Research Article

Spectrofluorimetric Method for the Determination of Doxepin Hydrochloride in Commercial Dosage Forms

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Abstract. A novel spectrofluorimetric method has been developed for the determination of doxepin hydrochloride in commercial dosage forms. The method is based on the fluorescent ion pair complex formation of the drug with eosin Y in the presence of sodium acetate–acetic acid buffer solution of pH 4.52 which is extractable in dichloromethane. The extracted complex showed fluorescence intensity at $\lambda_{em}=567$ nm after excitation at 464 nm. The calibration curve was linear over the working range of 0.1–0.8 µg ml^{-1}. Under the optimized experimental conditions, present method is validated as per International Conference on Harmonization guidelines. The limit of detection for the developed method is 2.95 ng ml^{-1}. The method has been successfully applied to the determination of doxepin hydrochloride in commercial dosage forms. The results are compared with the reference spectrofluorimetric method.

KEY WORDS: commercial dosage forms; doxepin hydrochloride; eosin Y; spectrofluorimetry; validation.

INTRODUCTION

Doxepin hydrochloride is chemically known as 1-propanamine, 3-dibenz[b, e]oxepin-11(6H)-ylidene-N, *N*-dimethyl-, hydrochloride (Chemical Abstract Service (CAS): 1229-29-4; molecular weight (MW): 315.84). It is a tricyclic antidepressant which is widely used for the treatment of depression and anxiety. It displays a potent central anticholinergic activity and can inhibit both norepinephrine and serotonin (5-HT) reuptake in synapses in brain (1). It is neither a central nervous stimulant nor a monoamine oxidase inhibitor. In general, lower dosages of doxepin hydrochloride are recommended. Where the presenting symptoms are mild in nature, it is advisable to initiate treatment at a dose of 10–50 mg daily. At high concentrations, severe adverse effects and toxicity can appear (2). Therefore, the analysis of doxepin hydrochloride is important for obtaining optimum therapeutic concentration and for quality assurance in pharmaceutical preparations.

The assay of doxepin hydrochloride in bulk and formulations is cited in the United States Pharmacopoeia (3) which is based on liquid chromatography. In view of the great importance in terms of its optimum oral dose and wide use of doxepin hydrochloride, various analytical methods have been reported which include high-performance thin layer chromatography (4), high-performance liquid chromatography (HPLC; 5,6), liquid chromatography coupled with mass spectrometry (7), capillary electrophoresis (8), electroanalytical method of analysis (9), spectrophotometry (10,11), and extractive spectrophotometry (12,13). Spectrofluorimetry is attractive because of sensitivity, speed, and simplicity. Most of the additives or excipients found in pharmaceutical preparations are not fluorescent in nature. Therefore, spectrofluorimetry is the good choice to analyze doxepin hydrochloride in pharmaceutical preparations. There are two methods available on spectrofluorimetry which are based on dichloromethane extractable ion pair complexes of the drug with 9,10-dimethoxyanthracene-2-sulfonate (14) and tetraiodofluorescein (15).

The aim of this study is to develop an optimized spectrofluorimetric method for the determination of doxepin hydrochloride in pharmaceutical formulations. The present spectrofluorimetric method is based on dichloromethane extractable ion pair complex formation of the doxepin hydrochloride with eosin Y at pH 4.52 (sodium acetate–acetic acid buffer solution). The extracted complex showed fluorescence intensity at 567 nm after excitation at 464 nm. The reaction conditions are optimized and validated as per International Conference on Harmonization (USA; 16).

MATERIALS AND METHODS

Apparatus

Fluorescence spectra and measurements were made on a F-2500 Hitachi fluorescence spectrophotometer (Tokyo, Japan) equipped with xenon lamp and 1-cm quartz cells. Excitation and emission wavelengths were set at 464 and 567 nm with the excitation and emission slit widths of 10 nm, respectively. All measurements were performed at 25±1°C.

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An Elico LI 120 pH meter (Hyderabad, India) was used for pH measurement.

Reagents and Standards

All reagents used were of analytical reagent grade.

Eosin Y disodium salt (CAS: 17372-87-1, MW: 691.85, Fluka Chemie AG, Switzerland) solution, 2.891×10^{-3} M (0.2%), was freshly prepared in distilled water, and buffer solutions ranging from 4.05 to 5.57 were prepared by mixing appropriate volumes of 0.2 M acetic acid and 0.2 M sodium acetate (17). Doxepin hydrochloride reference standard drug was purchased from Sigma Chemical Company (St. Louis, USA). Spectra-25 capsule (Rexcin Pharmaceutical Private Limited, Himachal Pradesh, India) and Doxetar-25 capsule (Torrent Labs Pvt. Ltd., Ahmedabad, India) are the commercial products of doxepin hydrochloride which were purchased from local market.

Doxepin hydrochloride test solution of 0.01 mg ml^{-1} was prepared in distilled water.

Procedure for the Determination of Doxepin Hydrochloride

Aliquots of 0.05–0.4 ml standard doxepin hydrochloride (0.01 mg ml^{-1}) aqueous solution corresponding to 0.1–0.8 μg ml^{-1} were pipetted into a series of 50-ml separating funnels. To each funnel, 3.2 ml of eosin Y (0.2%) aqueous solution and 2.5 ml of sodium acetate–acetic acid buffer solution of pH 4.52 were added and mixed well. The contents of the separating funnel were shaken vigorously with 5 ml of dichloromethane for 2.5 min and then allowed to separate the two layers. The fluorescence intensity of the organic layer was recorded at 567 nm after excitation at 464 nm. The amount of the drug was obtained either from the calibration graph or the regression equation.

Analysis of Doxepin Hydrochloride in Commercial Dosage Form

The powder contents of two commercially available capsules of 25 mg strength of doxepin hydrochloride were taken in 50 ml of distilled water and kept for 10 min for complete dissolution of the drug. The mixture was filtered through Whatmann no. 42 filter paper (Whatmann International Limited, Kent, UK) in 100-ml standard volumetric flask. The residue was washed well for three times with 10-ml portions of distilled water for complete recovery of the drug and diluted to volume with distilled water. This solution was further diluted to complete the analysis following the developed method for the determination of doxepin hydrochloride.

Procedure for Reference Method

Aliquots of 0.2–3.0 ml of 0.001% standard doxepin hydrochloride aqueous solution corresponding to 0.2–3.0 μg ml^{-1} doxepin hydrochloride were pipetted into a series of 50-ml separating funnel. To each funnel, 2.90×10^{-3} M 9, 10-dimethoxyanthracene-2-sulfonate in 0.2 M HCl was added and mixed well. The contents of the funnel were shaken with 10 ml dichloromethane for 2.5 min and allowed to stand for clear separation of the two phases. The

fluorescent intensity of the organic phase at $\lambda_{em} = 448$ nm after excitation at 265 nm was measured against the reagent blank prepared similarly except drug. The amount of the drug in a given sample can be estimated either from the calibration graph or the corresponding linear regression equation.

Validation

The present method has been validated for solution stability, specificity, linearity, precision, accuracy, robustness, and evaluation of bias.

The solution stability of aqueous doxepin hydrochloride was investigated by taking 200 μg ml^{-1} of doxepin hydrochloride. The drug solution was monitored spectrophotometrically by observing the ultraviolet (UV)–visible spectra for 6 h. The spots on thin layer chromatographic (TLC) plates for freshly prepared drug solution (aqueous) were monitored for 12 h at room temperature. The thin layer chromatography was performed using TLC plates coated with silica gel G (Merck Limited, Mumbai, India) and developed in the solvent system: benzene–dioxan–7.75 M ammonia solution (30%) in the ratio of 2.5:5.5:2.0 $v/v/v$. Then, the TLC plates were freed from mobile phase and dried, and spots were detected in iodine chamber.

The specificity and selectivity of the present method were ascertained by the analysis of placebo solution of doxepin hydrochloride by taking 1 mg of pure doxepin hydrochloride with 50 mg glucose, 80 mg fructose, 150 mg lactose, 100 mg starch, and 150 mg sodium benzoate in 100-ml standard volumetric flask and diluted up to the mark with distilled water. This solution was analyzed following the developed procedure for the determination of doxepin hydrochloride.

The linearity of the present method was evaluated at nine concentration levels of 0.10, 0.16, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, and 0.80 μg ml^{-1}. Each concentration level was analyzed repeatedly for five times.

The intraday (within day) and interday (between days) precisions were evaluated using standard doxepin hydrochloride aqueous solution at three concentration levels: 0.16, 0.40, and 0.80 μg ml^{-1} (50–150% of the nominal doxepin hydrochloride concentration). The intraday repeatability was assessed with five replicates for each of three working sample concentrations in a single day. The interday reproducibility was assessed with five replicates at each concentration over 5 days.

The accuracy of the procedure was evaluated by the standard addition technique. In this technique, aliquot of 0.1 ml (or 0.2 mL) of active drug solution (aqueous) of Spectra-25 capsule was spiked with 0, 0.05, 0.08, 0.1, and 0.15 ml of reference doxepin hydrochloride (0.01 mg ml^{-1}) into a 50-ml separating funnel. To each funnel, 3.2 ml of eosin Y (0.2%) aqueous solution and 2.5 ml of sodium acetate–acetic acid buffer solution of pH 4.52 were added and mixed well. The funnel was shaken vigorously with 5 ml dichloromethane for 2.5 min, and then allowed to stand for clear separation of the organic layer from the aqueous phase. The nominal value was determined by measuring the fluorescence intensity at each concentration level.

The robustness of the method was evaluated by observing the influence of small variations of experimental variables, i.e., concentration of eosin Y, volume of buffer solution of pH 4.52, shaking time, and solvent. The exactness of each operational parameter was checked by varying one exper-
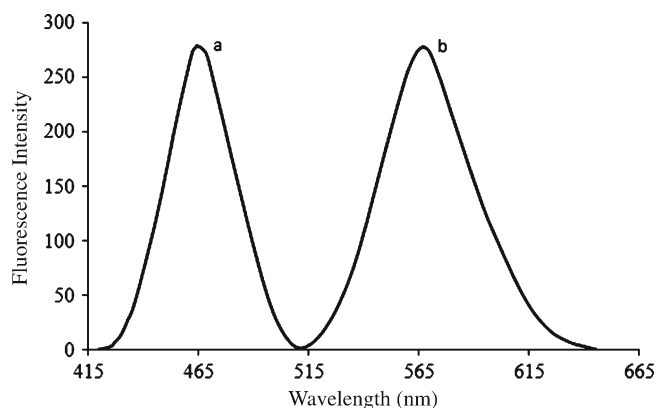
Fig. 1. Excitation and emission spectra of ion pair complex: 0.08 μg ml^{-1} doxepin hydrochloride + 3.2 ml of Eosin Y (0.2%) + 2.5 ml of buffer solution of pH 4.52. The complex was extracted in 5 ml dichloromethane

imental parameter at a time keeping the other parameters constant, and the percentage recovery ± reflex sympathetic dystrophy (RSD) of drug was calculated.

The bias has been evaluated by means of point and interval hypothesis tests (17). In interval hypothesis test, the test method is compared with the reference method and considered to be acceptable if the mean recovery is within ±2.0% of that of the reference method (18), i.e.,

$$0.98 < \mu_2/\mu_1 < 1.02$$

which can be generalized to

$$\theta_L < \mu_2/\mu_1 < \theta_U$$

where θ_L and θ_U are lower and upper acceptance limits, respectively. The limits of this confidence interval can be calculated using the following quadratic equation:

$$\theta^2\left(\overline{x_1^2} - S_p^2 t_{tab}^2/n_1\right) + \theta(-2\overline{x_1}\,\overline{x_2}) + \left(\overline{x_2^2} - S_p^2 t_{tab}^2/n_2\right) = 0$$

where $\overline{x_1}$ and $\overline{x_2}$ are mean values based on n_1 and n_2 measurements, respectively. S_p is the pooled standard deviation, and t_{tab} is the tabulated one-sided t value, with $n_1 + n_2 - 2$ degrees of freedom at 95% confidence level.

RESULTS AND DISCUSSION

Spectral Studies

The literature citation revealed that doxepin hydrochloride is not fluorescent in nature but, when reacted with 9, 10-dimethoxyanthracene-2-sulfonate (14) and tetraiodofluorescein (15), the fluorescent ion pair complexes are formed which are extractable in dichloromethane and thus, can be utilized for the estimation of doxepin hydrochloride in pharmaceutical formulations. In the similar manner, aqueous doxepin hydrochloride solution when treated with aqueous eosin Y disodium salt solution in the presence of sodium acetate–acetic acid buffer solution of pH 4.52, a fluorescent light pink ion pair complex is formed which is quantitatively extracted in dichloromethane. The fluorescent intensity was

measured at 567 nm after excitation at 464 nm. The reaction is confirmed as eosin Y in blank solution did not form ion pair complex in the absence of drug and cannot be extracted in dichloromethane. Figure 1 shows the excitation and emission spectra of ion pair complex.

Stoichiometry

The stoichiometric ratio between doxepin hydrochloride and eosin Y at pH 4.52 was evaluated by Job's method of continuous variations (19). For this, different volumes that is 0, 0.4, 1.2, 1.8, 2.0, 2.2, 2.6, 2.65, 2.67, 2.7, 3.2, 3.8, and 4.0 ml of 1.0×10^{-4} M doxepin hydrochloride aqueous solution were added with 4.0, 3.6, 2.8, 2.2, 2.0, 1.8, 1.4, 1.35, 1.33, 1.3, 0.8, 0.2, and 0 ml of 1.0×10^{-4} M aqueous eosin Y solution into a 50-ml separating funnel, and analysis was completed by following the recommended procedure. The fluorescence intensity was measured at 567 nm after excitation at 464 nm and plotted against the mole fraction of drug (Fig. 2). It is apparent from the figure that the combining molar ratio between doxepin hydrochloride and eosin Y is 2:1. The apparent formation constant and standard Gibbs free energy ($\Delta G°$) were calculated and found to be 5.65×10^{13} and -78.467 kJ mol^{-1}, respectively.

Mechanism of the Reaction

Doxepin hydrochloride as a derivative of dibenzoxepine possessing nitrogen atom of tertiary amine group in the aliphatic chain offers a basic characteristic to the drug. The drug is protonated in the presence of sodium acetate–acetic acid buffer solution of pH 4.52. Eosin Y is tetrabromofluorescein disodium salt and loses its two sodium ions in distilled water, thus 1 mol of eosin provides two negative sites and forms a fluorescent ion pair complex with 2 mol of protonated doxepin which is extractable in dichloromethane. The extracted dichloromethane ion pair complex fluoresces conveniently at 567 nm after excitation at 464 nm. Therefore, based on the literature background and our experimental findings, the reaction sequence is given in Fig. 3.
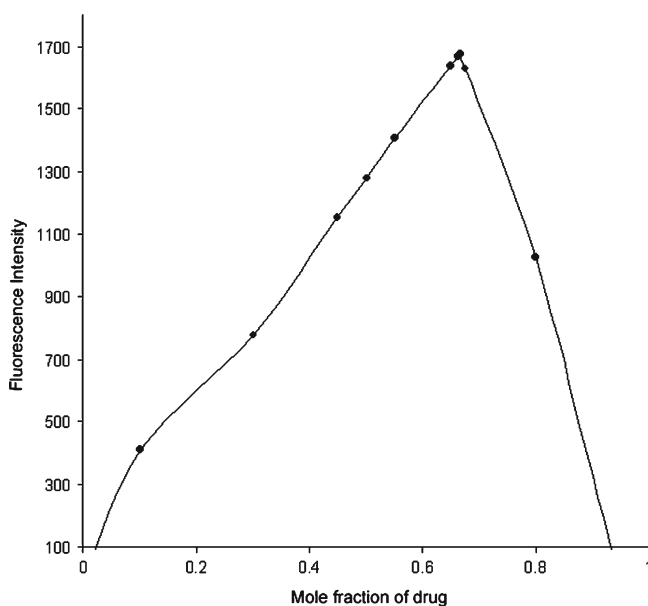
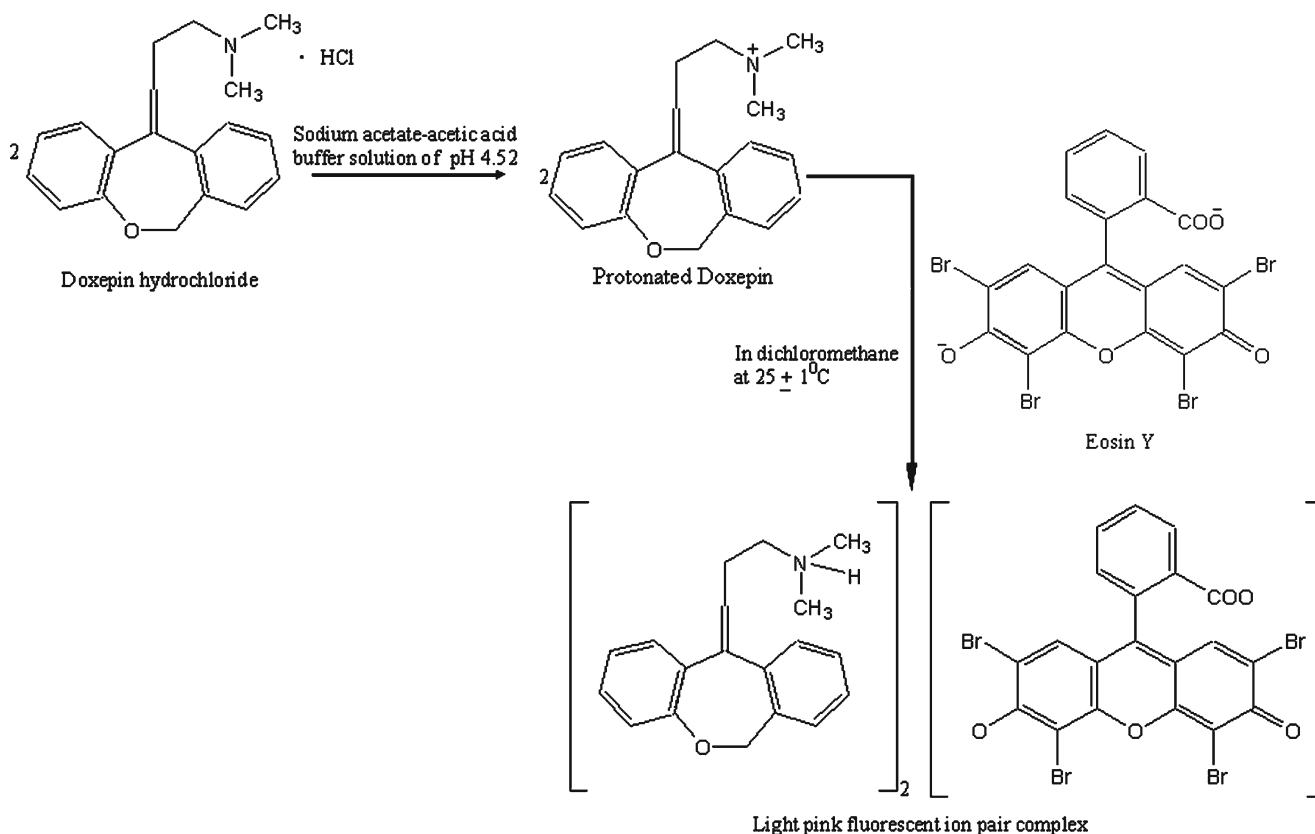**Fig. 2.** Job's plot to establish the stoichiometry of the reaction

Fig. 3. Reaction sequence of the developed method

Optimization of Variables

The optimization of variables for the present spectro-fluorimetric method was assessed by testing several parameters such as reaction time, concentration of eosin Y, buffer solutions of different pH, volume of buffer solution of pH 4.52, shaking time for extraction of complex, and solvents.

The effect of the reaction time on the development of fluorophore and its stability was investigated. The ion pair complex got stabilized immediately at $25 \pm 1°C$ and remained stable for at least 1 h.

The effect of the concentration of eosin Y on the fluorescence intensity of the ion pair complex was investigated in the range of 5.78×10^{-5}–2.08×10^{-3} M eosin Y. It was observed that the maximum fluorescence intensity was attained with 1.62×10^{-3} M eosin Y and remained constant up to 2.08×10^{-3} M eosin Y. Therefore, 1.85×10^{-3} M eosin Y was taken as the optimum concentration for the determination of the drug.

The influence of pH on the fluorescence intensity of the ion pair formation of doxepin hydrochloride (0.6 µg ml^{-1}) with eosin Y (1.850×10^{-3} M) has been studied using sodium acetate–acetic acid buffer solution in the pH range of 4.05–5.57. The results are shown in Fig. 4. It is evident from the figure that the maximum fluorescence intensity of the complex was found at pH 4.52. Therefore, all fluorescence intensity measurements were made at pH 4.52 in the determination process.

The effect of volume of pH 4.52 buffer solution of sodium acetate–acetic acid on the fluorescence intensity of the complex was studied in the range of 0.5–3.0 ml. The highest fluorescence intensity was obtained with 2.0 ml of buffer (pH 4.52) solution,

beyond this, further increase in the volume of buffer solution resulted in no change in the fluorescence intensity of the complex. Therefore, 2.5 ml of pH 4.52 buffer solution was adopted as an optimum volume for measuring fluorescence intensity in the determination procedure.

The effect of the shaking time for the extraction of the ion pair complex was studied in the range of 0.5–3.0 min. The maximum fluorescence intensity of the complex was obtained
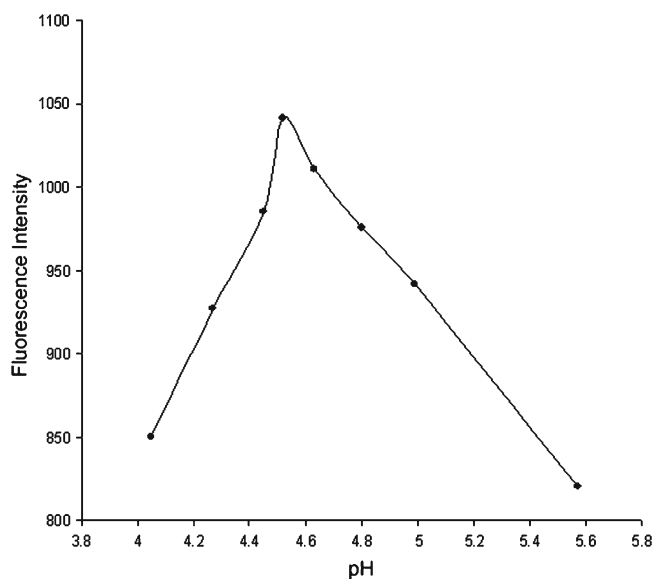

Fig. 4. Effect of pH on the flourescence intensity of the ion pair complex

Table I. Effect of Foreign Species on the Determination of 0.6 $\mu g\ ml^{-1}$ Doxepin Hydrochloride

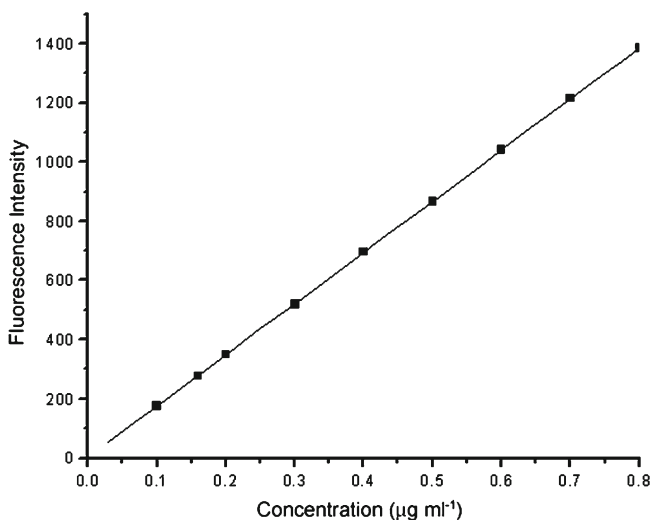| Sample number	Foreign species	Maximum tolerance limit ($mg\ ml^{-1}$)
1	Glucose	0.10
2	Fructose	0.16
3	Lactose	0.30
4	Starch	0.20
5	Sodium benzoate	0.30

at 2.0 min, and above this up to 3.0 min, the fluorescence intensity remained constant. Therefore, 2.50 min was used as an optimum shaking time throughout the determination process. The ion pair complex was quantitatively recovered in one extraction only and was stable for at least 1 h.

A number of organic solvents such as chloroform, carbon tetrachloride, dichloromethane, dichloroethane, and ethyl acetate were examined for extraction of the ion pair complex in order to provide an applicable extraction procedure and maximum fluorescence intensity. It was observed that the highest fluorescence intensity of the complex was attained in dichloromethane, and there is no extraction of the complex in carbon tetrachloride and ethyl acetate. Therefore, dichloromethane was selected as the best solvent for extraction of the complex.

Validation

The UV absorption spectrum of aqueous solution of doxepin hydrochloride was recorded in the wavelength range of 200–320 nm. It was found that the aqueous solution of drug (200 $\mu g\ ml^{-1}$) absorbs maximally at 238 nm (0.706 absorbance value), and the absorbance value remained constant up to 5 h 30 min. This was also confirmed as TLC plate showed a single spot with $R_f = 0.692$ up to the duration of 5 h 30 min. Hence, it is clear from the absorbance value and R_f value of the spot that the solution of doxepin hydrochloride in distilled water has considerable stability of 5 h 30 min.

Table II. Test of Precision

Parameters	Intraday assay			Interday assay		
Concentration taken ($\mu g\ ml^{-1}$)	0.160	0.400	0.800	0.16	0.400	0.800
Concentration found ($\mu g\ ml^{-1}$)	0.160	0.401	0.800	0.1601	0.401	0.800
Standard deviation[a] ($\mu g\ ml^{-1}$)	0.001	0.001	0.002	0.001	0.001	0.002
Recovery (%)	100.02	100.20	100.01	100.05	100.20	100.02
RSD (%)	0.58	0.32	0.23	0.59	0.33	0.25

[a] Mean for five independent determinations

The spectrofluorimetric conditions were found to be specific in the presence of tablet excipients (Table I). It is clear from the table that common excipients present in capsule formulations did not cause any significant interference.

Under the optimized experimental conditions, the fluorescence intensity was plotted against the concentration of doxepin hydrochloride and found to be linear over the concentration range 0.1–0.8 $\mu g\ ml^{-1}$ (Fig. 5). The statistical analysis of the experimental data yielded the regression equation: F.I. = 2.250 + 1,729.99C (C = concentration in microgram per milliliter). with correlation coefficient (r) of 0.9999. The detection and quantitation limits were found to be 2.95 and 8.93 $ng\ ml^{-1}$, respectively. The high value of correlation coefficient (0.9999) indicated excellent linearity of the calibration graph.

Fig. 5. Calibration curve for the determination of doxepin hydrochloride. ($\lambda ex = 464$ nm and $\lambda em = 567$ nm)
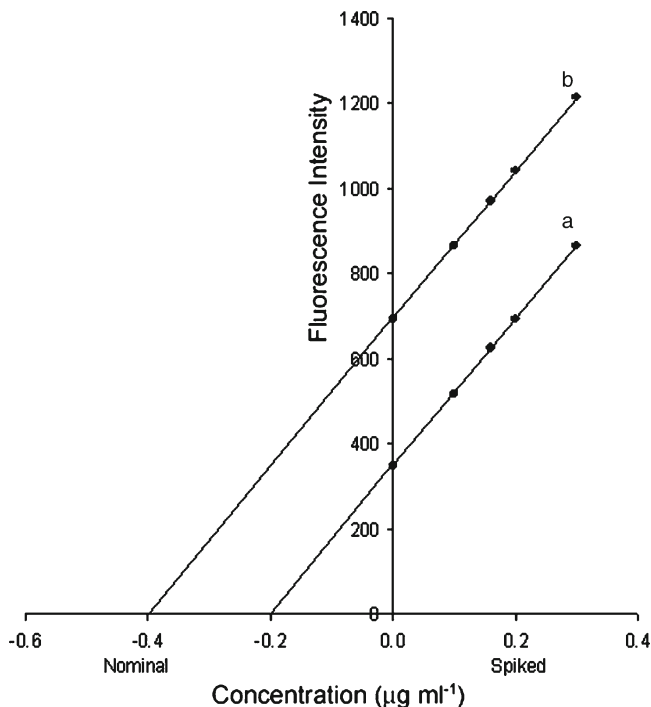
Fig. 6. Recovery of doxepin hydrochloride from Spectra-25 capsule by standard addition technique: 0.202 (*a*) and 0.399 $\mu g\ ml^{-1}$ (*b*)

Table III. Point and Interval Hypothesis Tests: Applicability of the Present Method in Pharmaceutical Formulations and Its Comparison with the Reference Method at 95% Confidence Level

Pharmaceutical formulations	Proposed method		Reference method				
Capsule	Recovery (%)	RSD^a (%)	Recovery (%)	RSD^a (%)	t and F values^b	$\theta_L{}^c$	$\theta_U{}^c$
Spectra-25 (Rexcin Pharma)	99.99	0.232	100.17	0.338	$t=0.983$ $F=1.458$	0.993	1.003
Doxetar-25 (Torrent Labs Pvt. Ltd)	99.99	0.236	100.17	0.338	$t=0.956$ $F=1.432$	0.995	1.002

^{a} Mean for five independent analyses
^{b} Theoretical t ($\upsilon=8$) and F values ($\upsilon=4, 4$) at 95% confidence level are 2.306 and 6.39, respectively
^{c} A bias, based on recovery experiments, of ±2% is acceptable

The intra- and interday precisions were evaluated by determining the concentration of doxepin hydrochloride at lower, middle, and upper concentration levels for five repeated times within the same day and on five consecutive days, respectively (Table II). It can be seen from the table that the percent recovery and percent RSD were in the ranges of 100.01–100.20% and 0.23–0.59%, respectively for intraday and interday precisions. The percent recovery and percent RSD values showed that the developed method is very precise and can be used to analyze doxepin hydrochloride in pharmaceutical formulations.

The accuracy of the present method was tested by performing recovery experiments through standard addition technique. The results of analyses are presented in Fig. 6. It is evident from the graph that the linearity of the regression lines for samples 1 and sample 2 were good ($r=0.9999$) with slope 1,731.55, 1,734.91 and intercept 347.21 and 693.90, respectively. The recovery of the doxepin hydrochloride ranged from 99.99% to 100.26%. Hence, the developed method being a precise one is an accurate too. The most attractive feature of the spectrofluorimetric method using standard addition method is its relative freedom from pharmaceutical additives and excipients. Mostly, the pharmaceutical additives and adjuvants are not fluorescent in nature and did not interfere with the determination procedure.

The robustness of the method was established by deliberately changing the reaction conditions. The operational parameters challenged to prove the robustness include: volume of 2.891×10^{-3} M eosin Y, 3.2 ml (±0.4 ml), volume of pH 4.52, 2.5 ml (±0.5 ml), and shaking time, 2.5 min (±0.5 min).

Under these optimal conditions, the doxepin hydrochloride aqueous solution containing 0.8 μg ml^{-1} (Spectra-25 capsule) was analyzed by the present method. The results

showed the mean percent recovery ± RSD of 99.99±0.23%. The results indicated the robustness of the method.

The applicability of the present method for the quantitative analysis of doxepin hydrochloride in Spectra-25 and Doxetar-25 capsule has been tested. The results of the present method were statistically compared with those of the reference spectrofluorimetric method (14) using point and interval hypothesis tests. Table III shows that the calculated t (paired) and F values at 95% confidence level are less than the tabulated t value (2.036 at $\upsilon=8$) and F value (6.39 at $\upsilon=4, 4$), thus confirming no significant difference between the performance of the developed method and the reference method. It is also clear from the table that the bias evaluated by interval hypothesis test by means of lower limit (θ_L) and upper limit (θ_U) are in the range of 0.98–1.02. Therefore, it is concluded that the present spectrofluorimetric method is applicable for routine quality control analysis of doxepin hydrochloride in commercial dosage forms with acceptable recovery results which are within the acceptable limit of ±2%.

The performance of the proposed method was compared with other existing methods (Table IV). As can be seen from the table, the proposed method is more sensitive with low limit of detection and RSD values as compared to the existing spectrofluorimetric (14) and spectrophotometric methods (10,12). Capillary electrophoresis is more sensitive but its limit of detection is comparable to those of the proposed method. HPLC provides a wider linear range with RSD less than 2%.

CONCLUSIONS

Doxepin hydrochloride is a nonfluorescent drug but displays fluorescence by means of the ion pair complex formation of the drug with eosin Y in the presence of sodium acetate–acetic

Table IV. Comparison of the Developed Method with Other Existing Methods for the Determination of Doxepin Hydrochloride

Techniques/reagents	Linear range (μg ml^{-1})	Limit of detection (ng ml^{-1})	RSD (%)	References
Spectrofluorimetry				
9,10-dimethoxyanthracene-2-sulfonate	0.25–3	220	2.1	(14)
Eosin Y	0.1–0.8	0.95	0.59	This work
Spectrophotometry				
Ti(IV) thiocyanate	5–50	340	0.43	(12)
Fe(III) thiocyanate	3–30	260	0.32	(12)
3-methylbenzothiazolin-2-one	0.8–10	–	–	(10)
HPLC	30–70	–	<2	(6)
Capillary electrophoresis	0.005–0.8	1	4.2	(8)

acid buffer solution of pH 4.52. The fluorescent behavior of the ion pair complex between drug and eosin Y is exploited for the analysis of the doxepin hydrochloride in commercial dosage forms. The present method was optimized and validated for precision and accuracy. The method has the advantage of having simple operation, high sensitivity, repeatability, and reproducibility. In addition, the present method has low limit of detection (2.95 ng ml^{-1}). Hence, the developed method can be used for the routine quality control analysis of doxepin hydrochloride in industries, research laboratories, and hospitals.

ACKNOWLEDGMENTS

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