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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF DOXOFYLLINE

Shraddha Jayant Bhangre[1]*, Suhas S. Siddheshwar[2] and Prof. Mahesh Kolhe[2]

[1]Department of Pharmaceutical Quality Assurance, Pravara Rural College of Pharmacy, Pravaranagar, Loni Bk., Tal. - Rahata, Dist. - Ahmednagar (M.S.).

[2]Department of Pharmacology, Pravara Rural College of Pharmacy, Pravaranagar, Loni Bk., Tal. - Rahata, Dist. - Ahmednagar (M.S.).

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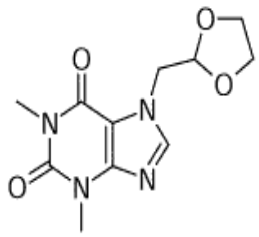
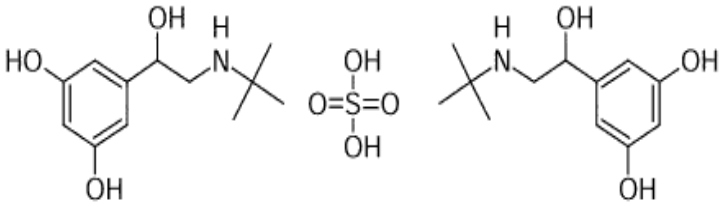
***Corresponding Author**
Shraddha Jayant Bhangre
Department of Pharmaceutical Quality Assurance, Pravara Rural College of Pharmacy, Pravaranagar, Loni Bk., Tal. - Rahata, Dist. - Ahmednagar (M.S.).

ABSTRACT

Doxofylline is an effective bronchodilator for relieving airway obstruction in patients with asthma or chronic obstructive pulmonary disease (COPD), and displays a better safety profle with respect to theophylline. Here, we performed a pairwise analysis of the currently available data to provide consistent and homogeneous fndings on the impact of this xanthine in COPD patients. Results obtained from 820 patients were selected from 20 clinical trials. Meta-regression was performed to examine the source of heterogeneity between-studies and identify potential confounder covariates. The quality of the evidence was assessed by the GRADE system. Doxofylline induced a signifcant ($P < 0.001$) increase in forced expiratory volume in 1s (FEV1) of 8.20% (95%CI 4.00–12.41; I2 93%) and 317ml (95%CI 19–439; I2 87%) compared with baseline. The total administered dose of doxofylline signifcantly ($P < 0.001$) interacted with the size of the effect estimates detected for FEV1. Doxofylline induced a signifcant ($P < 0.001$), although moderate, increase in adverse events (AEs) frequency (proportion 0.03, 95%CI 0.02–0.04; I2 88%), but only epigastralgia, nausea, dyspepsia and headache were statistically signifcant ($P < 0.05$). The GRADE analysis indicated high quality of evidence (++++) for the impact of doxofylline on FEV1, and moderate quality of evidence (+++) for the safety profle in COPD patients. Doxofylline is an effective and safe medicine when administered to patients with COPD and can be considered as an alternative to theophylline.



Doxofylline
7-(1,3-dioxolan-2-ylmethyl)-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione



Terbutaline Sulphate
5-[2-(*tert*-butylamino)-1-hydroxyethyl]-benzene-1,3-diol sulfate (2:1) (salt)

KEYWORDS: Doxofylline Chronic obstructive pulmonary disease Lung function Safety Pairwise.

INTRODUCTION

Doxofylline, [7-(1, 3-dioxolan-2-ylmethyl)-3, 7-dihydro-1, 3-dimethyl-1H-purine-2, 6-dione] is a new bronchodilator xanthine based drug which differs from theophylline by the presence of dioxalane group at position.[7] It is used in the treatment of bronchial asthma, chronic obstructive pulmonary disease (COPD), and chronic bronchitis.[49-51] The mechanism of action is similar to that of theophylline in that it inhibits phosphodiesterase (PDE-IV), thereby preventing breakdown of cyclic adenosine monophosphate (cAMP). Increase in cAMP inhibits activation of inflammatory cells resulting in bronchodilating effect.[52] In contrast to theophylline, doxofylline has very low affinity towards adenosine A1 and A2 receptors which explain its better safety profile.[53,54]

Doxofylline is a xanthine that is structurally di?erent from theophylline by having a dioxalane group at position 7 of the xanthine ring.[1] Consequently, it has mechanisms of action distinct from those of theophylline.[2–4] in that lacks adenosine receptor antagonism or the ability to inhibit any of the known PDE isoforms, which may contribute to the better safety role. Furthermore, unlike theophylline, doxofylline does not interact with histone deacetylases,[3] but is able to positively interact with f2-adrenoceptors.[5] The narrative analysis of literature has suggested that doxofylline is an effective bronchodilator for relieving airway obstruction in patients with asthma or chronic obstructive pulmonary disease (COPD) and displays a better safety profile with respect to theophylline, having a favourable risk-to-benefit ratio.[2,4,6] Unfortunately, narrative reviews primarily focused on the conclusions reached in various studies and, furthermore, mainly related with the researchers' personal preference.

Abbreviations

AE, adverse events; CI, confidence interval; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in 1s; GRADE, Grading of Recommendations Assessment; Development, and Evaluation; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; MCID, minimal clinically important difference; MD, Mean Difference; MRC, Medical Research Council; PLN, Logarithmic transformed Proportion; Pr, proportion; RCTs, randomized clinical trials; SE, standard error; SMD, Standardized Mean Difference.

Literature review of analytical methods

Although various bioanalytical methods for estimation of doxofylline in human serum.[55–58] stability indicating HPTLC method,[59] HPLC[60,61] and HPLC method for estimation of doxofylline in dosage form[62] are reported in the literature, there have been no reports of characterization of the degradation products. Therefore, in this chapter the stability properties of the drug doxofylline under different stress conditions were studied. Structure of degradation products formed under hydrolytic conditions was confirmed by IR, Mass and NMR spectra and mechanistic degradation pathways were proposed.

Experimental work

Prepration of parasample

Preparation of 0.1 N sodium hydroxide

0.4 g of sodium hydroxide (NaOH) was dissolved in water and made up to 100 mL to get 0.1 N sodium hydroxide solution.

Preparation of 0.1 N hydrochloric acid

0.85 mL of concentrated hydrochloric acid (HCl) was diluted with water and volume adjusted to 100 mL to get 0.1 N hydrochloric acid solution.

Preparation of 3% hydrogen peroxide

To 10 mL of hydrogen peroxide solution (30% w/v H2O2) water was added and volume adjusted to 100 mL to get 3% hydrogen peroxide solution.

Preparation of 0.01 M potassium dihydrogen orthophosphate buffer (pH 3)

1.36 g of KH2PO4 was dissolved in 1000 mL of water and pH was adjusted to 3.0 with ortho-phosphoric acid.

HPLC analysis

All the stressed samples were diluted with mobile phase to make concentration of 100?g mL-1 and injected on HPLC. Phenomenex Gemini C18 (250mm×4.6mm, 10?m particle size) column was used for analysis. Mobile phase containing 20 volumes of acetonitrile and 80 volumes of potassium dihydrogen orthophosphate(0.01M) previously adjusted to pH 3 with ortho-phosphoric acid was used. Analysis was carried out at 274nm. Flow rate of mobile phase was kept at 1mL min-1 and injection volume was 20L.

Another gradient method was developed in order to check the possibility of any late eluting degradation product. The mobile phase consist of gradient program of solvent A and B. Solvent A composed of mixture of potassium dihydrogen orthophosphate (10mM) with its pH adjusted to 3 with orthophosphoric acid and acetonitrile in ratio of 95:5 (v/v) . Acetonitrile and water in the ratio of 95:5 (v/v) was used as solvent B. The flow rate of the mobile phase was 1.0 ml/min with a linear gradient program of 0/20, 5/20, 7/30, 9/50, 16/75, 20/65, 24/55, 26/40, and 30/20 (time (min)/%B). Analysis was carried out at 274nm.

Validation of analytical method

1. Linearity and range

A stock solution of the drug was prepared at strength of 1mg/mL. It was diluted to solutions of concentration in the range of 5–30mg/mL of the drug. The solutions were injected in triplicate into the HPLC column, keeping the injection volume constant (20L).

2. Specificity

Specificity of the HPLC method was determined by spiking the degradation products at 0.15% level and determining the resolution. Peak purity of drug and its degradation product was determined by PDA detector to rule out overlap of peaks.

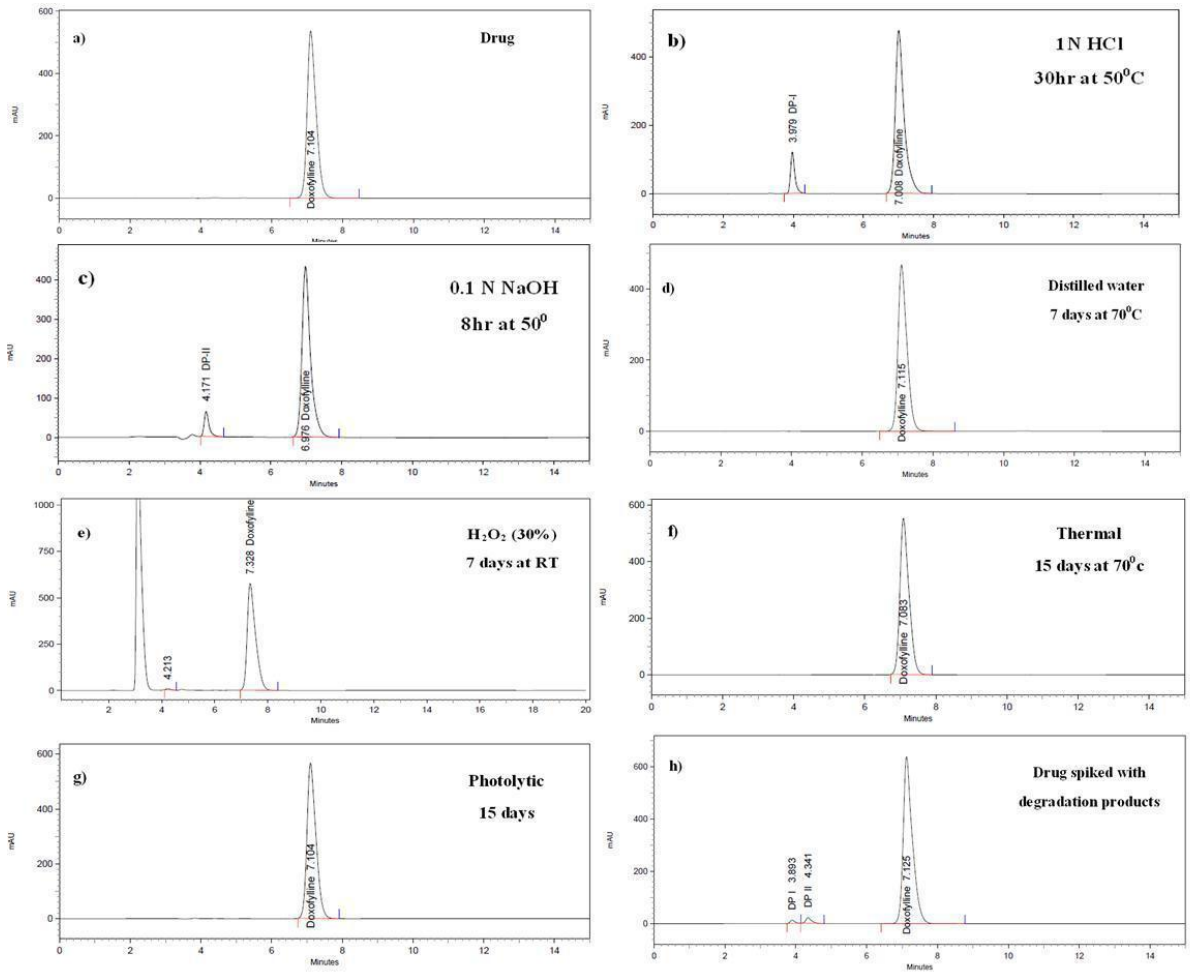
3. Precision

Six injections of three different concentrations each (10, 20 and 30) of bulk drug were injected and analyzed on the same day and the values of relative standard deviation (R.S.D.) were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision.

4. Accuracy

Accuracy was determined by spiking a mixture of stressed samples with three known

concentrations of the drug i.e., 10, 20 and 30g mL-1 in triplicate and % recoveries of the added drug were calculated.



Chromatograms of a) Doxofylline and its degradation under b) Acidic, c) Basic, d) Neutral e) Oxidative, f) Thermal, g) Photolytic and h) Drug spiked with degradation products using isocratic method.

DISCUSSION

Stress degradation study

The chromatogram of pure doxofylline shows retention time of 7 min is as shown in Fig 4.1a. Two degradation product peaks were observed, one peak DP-I in acid (10.92%) and another peak DP-II in basic conditions (7.62%) respectively (Fig. 4.1b, c). No degradation products were generated on heating the drug in water for 7 days at 700 C (Fig.4.1 d), indicating it to be stable under neutral condition. Negligible degradation occurred in oxidative stress (Fig. 4.1e) and no degradation observed in thermal (Fig. 4.1f) and photolytic condition (Fig. 4.1g). Stress study carried out on formulation under identical conditions showed no peak due to drug-

excipient interaction indicating there is no incompatibility between drug and excipients.

Summary

The present study revealed very important and useful information which has not yet reported in the literature of doxofylline. The result of the present work can be summarized as follows: The drug was found to be susceptible to base hydrolysis followed by acid hydrolysis and was stable in other stress conditions.

Methods Validation: Establishing documented evidence that provides a high degree of assurance that a specific method and the ancillary instruments included in the method will consistently yield results that accurately reflect the quality characteristics of the product tested.

Method development and validatiom

Method validation is an important requirement for any package of information submitted to international regulatory agencies in support of new product marketing or clinical trials applications.

Analytical methods should be validated, including methods published in the relevant pharmacopoeia or other recognized standard references. The suitability of all test methods used should always be verified under the actual conditions of use and should be well documented.

Methods should be validated to include consideration of characteristics included in the International Conference on Harmonization (ICH) guidlines1, 2 addressing the validation of analytical methods. Analytical methods outside the scope of the ICH guidance should always be validated.

ICH is concerned with harmonization of technical requirements for the registration of products among the three major geographical markets of the European Community (EC), Japan, and the United States (U.S.) of America. The recent U.S. Food and Drug Administration (FDA) methods validation guidance document, 3-5 as well as the United States Pharmacopoeia (USP), 6 both refer to ICH guidelines.

The most widely applied typical validation characteristics for various types of tests are accuracy, precision (repeatability and intermediate precision), specificity, detection limit,

quantitation limit, linearity, range, and robustness,

In addition, methods validation information should also include stability of analytical solutions and system suitability.

The first step in method validation is to prepare a proto-number of replicates may be reduced or increased based on protocol, preferably written, with the instructions in a clear scientifically sound judgmentstep-by-step formay, and approved prior to their initiation. This A test method is considered validated when it meets the approach is discussed in this paper. The suggested acceptance criteria of a validation protocol may be modified depending on method used, step-by-step practical guide for preparing protocols.

Methods validation by uv sprectoscopy

MATERIALS AND METHODS

Instruments

Absorbance measurements were made on double beam UV-Visible spectrophotometer, model 1800, Shimadzu, Japan with software UV Probe 2.10 and 1 cm matched quartz cells.

Chemicals

Gift samples of salbutamol sulphate and doxophylline were provided by Key Pharmaceuticals Limited, Ambala, Haryana, India. The pharmaceutical dosage form used in the study was Doxoril plus 4 (Macleods Pharmaceuticals Pvt Ltd). Each uncoated tablet contains 4 mg SBS and 400 mg DOX. All chemicals were of analytical reagent grade and solutions were prepared with double distilled water.

Preparation of standard stock solution

Standard stock solutions (20 g/ml) of both of SBS and DOX were prepared separately by dissolving accurately weighed (2.0 mg) quantity of pure SBS and DOX in 100 ml volumetric flask and diluting up to the mark with phosphate buffer (pH 7.4) to get working standard solution of each containing 20µg/ml of both SBS and DOX.

Preparation of working standard solutions

From the above stock solution desired concentrations were prepared by transferring specific volume to separate 10 ml volumetric flasks and volume was made up to 10 ml with phosphate buffer.

Determination of isoabsorptive point and absorption maxima

By appropriate dilution of standard solutions of SBS and DOX with phosphate buffer (pH 7.4), solutions containing 10 g/ml of both drugs were scanned separately in the range of 200-400 nm against phosphate buffer (pH 7.4) as blank. The overlaying spectrum was also obtained to determine isoabsorptive point and wavelength of maximum absorbance max of both the drugs.

Methods

Simultaneous equation method (Method A) 1µg/ml solutions of SBS and DOX were prepared separately in phosphate buffer (pH 7.4) and the solutions were scanned against blank in the entire UV range to determine the max values. Clear peaks were observed at 272 nm for SBS and 276 nm for DOX. Hence these wavelengths were chosen as max values for each drug respectively (fig. 1). Standard solutions of SBS and DOX in the concentration range 0.15g/ml were prepared in the phosphate buffer (pH 7.4) and the absorbance of these solutions was measured at 272 nm and 276 nm. Calibration curves were plotted to verify the Beer's law and the absorptivity values calculated at the respective wavelengths for both the drugs. Two simultaneous equations as below were formed using these absorptivity values A (1%, 1 cm).[21-24]

At λ1 $A1 = ax1bCx + ay1bCy$ …. (1)

At λ2 $A2 = ax2bCx + ay2\ bCy$……. (2)

For measurements in 1 cm cells b=1

Rearrange eq. (2)

$$Cy = \frac{A2 - ax2Cx}{ay2}$$

Substituting for Cy in eq (1) and rearranging

$$Cx = \frac{A2ay1 - A1ay2}{ax2ay1 - ax1ay2} \quad \text{……. (3)}$$

$$Cy = \frac{A1ax2 - A2ax1}{ax2ay1 - ax1ay2} \quad \text{…….(4)}$$

Where, Cx and Cy are the concentrations of SBS and DOX measured in gm/100 ml in sample solutions, A1 and A2 are absorbance of mixture at selected wavelengths 272 nm and 276 nm respectively.

Absorbance ratio method/Q-analysis (method B)

The absorbance ratio method is a modification of the simultaneous equation procedure. It depends on the property that for a substance, which obey Beer's law at all wavelength, the ratio of absorbance at any two wavelengths in constant value independent of concentration or path length. E. g. two dilutions the USP, this ratio is referred to as Q value. In the quantitative assay of two components in admixture by the absorbance ratio method, absorbance are measured at two wavelengths, one being the max of one of the component (λ2) and the other being wavelength of equal absorptivity of two components (λ1) i.e. an isoabsorptive point.

A series of standard solutions of SBS and DOX in the concentration range of 0.1-5 g/ml were prepared in phosphate buffer and the absorbance of these solutions was measured at 225 nm (isoabsorptive point) and 276 nm (max of DOX) (fig. 1). Calibration curves were plotted to verify the Beer's law and the absorptivity values calculated at the respective wavelength for both the drugs. The absorptivity values were reported in table 1.[21-24]

The concentration of two drugs in mixture was calculated by using the following equations:

$$C_x = \frac{Q_{M-QY} * A1}{ax - Q_Y \; AX1} \quad \ldots\ldots. (5)$$

$$C_{Y=} \frac{Q_M - Q_X * A1}{Q_Y - Q_X \quad AY1} \quad \ldots\ldots\ldots (6)$$

Where Qm = A2/A1, Qx=ax2/ax1, Qy=ay2/ay1

A1 is absorbance of mixture at isosbestic point i. e 225 nm.

A2 is absorbance of mixture at 276 nm λmax of DOX.

ax1 and ax2 represent absorptivities of SBS at 225 nm and 276 nm.

ay1and ay2 denotes absorptivities of DOX at 225 nm and 276 nm.

Cx and Cy are the concentration of SBS and DOX.

Validation of proposed method (Method A and B)

The method was validated according to ICH guidelines for validation of analytical procedures in order to determine linearity, sensitivity, accuracy and precision for each analyte.[20]

Linearity

Appropriate dilutions of working standard solutions for SBS and DOX were prepared in the concentration range of 0.1-5µg/ml and 0.1-3µg/ml, respectively and analyzed as per the developed methods A and B. Calibration curves were generated and the linearity was

evaluated by the least square regression method.

Accuracy (Recovery studies)

To ascertain the accuracy of the proposed methods, recovery studies were carried out by standard addition method at three different levels according to ICH guidelines. A series of solutions of SBS and DOX at 80%, 100%, and 120% of the standard preparation in the ratio of the formulation were prepared and checked for accuracy by determining the absorbance values at λmax of 272 nm and 276 nm (Method A) and 225 nm and 276 nm (method B) respectively. To a fixed concentration of the formulation, varying concentrations of pure drug solutions were added and percentage recoveries calculated.

Precision

Precision is the degree of repeatability of analytical method under normal operational conditions. The precision of the assay was determined by repeatability (intraday) and intermediate (interday) and reported as %RSD for a statistically significant number of replicate measurement. The intermediate precision was studied by comparing the assays on three different days and the results documented as standard deviation and %RSD.

Precision studies were performed in triplicate at three different concentration levels covering the entire linearity range for SBS and DOX.

Limit of detection (LOD) and limit of quantification (LOQ)

The detection limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be detected but not necessarily quntitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ of the proposed method were determined by using calibration curve:

$$LOD = \frac{3.3\sigma}{s}$$

$$LOQ = \frac{10\sigma}{s}$$

Where, σ is mean standard deviation of y-intercepts of regression lines, s is slope of the standard curve.

Assay of tablets formulation

For estimation of drugs in the commercial formulations, twenty tablets containing 400 mg DOX and 4 mg of SBS were weighed and average weight was calculated. The tablets were crushed and powdered in glass mortar. For the analysis of drugs, quantity of powder equivalent to 1 mg of SBS and100 mg of DOX was transferred to 100 ml volumetric flask and dissolved in sufficient quantity of phosphate buffer. It was sonicated for 30 min and volume was made upto obtain a stock solution 10µg/ml of SBS and1000µg/ml of DOX. This solution was then filtered through watmann filter paper #42. Further dilutions were made from this stock solution to get required concentration. In method A, the concentration of SBS and DOX was determined by measuring absorbance of sample solutions at 272 nm (λmax of SBS) and276 nm (λmax of DOX) using simultaneous equation. In method B, the concentration of both SBS and DOX was determined by measuring absorbance of sample solutions at 276 nm (λmax of DOX; λ2) and 225 nm (isosbestic point of both drugs; λ1). The results of recovery studies conducted by the addition of different amount of pure drugs at different levels to a tablet solution were found to be satisfactory.

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RESULTS AND DISCUSSION

The simultaneous equation method is generally used to estimate two absorbing substances

(SBS and DOX) each of which absorbs at the wavelength of the other drug. By constructing and placing values in simultaneous equations 3 and 4 the concentration of two drugs was determined. The absorption ratio method generally used to estimate two absorbing substances (SBS and DOX) each of which absorbs at the wavelength of the other by constructing and placing values in absorption ratio equation 5 and 6 to determine the concentration of SBS and DOX.

SBS and DOX exhibited maximum absorption at 272 nm and 276 nm (Method A), so using these wavelengths simultaneous equation method for analysis of SBS and DOX in combine form was developed. For Q-absorption method (Method B) of simultaneous analysis of SBS and DOX in combine form, 225 nm (iso-absorptive point) and 276 nm (λmax of DOX) was used. Beer's law were found to be obeyed in the concentration range between 0.2-1.6g/ml and 0.1 to 3.5µg/ml at 276 nm; 0.2-1.6 µg/ml and 0.1-4.5 µg/ml at 272 nm and 0.2 to 2.0µg/ml and 0.2 to 3.5µg/ml at iso-absorptive point 225 nm for SBS and DOX respectively (Method A and B). Calibration curves were prepared for both the drugs at 276 nm, 272 nm and 225 nm (fig. 4-6, table 1, 5). The overlain UV-absorption spectra of SBS (272 nm) and DOX (276 nm) showed isoabsorptive point (225 nm) in ethanol is shown in fig. 3. All calibration curve obtained was linear with coorelation coefficient (r2) greater than 0.998. Hence the relationship between the concentrations and absorbances of SBS and DOX showed linearity.

The validation parameters were studied on marketed formulation at all the wavelengths for the proposed methods. As per IP, tablets should contain not less than 95.0% and not more than 105.0% of active ingredients of the stated amount. The average % drug content was found to be of 97-98% and 96.89-98.16% for SBS and DOX by Method A and Method B respectively, which was found to be within the acceptance limit with %RSD values less than the limit of 2%. Accuracy was determined by calculating the recovery by standard addition method. Results revealed percentage recovery more than 97.50% with % RSD value within the accepted limit for both the components by both methods at all the three levels of recovery analysis. Hence both the proposed methods were found to be accurate for estimation of SBS and DOX in tablet formulation. Both the methods were subjected for study of repeatability, intraday and interday precision for both the drugs. % RSD values for repeatability, intraday and interday precision were calculated and found to be well below the specified limit of 2% (%RSD<2) indicating good precision in the specified range. The sensitivity of the proposed

methods was determined in terms of limit of detection (LOD) and limit of quantitation (LOQ). LOD values for SBS and DOX were found to be 0.048 and 0.064 µg/ml at 276 nm; 0.048 and 0.013 at 272 nm and 0.015 and 0.057 µg/ml at 225 nm. LOQ values for SBS and DOX were found to be 0.148 and 0.195 µg/ml at 276 nm; 0.158 and 0.042 µg/ml at 272 nm and 0.047 and 0.173 µg/ml at 225 nm.

Therefore the result shows that both the proposed methods are specific, accurate and precise as indicated by good recovery results and within acceptance limit relative standard deviation (RSD) values for simultaneous quantitation of SBS and DOX in bulk drug and combined dosage form. Overall proposed methods were found to be suitable for simultaneous quantitative estimation of both the drugs in pharmaceutical dosage form.

CONCLUSION

Two new, simple, sensitive and economical UV spectrophotometric methods were developed for the simultaneous analysis of SBS and DOX in bulk and in pharmaceutical formulations. The developed methods were validated and from the statistical data, it was found that the methods were linear, accurate and precise and can be successfully applied for the analysis of pharmaceutical formulations without interference of excipients.

The UV spectrophotometric simultaneous equation method and Qabsorption ratio method was developed and validated for the simultaneous analysis of SBS and DOX. The results together established that the methods are simple, accurate, precise, reproducible, rapid, and sensitive. The method could be applied successfully and economically for the simultaneous estimation of SBS and DOX in laboratory samples for efficient data generation and for combination formulations of these two drugs in the future.

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