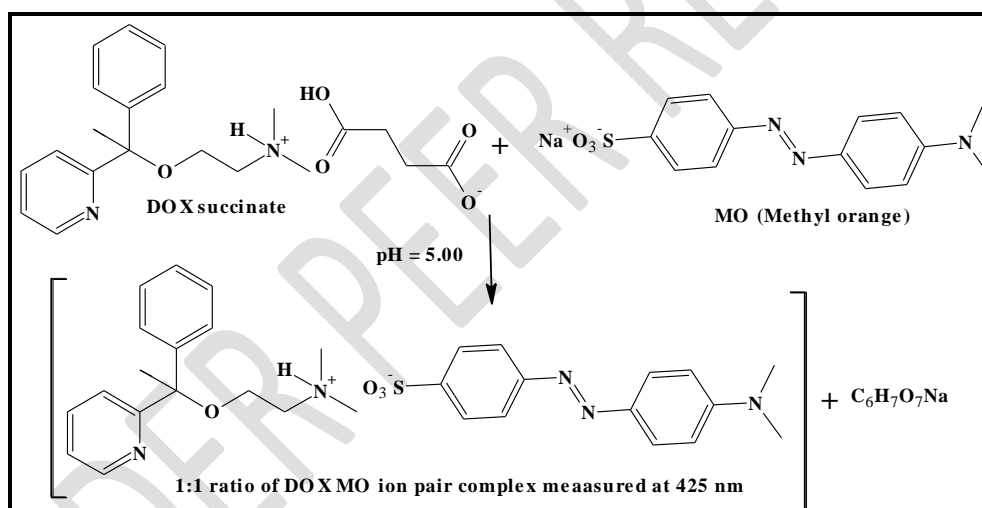
2.9.6 Ruggedness: It was validated with a degree of reproducibility of test results. The results were obtained by analyzing the samples under varied test conditions such as different analysts, laboratories, instruments, etc. The sample solution of 20 µg/mL of DOX succinate was taken and followed the general

method of DOX-MO complex formation and determined in triplicates at variable conditions. The % RSD of the results for the absorbances was calculated.

2.9.7 LOD and LOQ: In general, the limits are expressed with the help of a signal-to-noise ratio (S/N). Analysts often use S/N of 2:1 or 3:1 ($LOQ = 10 \sigma / S$) to measure the LOD, while an S/N of 10:1 ($LOQ = 10 \sigma / S$) is often considered for the LOQ.

3. RESULTS AND DISCUSSION

3.1 Absorption maxima: The spectroscopic conditions were successfully optimized for assay of DOX succinate in its tablet dosage form and human plasma. DOX succinate was allowed to react with MO dye in a buffer solution (pH = 5.00) which was converted into a yellow-colored product (DOX-MO complex) (**Scheme 1**), further it was extracted with chloroform. The maximum absorbance for the complex was measured at 420 nm (**Fig. 1**), where the reagent blank had no absorbance in this region.



Scheme 1: The probable reaction pathway for DOX-MO complex formation.

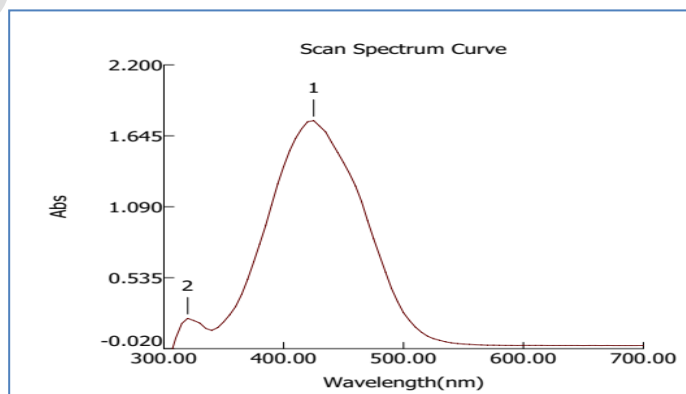


Fig. 1: Absorption spectrum for DOX-MO complex Of standard (λ_{max} 420 nm)

3.2 Optimization of experimental variables: As per the optimized conditions, MO dye is used as an ion-pairing reagent, acetate buffer (pH 5.00) was used as a solvent medium for allowing reaction, and chloroform was used as an extracting solvent. Preliminary experiments were conducted to know the effect of different variables on DOX-MO complex formation such as pH, type of extracting solvent, and temperature for extraction of complex.

3.3 Selection of extraction solvent: The extraction of DOX-MO complex was tested with ethyl acetate, dichloromethane, chloroform, and carbon tetrachloride. In these studies, chloroform was found a better extracting solvent as it has enhanced efficacy for color intensity and provided selective extraction to that of other solvents. Moreover, as per the standard protocols, a single extraction solvent could give the quantitative recovery for all the complexes.

3.4 Effect of pH: The optimum pH conditions for the formation of the DOX-MO complex was adjusted with the help of varying pH ranges (pH: 3.2-5.2) of the buffer solution. The pH was optimized at 5.00 as it showed the maximum absorbance.

3.5 Effect of dye concentration: The concentration of MO dye is one of the crucial parameters in adjusting the complexes' colour intensity; it was achieved by varying the dye concentration (0.01-0.1 %) at the detection wavelength (420 nm). The colour intensity of the DOX-MO complex was compatible with 0.05% MO dye.

3.6 Effect of shaking mode and time: Deferent approaches were employed to extract the DOX-MO complexes using a separating funnel, a magnetic stirrer, and a vortex mixture. Among this vertex, the mixture gave a little higher absorbance value. Further, shaking time was optimized (for 1.0 to 5.0 min) and there was no appreciable improvement in extraction after 1.0 min.

3.7 Effect of temperature on the stability: Temperature variation affected the stability of the DOX-MO complex, which was tested at 25, 30, and 35°C. The results exhibited that 30°C was found optimum with a minute change in the absorbances. Moreover, as the extracting solvent was volatile at tested temperatures, there was a slight increase (~1–3%) in the absorbance of the drug-dye complex.

3.8 Stoichiometry of DOX-MO complex: It was established by Job's method of continuous variation, in which the composition of the DOX-MO complex was measured (Job, 1928). In this study, the

test solutions were prepared in identical concentrations of the drug (1.5×10^{-3} M). Both the solutions were mixed in varying volume ratios by adjusting the total volume of each solution as constant. The resulting complexes (with varied volume ratios of drug and dye) were measured for absorbance at 420 nm. In a plot of absorbance against the mole fraction of the drug, these results showed the 1:1 stoichiometric ratio of drug and dye showed a stable DOX-MO complex in **Fig. 2**.

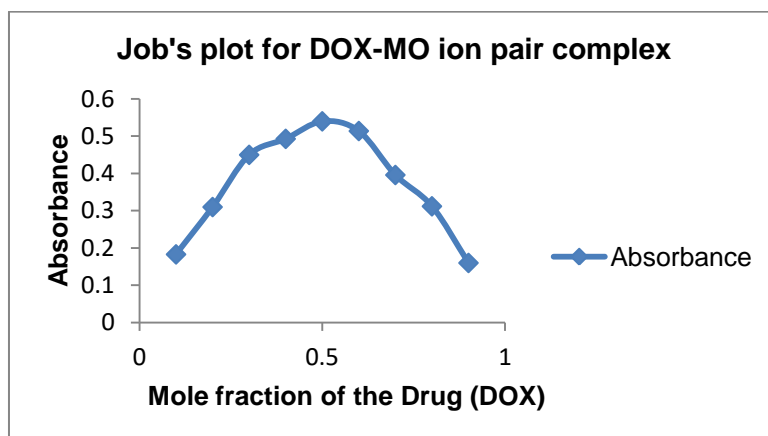


Fig. 2: Job's plot for DOX-MO complex.

3.9 Assay: The standard and sample solutions of DOX succinate were prepared separately in appropriate concentrations as per the general method. The sample solution containing nearly equivalent to the standard amount (as per the label claim) was prepared and scanned to measure absorbances in triplicate at 420 nm (**Table 1**). The mean absorbance values for standard and sample solution were found as 0.826 and 0.822, respectively. The amount of drug present was found to be 99.25 %, which was found as per the label claim amount. The absorption spectrum for standard and sample solution was shown in **Fig. 3 & 4**.

Table 1: The Absorbance values of the DOX-MO complex

S. No.	Analyte (DOX-MO complex)	Absorbance 420 nm			Mean Absorbance \pm SD
		A ₁	A ₂	A ₃	
1	Standard	0.827	0.827	0.826	0.826 \pm 0.001
2	Sample	0.822	0.820	0.826	0.822 \pm 0.003

Note: A = Absorbance

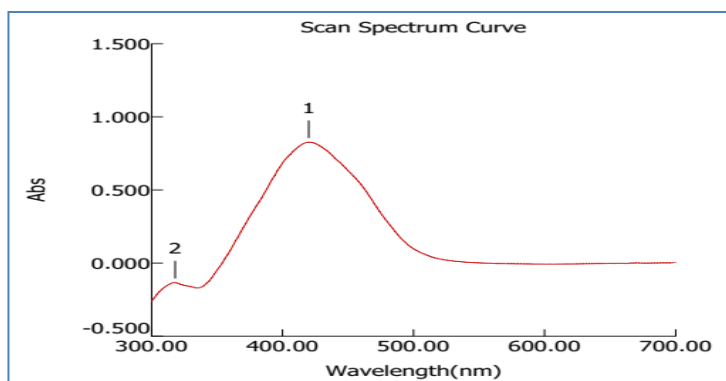


Fig. 3: Absorption spectrum for DOX-MO complex of standard.
(At 10 $\mu\text{g}/\text{mL}$ of DOX, λ_{max} 420 nm)

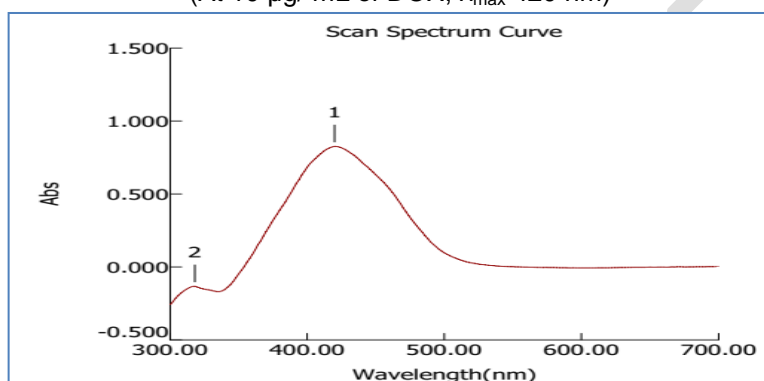


Fig. 4: Absorption spectrum for DOX-MO complex of sample
(At 10 $\mu\text{g}/\text{mL}$ of DOX, λ_{max} 420 nm)

3.10 Method validation

3.10.1 Specificity

Identification of DOX-MO complex: Standard and sample solutions were prepared and analyzed as per test procedure and scanned under UV-visible spectrophotometer (**Fig. 5 & 6**). The absorption spectrum showed that both standard and sample spectrums for DOX-MO complex were similar and no interferences of excipients were observed. The absorption maximum was observed for the DOX-MO complex at 420 nm.

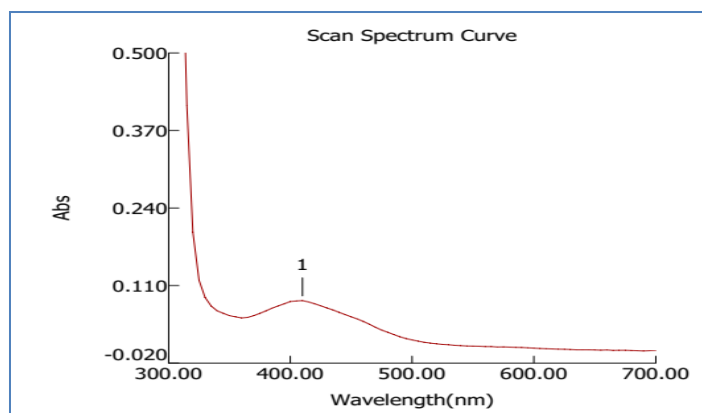


Fig. 5: Absorption spectrum for DOX-MO complex of sample.

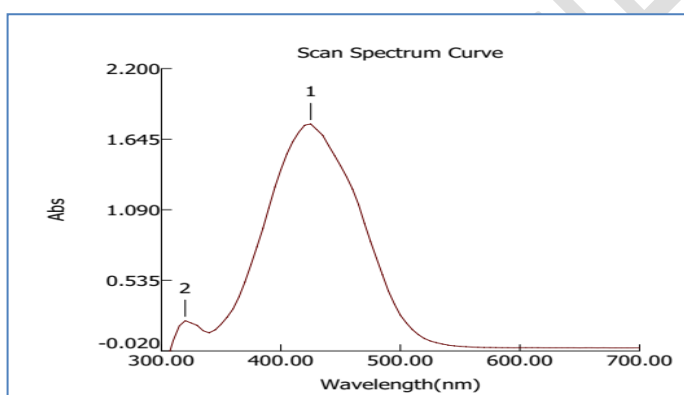


Fig. 6: Absorption spectrum for DOX-MO complex of standard.

Blank determination: The extracting solvent (CHCl_3) with reagent blank was scanned in the UV-visible region (200-800 nm) as per optimized conditions (**Fig. 7**). The blank spectrum indicated that there was no interference of solvent or dye at the detected wavelength.

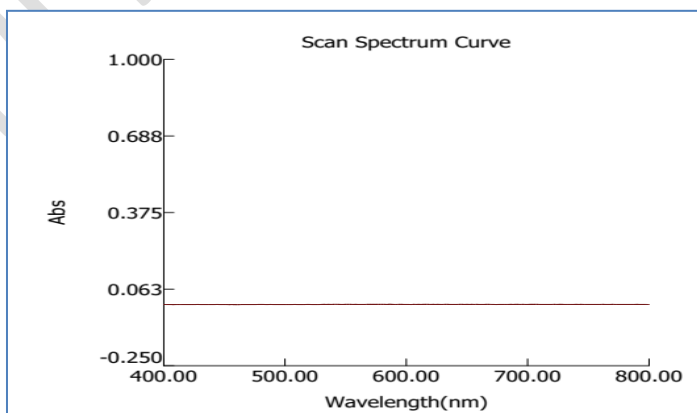


Fig. 7: Absorption spectrum for reagent blank without DOX.

3.10.2 Linearity: Five concentrations (5-25 µg/mL) of DOX succinate with 2 mL MO dye were freshly prepared for linearity studies and measured the absorbance at 420 nm. The overlain spectrum was drawn for five individual concentrations in triplicates (**Fig. 8**). The absorbances were recorded and the calibration curve was plotted against concentration v/s absorbance's (**Fig. 9**). Results showed a good linear relationship in the absorbance versus concentration with a regression equation of $y = 0.030x + 0.122$ for the DOX-MO complex (**Table 2**). The correlation coefficient ($r^2 = 0.992$) found was within the prescribed range. Hence the concentration range from 5 to 25 µg/mL showed linearity in the responses.

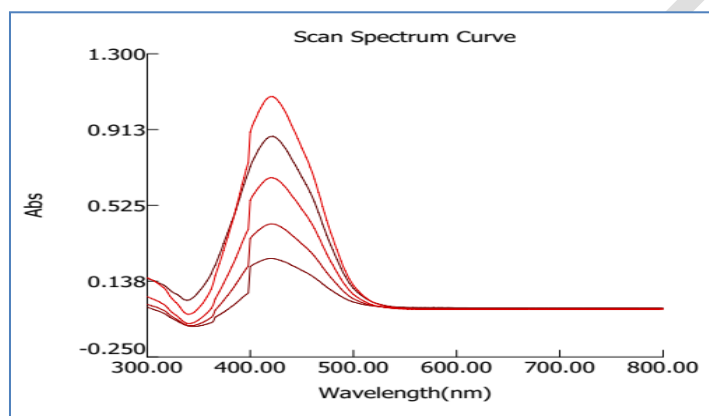


Fig. 8: Overlain spectrum of DOX-MO complex at linearity ranges.

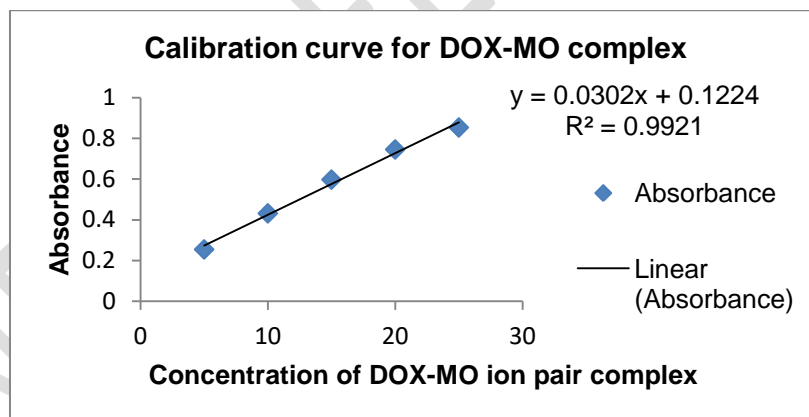


Fig. 9: Calibration curve for DOX-MO complex (5-25 µg/mL).

Table 2: Linearity data of DOX-MO complex

Sample	Concentration of analyte (µg/mL)	Absorbance	Linear regression equation
DOX-MO complex	5	0.254	$y = 0.030x + 0.122$ $r^2 = 0.992$
	10	0.431	
	15	0.667	
	20	0.745	
	25	0.853	

3.10.3 Accuracy: The percent analyte recovered was calculated at each level, in which the recovery was found as 99.09-100.6 % (**Table 3**). The % RSD values were within 2.0 % for each level, which revealed that good agreement with accuracy (**Fig. 10-12**).

Table 3: Accuracy data of the DOX-MO complex (n = 3)

S. No.	Spiked level	Absorbance	% Recovery	SD	% RSD	% Mean recovery \pm SD
1	50 %	0.693	101.0	0.001	0.324	100.6 \pm 0.65
	50 %	0.686	100.4			
	50 %	0.685	100.6			
2	100 %	0.818	100.3	0.002	0.324	100.2 \pm 0.45
	100 %	0.811	99.7			
	100 %	0.809	100.8			
3	150 %	0.942	99.15	0.0026	0.21	99.09 \pm 0.081
	150 %	0.933	99.12			
	150 %	0.928	99.01			

n= number of determinations; SD = Standard deviation; % RSD = % Relative standard deviation

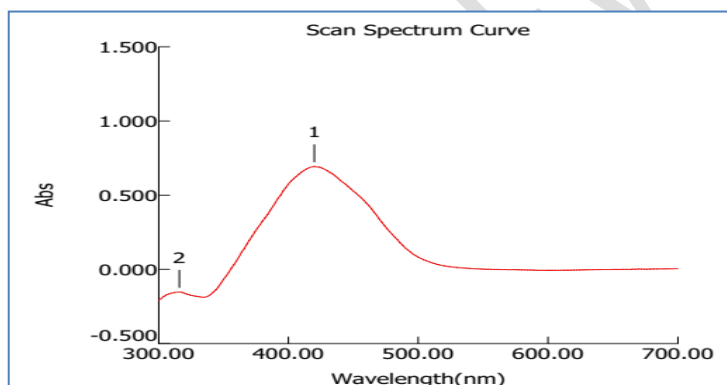


Fig. 10: Spectrum at 50% accuracy level.

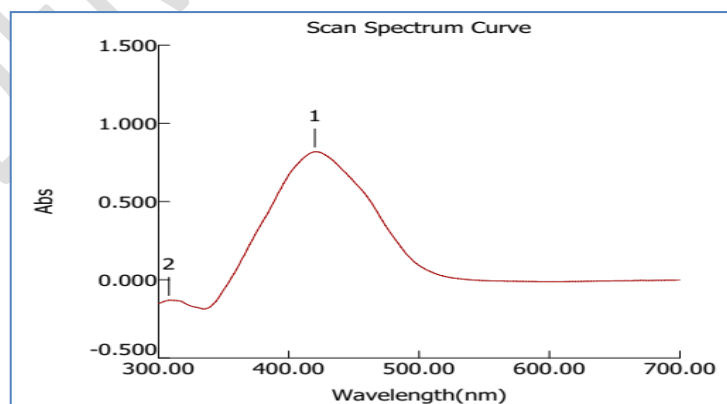


Fig. 11: Spectrum at 100% accuracy level.

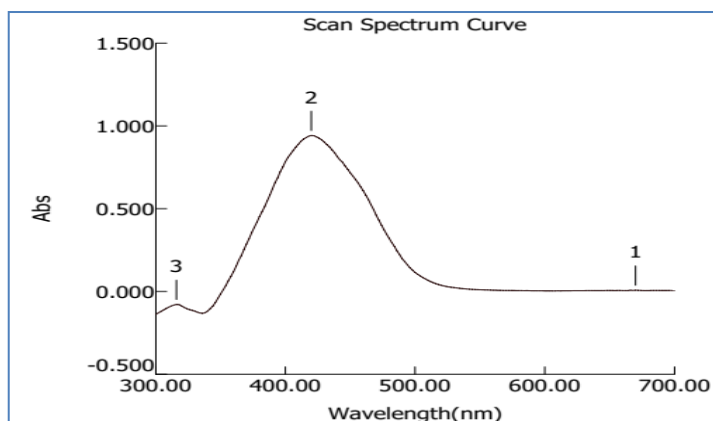


Fig. 12: Spectrum at 150% accuracy level.

3.10.4 Precision: Precision was determined as system precision and method precision, and the % RSD results obtained were within acceptance criteria which indicated that the method was highly precise. The system precision was established by measuring the absorbances at 420 nm for five replicates of standard solutions at optimized spectroscopic conditions (**Table 4**).

Table 4: System precision data (n =3)

S. No.	Concentration (µg/mL)	Absorbance
1	20	0.839
2	20	0.840
3	20	0.839
4	20	0.836
5	20	0.835
Mean	-	0.837
SD	-	0.0021
%RSD	-	0.25

n= number of determinations; SD = Standard deviation; % RSD = % Relative standard deviation

The system precision was established by measuring the absorbance at 420 nm for five replicates of sample solutions at optimized spectroscopic conditions (**Table 5**). The % assay (98-102%) and % RSD (>2%) results indicated the method falls under acceptable limits. Hence the method is called to pass repeatability.

Table 5: Method precision data (n = 3)

S. No.	Concentration (µg/mL)	Absorbance	Assay (%)
		420 nm	DOX-MO complex
1	20	0.842	99.8
2	20	0.838	99.1
3	20	0.844	99.5
4	20	0.837	99.6
5	20	0.838	99.5

Mean	-	0.839	-
SD	-	0.0031	-
% RSD	-	0.369	-

n= number of determinations; SD = Standard deviation; % RSD = % Relative standard deviation

3.10.5 Robustness: In varying buffer content, the % RSD was found as 0.878 for DOX succinate (**Table 6**). A slight variation in dye content does not show any drastic changes in absorbance. The % RSD was found as 1.150 (**Table 7**), which indicated the method was robust by marginal variation in buffer content and dye content.

Table 6: Robustness data change in buffer content

S. No	DOX succinate was taken ($\mu\text{g/mL}$)	Change in buffer content	The absorbance of DOX-MO complex	% RSD
1	20	Low (1.5 mL)	0.846	0.878
2	20	Original (2 mL)	0.876	
3	20	High (2.5 mL)	0.912	

Table 7: Robustness data change in dye content

S. No	DOX succinate was taken ($\mu\text{g/mL}$)	Change in dye content	The absorbance of DOX-MO complex	% RSD
1	20	Low (0.5 mL)	0.858	1.150
2	20	Original (1 mL)	0.876	
3	20	High (1.5 mL)	0.882	

3.10.6 Ruggedness: In the ruggedness studies, the sample solution (20 $\mu\text{g/mL}$) was analyzed in triplicate, and their relative absorbance was measured at variable conditions. The % RSD of the results found for the different analysts, for different laboratories, and for different instruments as 1.32 %, 1.65 %, and 1.59%, respectively which indicates the method follows ruggedness.

3.10.7 LOD and LOQ: The LOD for the DOX-MO complex was found as 0.31 $\mu\text{g/mL}$ whereas the LOQ was found at 0.939 $\mu\text{g/mL}$ (**Fig. 13-14**) of the analyte. The method gave a good sensitivity for the drug-dye complex.

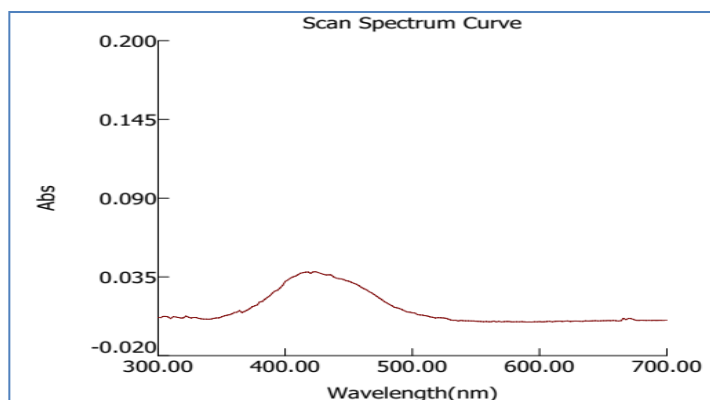


Fig. 13: LOD spectrum for DOX-MO complex.

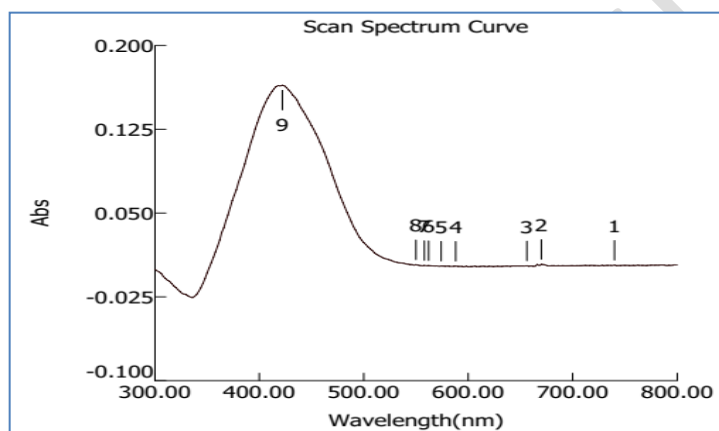


Fig. 14: LOQ spectrum for DOX-MO complex.

4 CONCLUSIONS

An ion-pair method was developed to estimate DOX succinate in its tablet dosage form and human plasma using UV-visible spectrophotometry. Acetate buffer (1 M; pH: 5.00) was used as a solvent system at temperature conditions $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$. MO (0.05 % w/v) was used as a complex-forming agent. An absorbance maximum for the DOX-MO complex was measured at 420 nm. Furthermore, the method was validated for specificity, linearity, accuracy, precision, ruggedness, robustness, LOD, and LOQ according to ICH (Q₂R₁) guidelines. The proposed method was simple, specific, and requires a short analysis time, and it could be easy and economical to perform. Hence it was concluded that the developed method was well suitability for routine analysis of DOX succinate in its dosage forms and biological samples.

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COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly used products in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

AUTHORS' CONTRIBUTIONS

'Dr. Narender M' designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. 'Sowjanya R and 'Lakshmi Prasanna P' managed the analyses of the study. 'Narayana Rao A and Ankarao A' managed the literature search. All authors read and approved the final manuscript."

Ethical Approval:

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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