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STATISTICAL MODEL SELECTION FOR DETERMINING THE STANDARD CURVE OF DOXORUBICIN BY SPECTROFLUOROPHOTOMETRIC METHOD

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ABSTRACT

Objective: The present article was undertaken to develop a simple, precise, accurate and reproducible spectroscopic method for assay development of standard curve of doxorubicin in micellar dosage form by considering statistical model selection.

Method: The developed method was based on the comparison of DOX free base and its micellar-encapsulated formulation with Kolliphor RH40 surfactant through UV-visible spectrophotometry and spectrofluorophotometric method. For assay development of appropriate standard curve, model selection and weight selection method (acceptable to the USFDA) were considered.

Results: UV-visible spectrophotometric analysis of DOX and its formulation shows that, the λ_{max} values do not show consistency in any case whether prepared by using same or different solvent system. While on the other hand, by spectrofluorophotometric analysis, the estimated excitation

wavelength (470nm) and emission wavelength (591nm) of doxorubicin free base and its formulation remains consistent along with change in solvent system.

Conclusion: So, it is not wrong to say that UV-visible spectrophotometry was not considered a better technique for DOX analysis as compared with spectrofluorophotometry. For assay development of doxorubicin standard plot, various statistical models were considered and out of them model 1 (linear model) was considered appropriate on the basis of fitting coefficient values, p values, and R square values. The different experimental parameters affecting the development and stability were studied carefully and optimized.

Keywords: Doxorubicin analysis, spectrofluorophotometry, assay, statistical models, Detection and Quantitation limits

1. INTRODUCTION

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most popular anticancer drugs. DOX is used for the treatment of human cancers including a variety of soft and solid tissue cancers. These include breast cancer, acute lymphoblastic leukemia, acute myeloblastic leukemia, hodgkin lymphoma, neuroblastoma, soft tissue sarcoma, bone sarcoma, ovarian carcinoma, gastric carcinoma and thyroid carcinoma etc. DOX interacts with both healthy and cancerous cells causing undesirable side effects. Any technique that could increase the activity of DOX by maximizing its effect by concentrating it at the cancerous tissue and minimizing its damage to healthy sites is highly desirable. Nanoparticles decorated with cancer specific ligands have been the center of choice of many researchers. Doxil® an approved formulation by USFDA is also a nanoparticle (liposomal) formulation having much reduced side effects, i.e., no vomiting and hair falls, etc. In order to further reduce the size, micelles have been used for delivery of DOX but still none of its formulation is approved and many researches are trying to develop micellar formulation of DOX.

DOX is available in market in injection form; liposomal form Doxil®, Caelyx® and Myocet® **[1]**, and plain injection Adriamycin®, Rubex® having DOX as DOX hydrochloride (DOX.HCl) salt. DOX.HCl is water soluble and can be enclosed in core of liposomes or niosomes, but its availability in micelles is limited because of hydrophobic environment in the micellar core. Therefore, for the purpose of enhancing it's loading in micelles and for the improvement of its efficacy it is first converted into DOX free

base **[2, 3]** and then enclosed in small vesicles like micelles. As DOX free base is water insoluble, it is impossible to analyze it in aqueous solvents or buffers. By preparing its various concentrations in DMSO (Dimethyl sulfoxide) or any other suitable solvent, because in these solvents encapsulated drug get solubilized, it can be analyzed **[3]**.

A number of methods are available for DOX.HCl analysis in various formulations. These include, electrophoresis **[4, 5]**, HPLC **[6]**, voltammetry **[7, 8]**, spectrofluorometry **[9]** and UV-visible spectrophotometry **[10, 11]**. Several of these methods require the use of hazardous and expensive chemicals, which make the process not only a challenge for the environment but also complicated, time consuming and costly.

The aim of the present study is to investigate an economical, simple, sensitive and accurate spectrophotometric method for the estimation of DOX free base in micellar dosage forms. These methods could overcome nearly all problems along with achieving an extended range of calibration and lower detection limits. The validity of the method has also been checked by comparison of its results with those obtained by some other method reported earlier. Therefore, the present study has been undertaken to develop and validate a simple, sensitive, accurate, precise, and reproducible spectrophotometric method for the estimation of DOX free base.

2. MATERIAL AND METHODS

2.1. Materials

Doxorubicin hydrochloride (DOX.HCl) (Assay= 99.9%) was obtained from Beijing Mesochem Technology Co Ltd., China; Kolliphor RH- 40 (KRH-40) from Sigma-Aldrich, Germany; Cholesterol (CHOL) from Titan Biotech Ltd., India; Dimethyl sulphoxide (DMSO) from Central Drug House (P) Ltd., India; Triethylamine (TEA) from Lobal Chemie, India; Acetic acid glacial from RFCL Limited, India; Sodium acetate from Titan Biotech Ltd., India; Hydrochloric acid (HCl) from Linco Scientific Instruments and Chemicals Pvt. Ltd., India; and RO water from in-house lab were used for preparation of all solutions and dilutions. All other chemicals and reagents used were of analytical grade.

2.2. Preparation of DOX free base

For drug loading, the DOX.HCl was first converted to DOX free base by treating it with 5/3 molar ratio of TEA/DMSO along with orbital shaking. TEA being basic compound converts DOX.HCl to DOX free base **[3]**. After overnight shaking, the DMSO layer containing DOX free base was removed by micropipette and preserved in amber colored stoppered container. The DOX free base was

assayed for DOX content using standard DOX free base.

2.3. Preparation of DOX formulation

As in case of hydrophilic surfactants the inner core of the micelles is hydrophobic, therefore, the hydrochloride salt of DOX was first converted to DOX free base. The DMSO layer (3ml) containing DOX free base (as described in above section 2.2) was mixed with 3ml DMSO solution containing Kolliphor RH40 (96mg) and cholesterol (32mg). The above mixture was transferred to a pre-swollen dialysis membrane (MW 12000-14000 Da; Make: Himedia, India) and the dialysis was carried out for 24 hours against 900 ml water. The water is exchanged every 3 hours for total 10 cycle changes. After dialysis, the micelle solution was filtered through 0.45 µm membrane filter, and DOX formulation is preserved for further experiments.

2.4. Preparation of buffer

Initially, the analysis was planned in aqueous acetate buffer (pH 5.0), but DMSO being a universal solvent, analysis was also designed in this solvent. The acetate buffer (pH 5.0) was prepared by adding 5.77g of sodium acetate in 800ml of RO water followed by addition of 1.77g acetic acid. The pH was adjusted to 5.0 using hydrochloric acid and the final volume was adjusted to 1000 ml. If required, the buffer was also preserved in stoppered container.

2.5 Preliminary selection of analytical method

The preliminary selection among the UV-visible or spectrofluorometric methods was done on the basis of simple considerations. First, the method showing stable wavelength maxima (λ_{max}) values at various concentrations can be considered suitable. Second, the method giving the lower detection and quantitation limit is best.

2.5.1 UV-visible scanning: The first step in UV-visible analysis was scanning of DOX solutions and formulation for the purpose of selecting wavelength maxima (λ_{max}). Various concentrations of DOX free base (ca. 0.5 - 62.5 ppm) and DOX formulation (ca. 1.25% v/v (eq. to 0.237 ppm) - 6.25% v/v (eq. to 1.243 ppm); Assay = 19.883 ppm (as found in inhouse laboratory)) were prepared separately in DMSO and acetate buffer (pH 5.0). The UV- visible scans were taken in the wavelength range of 300 – 700 nm using UV-visible spectrophotometer (Make: LabIndia 3000+, LabIndia, India). For the purpose of analysis, λ_{max} selectivity of DOX free base as well as DOX formulation is important, therefore, scans were looked for λ_{max} values.

2.5.2 Spectrofluorescence scanning: For the spectrofluorophotometric analysis, the emission spectra of the DOX free base and DOX formulation were obtained with a spectrofluorophotometer (Model: RF- 5301 PC, Shimadzu, Japan). The preliminary selection of excitation wavelength (λ_{max}^{ex}) was done on the basis of absorption peaks in UV-visible scans of three conc. (ca. 0.5 ppm, 6 ppm and 50 ppm) of DOX free base and DOX formulation. Finally, the emission spectra were taken at selected λ_{max}^{ex} and analyzed for best emission wavelengths (λ_{max}^{em}) for both DOX free base and DOX formulation.

2.5.3 Detection and Quantitation limits (LOD and LOQ): LOD and LOQ are the terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure. Sufficient analyte concentration must be present to produce an analytical signal that can reliably be distinguished from analytical noise. Appropriate amount of DOX free base was dissolved in DMSO to prepare working standard solutions of 0.5- 8ppm. Then the standard solutions were quantified by UV-visible spectrophotometry and spectrofluorophotometry. From mentioned two methods one is selected on the basis of LOD and LOQ.

2.6. Standard Curve

The standard curve of DOX free base is the main part of any assay development. After preliminary screening for analytical method, the DOX free base concentrations (ca. 0.5– 8 ppm) were subjected to absorbance and emission intensity studies. The method is repeated in triplicate for getting the average absorbance or intensity valves. The standard curve generation is further followed by model selection and weight selection procedures for development of appropriate standard curve and removing any heterogeneity in the variance of the responses, i.e., absorbance or emission intensities, at various concentrations. A brief description about model selection and weight selection procedure can be followed as:

2.7.1. ***Model selection***

For the estimation of unknown concentration by standard curve, the FDA has approved five commonly used statistical models in assay development **[12]**. These are;

Model 1: $Y_i = \alpha + \beta_1 X_i$ (follow simple linear regression model)

…(i)

Model 2: $Y_i = \beta_1 X_i$ (standard curve passes through the origin)

…(ii)

Model 3: $Y_i = \alpha + \beta_1 X_i + \beta_2 X_i$ (a quadratic relationship between Xi and Yi) …(iii)

Model 4: $Y_i = \alpha X_i^{\beta_1}$ (this model is equivalent to simple linear regression model after a logarithmic transformation)

$$log(Y_i) = log(\alpha) + \beta_1 log(X_i) \quad \ldots(iv)$$

Model 5: $Y_i = \alpha e^{\beta_1 Xi}$ (this model can also be linearized by taking a log transformation)

$$log(Y_i) = log(\alpha) + \beta_1 X_i \quad \ldots(v)$$

The selection of appropriate statistical model starts from polynomial equation, $Y_i = \alpha + \beta_1 X_i + \beta_2 X_i^2 + \beta_3 X_i^3 + \beta_4 X_i^4$. Depending upon the values of fitting coefficients the polynomial equation may get reduce to any of these models among eq. (i) to eq. (v). For example, if $\beta_4 = \beta_3 = \beta_2 = 0$ then polynomial equation reduces to model 1 and if α also reduces to zero, the polynomial equation becomes model 2. In case, if only β_3 and β_4 are equal to zero, then the finalized model is 3. Selection among model 4 or 5 is done only when none of the fitting coefficients is equal to zero, i.e., $\beta_1 \neq \beta_2 \neq \beta_3 \neq 0$. In such a case, final decision is made on the basis of residual sum of square (RSS) values for model 4 and model 5. If RSS of model 4 (RSS_4) is found smaller than the RSS of model 5 (RSS_5), the model 4 is selected, otherwise model 5 is applicable.

2.7.2. *Weight selection*

As heterogeneity of the response variability (calculated as standard deviation; SD) may occur in relation with concentration, so it is required to make it homogeneous. Weight method can be used to reduce such heterogeneity. For the selection of appropriate weight, another equation relating SD vs concentration is used as; $SD(Y_i) = \beta o + \beta_1 \sqrt{X_i} + \beta_2 X_i$. Where $SD(Y_i)$ is the standard deviation of the response "Y_i" at standard concentrations "X_i". For selection of weights again the criteria depends upon fitting coefficient values. The weight = 1, if $\beta_1 = \beta_2 = 0$ and in such a case, the $SD(Y_i)$ becomes independent of concentration automatically. If $\beta_1 \neq \beta_2 \neq 0$, then selection of weight is done on the basis of extra sum of square (SS) values. For example, if SS ($b_2|b_0,b_1$) > SS ($b_1|b_0,b_2$) then weights = $1/X^2$ is chosen, and if SS ($b_2|b_0,b_1$) < SS ($b_1|b_0, b_2$) then weights = $1/X$ is selected for data. Where SS ($b_2|b_0,b_1$) denotes extra sum of squares due to inclusion of term $\beta_2 Xi$ while excluding $\beta_1 \sqrt{Xi}$ from the $SD(Y_i)$ equation, and SS ($b_1|b_0,b_2$) represents extra sum of squares due to inclusion of $\beta_1 \sqrt{Xi}$ while excluding $\beta_2 Xi$ from the $SD(Y_i)$ equation. The smaller the extra SS the better is the equation but it should also satisfy significant p- level.

3. RESULTS

3.1. Preliminary selection of analytical method

3.1.1 UV -visible scanning: The UV-visible scans of DOX free base and DOX formulation in DMSO and acetate buffer (pH 5.0) have been given in **Figure 1**. For Dox free base and DOX formulation in acetate buffer, the λ_{max} has appeared at 485 nm **(Figure 1A)** and 510 nm **(Figure 1C)**, respectively. These different λ_{max} values suggest some sort of interactions of DOX in formulation. Further, in the same scans the absorbance of DOX free base is ~14 - folds higher than the absorbance of DOX formulation for a concentration difference of ~40 – folds between DOX free base (ca. 50 ppm) and DOX formulation (ca. 6.25% v/v equivalent to 1.243 ppm DOX). The shift of λ_{max} and reduced absorbance in case of DOX formulation can be attributed to some sorts of interactions, shielding of the drug by micellar shell **[13, 14]**, and/or aggregation of DOX in the micellar cores **[15, 16]**, etc.

For DOX free base and DOX formulation in DMSO, the λ_{max} has been observed at 540 nm **(Figure 1B)** and 575nm (Figure 1D), respectively. Again the different value of λ_{max} justifies some sort of interactions, which may have caused shift in λ_{max} values. Due to these shifts the usefulness of the λ_{max} seems to be less at preliminary levels for predicting the DOX concentrations in micelle formulations. Further, the absorbance of DOX free base (ca. 8 ppm) at its peak is ~12 - folds higher than the absorbance of DOX formulation (ca. 6.25% v/v) corresponding to a concentration change of ~6 - folds. The lesser reduction in absorbance of DOX formulation vs DOX free base in DMSO as compared to that seen in acetate buffer pH (5.0) could be attributed to the dissolution of micelles in DMSO layer.

By comparing **Figure 1A and Figure1B**, it is clear that λ_{max} of the neat drug DOX free base has changed with the solvent system. The changes in chromophore of the DOX with solvent system may be the reason for such shifts in the λ_{max}. Similarly, the λ_{max} of the DOX formulation has also shown shift in λ_{max} with change in solvent system **(Figure 1C and Figure 1D)**. The changing λ_{max} values for DOX with solvent system and after encapsulation in micellar formulation suggests that the UV-visible spectrophotometric methods are not good option for DOX analysis. This implies that, the UV- spectrophotometric analysis cannot be used for DOX predictions in DOX formulations due to lack of specificity of the method.

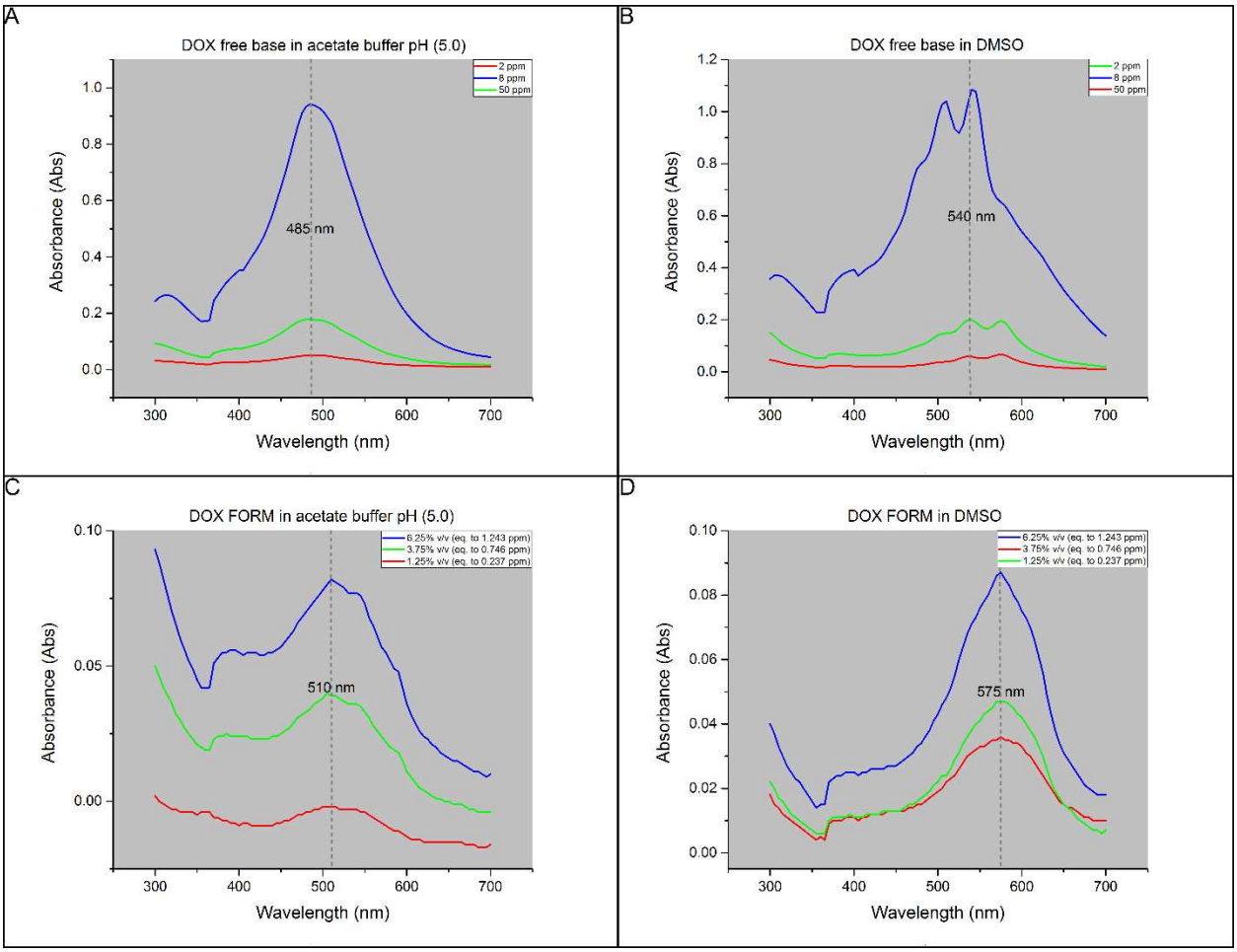

Figure 1: UV-visible scans of DOX free base and DOX formulation in acetate buffer pH (5.0) and in DMSO; a) DOX free base in acetate buffer, b) DOX free base in DMSO, c) DOX formulation in acetate buffer, d) DOX formulation in DMSO

3.1.2 Fluorescence scanning:

Looking into the UV-visible scan **(Figure 1A and 1B)** of DOX free base (ca. 50 ppm) in acetate buffer pH (5.0) and DMSO, it is evident that DOX has UV-visible spectral absorbance property and shows two absorption bands; one below 350 nm and second above 350 nm in both medium. The band below 350 nm constitutes single peak at 320 nm while second band (total bandwidth ~200 nm) consists of peaks at 380 nm, 470 nm, 510 nm, and 540 nm. Therefore, excitation wavelength (λ_{max}^{ex}) has to be chosen among these values for the spectrofluorophotometeric analysis. Out of these, the peak values of 510 nm and 540 nm are not suitable because the absorption-emission scans of DOX free base (ca. 50 ppm) are majorly overlapping around 500 - 600 nm **(Figure 2)**. This may lead to escalated emitted intensity due to reabsorption event. These results are consistent to the findings of Motlagh and coworkers **[16]** who has reported about the mixing of absorption and emission

bands causing reabsorption of emitted lights. For remaining wavelengths it is pertinent to mention here that these produce multiple emission bands. Referring to Figures 3A and 3B, the excitation at 320 nm, i.e., λ_{max}^{ex}= 320 nm, is causing three emission peaks at 350, 591, and 640 nm. At λ_{max}^{ex} = 380 nm, the emission peaks appeared at 430 and 591nm, and for λ_{max}^{ex}= 470 nm, the single predominant emission peak has been found at 591 nm. It is also clear that all these excitation wavelengths, i.e., λ_{max}^{ex}= 320 nm, 380 nm, or 470 nm have common emitted wavelength band between 525-625 nm with a common emission peak at 591 nm. These figures suggest that the emission peak at 591 nm is consistent and independent of selection of λ_{max}^{ex}. Further, it is interesting to note that the emission intensities at 591 nm (E_{591}^{em}) are maximum at λ_{max}^{ex} = 470 nm. The higher E_{591}^{em} for the λ_{max}^{ex} = 470 nm suggests that 470 nm is a suitable excitation wavelength for DOX analysis. Further, the λ_{max}^{ex}= 320 nm and 380 nm are giving relatively small E_{591nm}^{em} values **(Figure 3A)** attesting the usefulness of the 470 nm wavelength for the spectrofluorophotometric analysis of DOX.

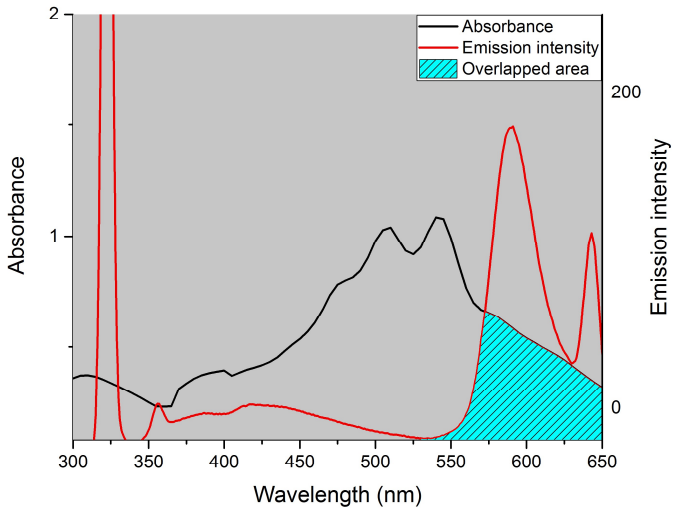

Figure 2: Absorption (black trace)-emission (red trace) spectra of DOX free base in DMSO

Since, the emission band is between 525-625 nm, the selection of λ_{max}^{ex} at 510 nm and 540 nm for DOX analysis is ruled out because these will become very close to the emission band and becoming cause for reabsorption event to happen.

Figure 4A and 4B suggests that upon excitation of DOX formulation (1.25%v/v, 3.75% v/v, and 6.25% v/v), the peak emission

maxima at 591nm does not change with change in excitation wavelength λ_{max}^{ex} from 320 nm to 380 nm or to 470 nm, and the E_{591}^{em} varies linearly with concentration. Figure 4C, Justifies the consistent peak emission wavelength at 591 nm while taking into account the changes of E_{591}^{em} with concentrations. Further, comparing DOX free base with DOX formulation in **Figures 2D and 3C** suggests that the E_{591}^{em} of DOX free base (ca. 6 ppm) is ~6 - folds higher than the intensity of DOX formulation (ca. 6.25% v/v equivalent to 1.246 ppm DOX), which corresponds to a conc. change of ~5 - folds. It is very interesting to note here that variation in intensity is almost equal to variation in concentration, which was not the case in UV-visible analysis. Therefore, in preliminary selection we suggest that spectrofluorophotometric method can be used for development of assay of DOX in micelles formulation.

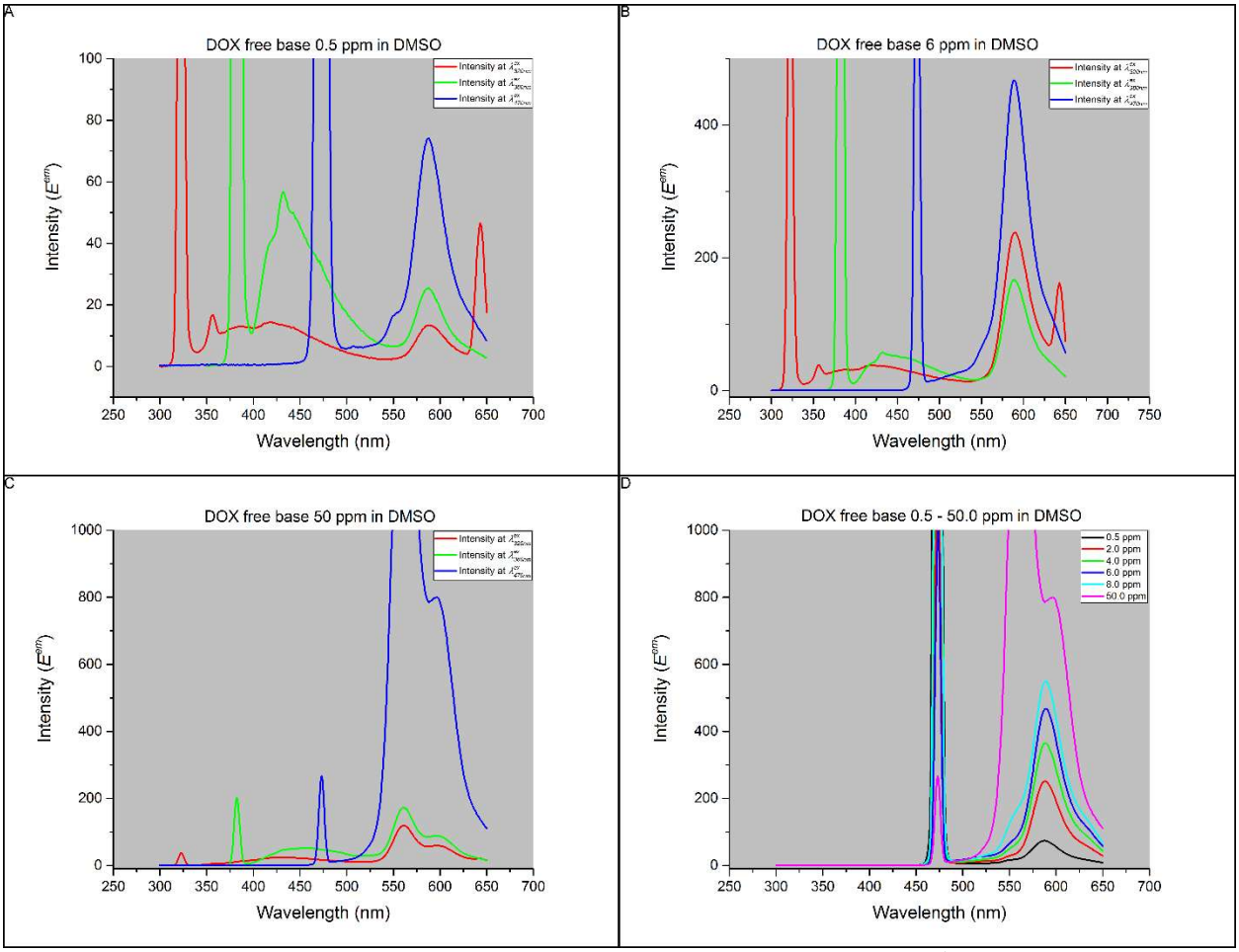

Figure 3: Fluorescence scans of DOX free base; a) DOX 0.5 ppm at E_{ex}^{320}, E_{ex}^{380}, E_{ex}^{470} and E_{em}^{591}, b) DOX 6 ppm at E_{ex}^{320}, E_{ex}^{380}, E_{ex}^{470} and E_{em}^{591}, c) DOX 50 ppm at E_{ex}^{320}, E_{ex}^{380}, E_{ex}^{470} and E_{em}^{560}, and d) DOX 0.5 - 50 ppm at E_{ex}^{320}, E_{ex}^{380}, E_{ex}^{470} and E_{em}^{591}, E_{em}^{560}

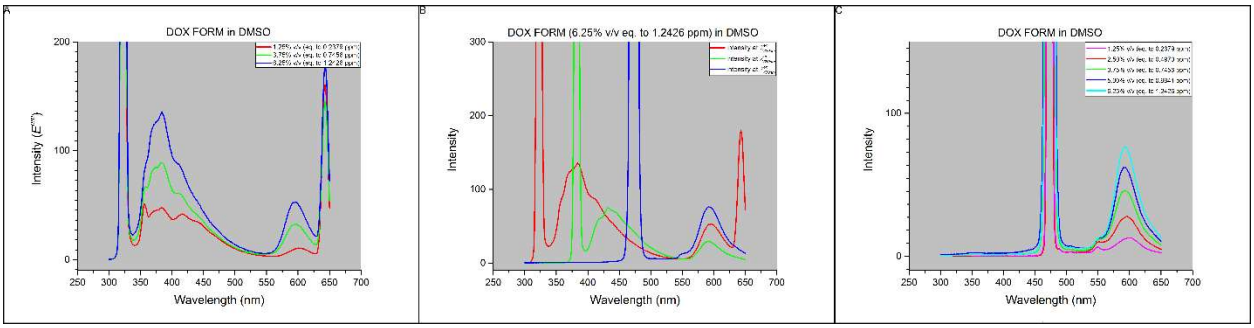

Figure 4: Fluorescence scans of DOX formulation in DMSO. a) DOX 1.25% v/v, 3.75% v/v, 6.25% % v/v at λ_{max}^{ex}= 320 nm, b) DOX 6.25% v/v at λ_{max}^{ex}= 320 nm, λ_{max}^{ex}=380 nm, λ_{max}^{ex}= 470 nm, c) DOX 1.25% v/v, 2.50% v/v, 3.75% v/v, 5.00% v/v and 6.25% v/v at λ_{max}^{ex}= 470 nm

3.1.3 Detection and Quantitation limits (LOD and LOQ): The LOD and LOQ, by UV-visible spectrophotometric method at estimated Λ_{max} (540nm) for DOX free base (0.5 ppm) in DMSO was 0.011 whereas when spectrofluorophotometry done at E_{591}^{em} , predicts the intensity of tested lowest concentration (0.5 ppm) to be 64.3967. So, according to the values obtained spectroflurophotometric method is considered best.

3.2 Standard curve

Since during preliminary screening, the spectrofluorometric method has been found suitable for DOX analysis the standard curve has been generated for this method only.

From various spectra of DOX free base (0.5, 2, 4, 6 and 8 ppm) in DMSO, the λ_{max}^{ex} at 470 nm and corresponding emission wavelength at 591 nm have led to the E_{591}^{em} values as given in **Table 1**. The concentration range of 0.5 – 8 ppm is applicable at all λ_{max}^{ex}, but the λ_{max}^{ex} = 470 nm has been chosen because the E_{591}^{em} has been found to attain maximum values at this excitation wavelength only, irrespective of the solvent system. The method has been repeated five times and the RSD has also been given in **Table 1**. The calibration data of the DOX free base for various concentration was further subjected to model selection and weight selection scheme acceptable with USFDA as given below:

3.3.1 ***Model selection:*** As summarized in section 2.7.1, the **Table 2** shows the results from the model selection scheme for the data set in **Table 1**. Starting from a polynomial relationship between E_{591}^{em} with concentrations, the values of fitting coefficients β_4, β_3 and β_2 are only equal to zero, while α and β_1 are not equal to zero. This suggests that polynomial relationship can reduce to the model 1, which can be further used for generating standard plot. The p-values of the fitting coefficients have been given in **Table 3**, which confirms the above findings correct on the basis of p- and R^2

values. The p- value is significant only for linear model, therefore, linear model is confirmed for the final standard curve.

3.3.2 ***Weight selection:*** As with increase in concentration the heterogeneity in response also maximizes. Therefore, weighted method has been applied but result shows that the p- value for the null hypothesis ($Ho: \beta 1 = \beta 2 = 0$) is more than level of significance. This implies that in this case, the response is not correlated with the conc. Therefore, weight is not required to stabilize the heterogeneity of the response.

Table 1: DOX free base

Conc. (ppm)	E_{591}^{em}	S.D.	% C.V.
0.5	64.3967	2.8332	4.3996
2	222.4515	12.7578	5.7351
4	336.1795	11.6027	3.4513
6	417.8267	24.4855	5.8602
8	490.2158	28.1570	5.7438

Note: *The intensity values are average of n= 3 values

Table 2: Coefficient statistics of various models:

Model	Fitting Coefficients (p- valves)				
	Intercept (α)	β_1	β_2	β_3	β_4
Quartic	-13.5192 (—)	171.9898 (—)	-34.2663 (—)	3.9827 (—)	-0.1754 (—)
Cubic	-1.3923 (0.9191)	143.0017 (0.0558)	-18.5376 (0.1202)	1.0444 (0.1630)	—
Quadratic	27.3579 (0.3399)	98.2833 (0.0165)	-5.1603 (0.0720)	—	—
Linear	82.7388 (0.09181)	54.5060 (0.0042)	—	—	—

Note: The values in parenthesis are corresponding p- values
***In none of the case, the standard error (SE) was greater than the corresponding coefficient's value, except α in case of cubic spline model where it was SE=10.9020.**

Table 3: Model fit summary

Model	p- value	Adjusted R square	Predicted R Square
Linear	0.0042	0.9390	0.8186
Quadratic	0.0720	0.9873	0.9075
Cubic	0.1630	0.9984	0.9475
Quartic	—	—	—

Note: *p- values less than 0.0500 indicate model terms are significant.
***Sequential p- value represent significance of highest polynomial term in equation on the basis of regression sum of squares**
***Difference between predicted R^2 and adjusted R^2 should be less than 0.2.**

Table 4: Coefficient statistic of weighing model:

Fitting coefficients	Coefficient values	p- values
Intercept (α)	0.4662	0.9714
β_1	2.4889	0.6047
β_2	2.8173	0.8650

Note: *p- values less than 0.0500 indicate coefficient terms are significant.

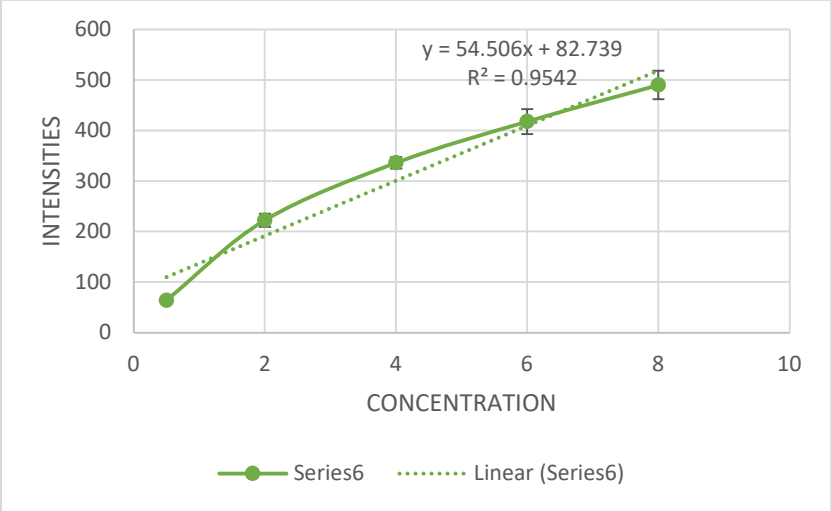

Figure 5: Standard curve of DOX free base (EX 470nm; EM 591nm)
Fluorescence intensity was measured at the E^{ex}_{max}= 470 nm and E^{em}_{max} = 591 nm

4. DISCUSSION

The UV absorption spectra determine the transition bands. **Figures 1A, 1B, 1C and 1D** demonstrate the spectral bands of DOX free base and formulation in DMSO and acetate buffer characterizing a couple of peaks at 485, 510, 540 and 575nm, respectively.

During our study on spectrofluorophotometer (Model: RF- 5301 PC, Shimadzu, Japan), we have characterized the fluorescence properties of doxorubicin in a hope to develop fluorescence- based assay for detecting doxorubicin as a function of the fluorophore. As shown in **Figures 3 and 4**, dox free base and dox formulation exhibits characteristic fluorescence spectra of emission with λ^{ex}_{max} and λ^{em}_{max} being at 470nm and 591nm, respectively. Notably, the λ^{ex}_{max} is similar to that of ROS- Fluorescence- based assay **[17]**. The emission spectra usually experiences a sharp rise and them follows a smooth decay surpassing concentration peak C_p. C_p is defined as a concentration at which maximum intensity signal occurs. The fluorescence signal arises from couple of molecular transitions and the decrease of amplitude is due to static and dynamic quenching including self- quenching, collisional effects and aggregation of monomers to dimers. It can be expressed by Stern- Volmer equation:

$$Fo/F = 1 + K[\,Q\,]$$

where Fo(F), K and [Q] refers to fluorescence intensity in the absence / presence of quencher, quenching constant and quencher density respectively. With the above E^{470}_{ex} and E^{591}_{em}, the amount of dox free base and dox formulation could be sensitively measured.

As **Figure 2** display the absorption- emission spectra and the corresponding overlapping

area of DOX free base in DMSO. Here, the emissive wavelength (591nm) shows a rise in curve due to the reabsorption events and it follows a plateau at higher fluorophore concentrations.

The best fitting of standard curve is performed on the scattered experimental data on the basis of USFDA approved statistical methods. Chow (1989) and Ju and Chow (1994a) the criterion for selecting an appropriate statistical model. As model 1 to 4 are polynomial and model 5 can be approximated to polynomial. By using these methods various coefficients like α, β, p and R square values as shown in **Table 2, 3 and 4** are obtained. By considering them, assay development of standard curve is done. And further we have obtained linear relationship between the concentration and fluorescence intensity, from the best fitted linear curve regression coefficient was found to be 0.9542 as shown in **Figure 5**. The relative standard deviation values were low that indicates the reproducibility of the method.

4. CONCLUSION

A spectrofluorophotometric method for quantifying DOX in formulation samples has been developed and validated. The proposed method for DOX determination has lots of advantages over other analytical methods like UV- visible spectrophotometric. It was concluded that the assay is selective, precise, accurate and linear over the conc. range studied. In summary, the proposed spectrofluorophotometric method can be used for DOX analysis in routine quality control compared with UV- visible spectrophotometric method.

Abbrevations

DOX: Doxorubicin

DOX.HCl: DOX hydrochloride

DMSO: Dimethyl sulfoxide

KRH-40: Kolliphor RH- 40

TEA: Triethylamine

λ_{max}: Wavelength maxima

λ_{max}^{ex}: Excitation wavelength

λ_{max}^{em}: Emission wavelengths

LOD and LOQ: Detection and Quantitation limits

SD: Standard Deviation

Cp: Concentration peak

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