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Research Article

Validated Analytical Spectrofluorimetric Method for Quantitation of Diphenhydramine HCl in Commercial Dosage Forms

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Abstract

Diphenhydramine HCl is a weakly fluorescent drug having a tertiary amine group forming an ion pair complex with eosin Y in dichloromethane at pH 5 in disodium hydrogen phosphate-citric acid buffer solution. The complex formation was the basis for the development of a new analytical method for determination of active diphenhydramine in pharmaceutical formulations. The stoichiometric ratio between diphenhydramine and eosin Y was studied by mole ratio method and found to be 2:1. The ion-pair complex showed maximum fluorescence emission intensity at 554 nm with excitation at 259 nm. The linear dynamic range was obtained in the concentration range of 2-22 µg mL^{-1} with a linear equation of FI = 0.361 + 13.675 C. The apparent Gibb's free energy (ΔG^{o}) was calculated and found to be -80.783 KJ mol^{-1}, confirming the feasibility of the reaction. The proposed method was successfully applied to the determination of diphenhydramine HCl in pharmaceutical formulations and in good agreement with the reference method.

Keywords:
diphenhydramine HCl; spectrofluorimetry; validation; pharmaceutical formulations

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Introduction

Diphenhydramine hydrochloride is chemically known as 2-(Diphenylmethoxy)-N,N-dimethylethylamine hydrochloride (CAS: 147-24-0; M.W.: 291.82). Figure 1 represents 3d structure of diphenhydramine. It belongs to the class of antihistamines, competitively blocks the effects of histamine at H_1-receptor sites, and has atropine-like, antipruritic and sedative effects [1]. It is approved to treat the

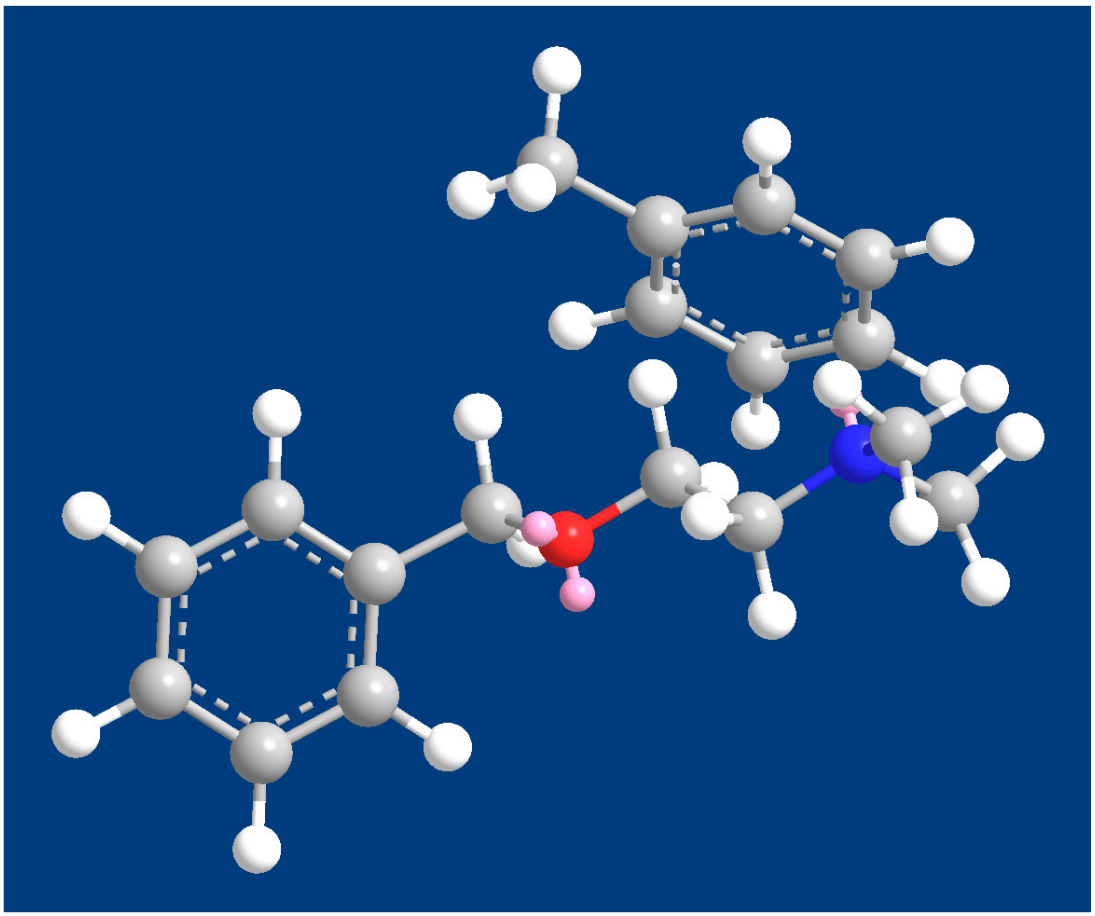


Figure 1. 3D structure of diphenhydramine.

symptoms of motion sickness, sneezing, runny nose, watery eyes, hives, skin rash, itching and other cold or allergy symptoms. The usual recommended dose of the active drug for patients is 25-50 mg for every 4-6 hr, not to exceed 300 mg per 24 hr. The assay of diphenhydramine hydrochloride is described in British Pharmacopoeia [2], United States Pharmacopeia [3] and Indian Pharmacopoeia [4] based on potentiometric titration, high performance liquid chromatographic method and volumetric titration, respectively. Here, the developed method is simple and more convenient based on spectrofluorimetric method. The drug is important and hence the safety, quality, quantity and purity of drug are on high demand [5]. In view of the great importance of the drug in terms of its optimum oral dose and wide use, it is important to analyze the drug in pharmaceutical preparations. With increasing regulatory strictness and owing to the reason of quality control, various analytical methods have been developed in pure and dosage forms including titrimetry [6], high performance thin layer chromatography [7], capillary electrophoresis [8], gas chromatography [9], high performance liquid chromatography [10], electrochemical analysis [11], [12] and spectrophotometry [13]. Analytical methods based on fluorescence spectroscopy are methods of good choice because of high degree of selectivity, sensitivity and wide linear dynamic range. Eosin Y is a most widely used reagent in drug analysis because it is fluorescent in nature [13], [14], [15], [16], [17]. Eosin Y is a xanthene polyprotic acid dye which can be dianionic in nature having pK_a of 2 and 3.8 in water [18]. In this research, eosin Y acted as a fluorescent probe and formed a fluorescent ion-pair complex with diphenhydramine in the presence of disodium hydrogen phosphate-citric acid buffer solution of pH 5.0. The complex was extracted in dichloromethane and provided fluorescence emission intensity at 554 nm after excitation at 259 nm. As per literature survey and gathered information, there is no spectrofluorimetric method based on the extraction of complex in dichloromethane for quantitation of diphenhydramine. Extraction in dichloromethane improved sensitivity and selectivity of the method. The reaction conditions helping the formation of ion-pair complex between eosin Y and diphenhydramine are optimized and validated as per International Conference on Harmonisation guidelines (USA) [19].

Experimental

Reagents and Materials

Fluorescence spectra and all measurements were done on Agilent's Cary Eclipse Fluorescence Spectrophotometer (Thermo Scientific, Australia) equipped with a xenon 150 W arc lamp and 1-cm quartz cells. Excitation and emission wavelengths were set with slit widths of 10 nm.

Hanna pH meter (USA) was used for pH measurements. IR spectra were recorded on IR Affinity-1 spectrophotometer (Shimadzu, Kyoto, Japan) in the range of 4000-400 cm^{-1} using KBr pellet technique.

All reagents and solvents were AR grade. 0.02% eosin Y disodium salt (CAS: 17372-87-1, M.W.: 691.85, Fluka Chemie AG, Switzerland) solution was freshly prepared in distilled water. McIlvaine Na_2HPO_4-citric acid buffer solutions of different pH ranging from 2.2-6.6 were prepared [20] and investigated for ion-pair complex formation. The pH of the solution was monitored using Hanna pH meter. All experiments were performed at a temperature of 25±1 ℃ and pressure of 760mm Hg.

Pure diphenhydramine hydrochloride reference drug was gifted by National Pharmaceutical Industries Company, Oman. 0.02% of pure diphenhydramine hydrochloride solution was tested for linear dynamic range and other validation parameters.

Marketed products of diphenhydramine hydrochloride such as Stopkof syrup 100 mL (National Pharmaceutical Industries Company, Oman), Histalix syrup 150 mL (Wallace manufacturing chemists Ltd, UK) and Amydramine syrup 120 mL (Julphar, UAE) were investigated for active diphenhydramine HCl in the presence of pharmaceutical excipients.

Procedure for the Determination of Diphenhydramine Hydrochloride by Proposed Method

Into a series of 10 mL volumetric flasks, varying aliquots (0.1-1.1 mL) of 0.02% diphenhydramine hydrochloride equivalent to 2-22 µg mL^{-1} were pipetted with 1.4 mL of 0.02% eosin Y and 2.0 mL of Na_2HPO_4-citric acid buffer of pH 5. The contents of the flask were diluted with distilled water at 25±1 ℃ and transferred into the separating funnel with 10 mL dichloromethane. The solution was shaken and mixed for 2 min for the separation of organic layer. The layer was separated and treated with anhydrous

sodium sulphate to remove any traces of water. The fluorescence emission intensity of the associated ion pair complex in the organic layer was recorded at 554 nm after excitation at 259 nm and the linear regression equation was developed for the estimation of active diphenhydramine in pharmaceutical formulations.

Procedure for the Determination of Diphenhydramine Hydrochloride by Reference Method [21]

Into a series of 10 mL standard volumetric flasks, different volumes (0.05, 0.1, 0.15, 0.2, and 0.25 mL) of 0.02% pure diphenhydramine HCl were taken and diluted up to the mark with distilled water at 25±1 ℃. The fluorescence emission intensity at 286 nm was recorded after keeping excitation wavelength fixed at 207 nm against distilled water as a blank solution. The calibration graph was constructed by plotting fluorescence emission intensity versus the concentration of diphenhydramine HCl in μg mL^{-1}. Alternatively, the linear regression equation was generated for finding out the concentration of active diphenhydramine HCl in pharmaceutical preparations.

Procedure for the Determination of Diphenhydramine Hydrochloride in Pharmaceutical Formulations by Proposed and Reference Methods

3 commercially available bottled syrups i.e. Stopkof, Histalix and Amydramine were shaken and poured each into a 50 mL beaker. 7.4, 7.2 and 7.4 mL of the respective syrups equivalent to 20 mg active diphenhydramine HCl were transferred into a 100 mL volumetric flask and diluted up to the mark with distilled water. The recommended procedures were then followed for determination of active diphenhydramine HCl in commercial dosage forms.

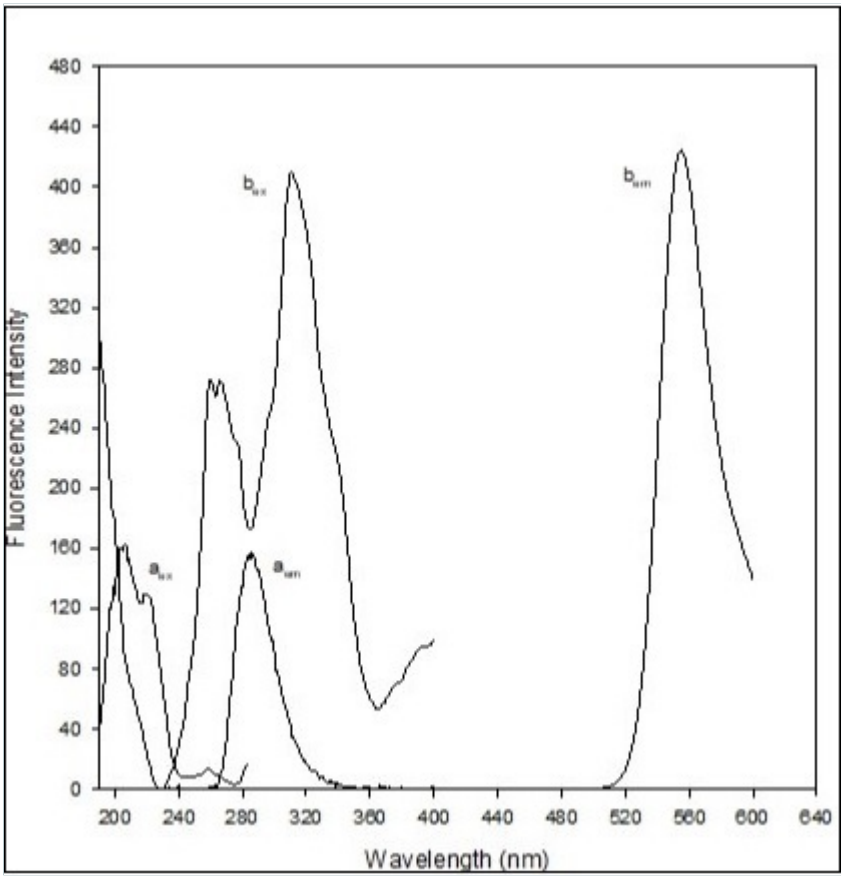


Figure 2. (a) Excitation and emission spectra of diphenhydramine HCl (0.4 mL of 0.01% diphenhydramine HCl in 10 mL distilled) and (b) Excitation and emission spectra of fluorescent ion pair complex (1.5 mL of 0.02% diphenhydramine + 1.3 mL of 0.02% eosin Y + 2.0 mL of buffer solution of pH 5, extracted in 10 mL dichloromethane).

Results and Discussion

In the literature, it was reported that chlorpheniramine [14], doxepin HCl [15], citalopram HBr [16] and imipramine [17] possessed a tertiary amine group. 2 protons of carboxylic and phenolic groups of eosin Y were ionized forming dianionic eosin Y which exhibited $\lambda_{emission}$ of 544.02 nm when excited at 257.96 nm [18]. The most important point is that there was no extraction of dianionic eosin Y in chloroform/dichloromethane in the presence of acidic buffer solution alone. 2 moles of protonated drugs (cation) binded with dianionic eosin Y, thus forming a drug-eosin Y ion-pair complex, extractable in chloroform/dichloromethane. Here in Na_2HPO_4-citric acid buffer of pH 5, eosin Y ionized 2 protons which was protonated to 2 diphenhydramine molecules through nitrogen as basic centre. Finally, dianionic eosin Y binded with protonated diphenhydramine and formed a fluorescent ion pair complex with $\lambda_{emission}$ of 554 nm when excited at 259 nm. At the same time, dianionic eosin Y with acidic buffer solution (in the absence of diphenhydramine) was not extracted in dichloromethane. The aqueous solution of diphenhydramine showed an emission wavelength of 286 nm when excited at 207 nm. The spectrofluorimetric spectra of aqueous diphenhydramine and ion-pair complex were given in Figure 2.

The molar combining ratio between diphenhydramine HCl and eosin Y was investigated by mole ratio method [22]. In the acidic condition, 1 mole of eosin Y (5.0×10^{-4} M) was combined with 2 moles of diphenhydramine (5.0×10^{-4} M) provided ion-pair complex. The fluorescence emission intensity of the complex was recorded at 554 nm after excitation at 259 nm and plotted against the mole ratio of drug to Eosin Y. The apparent formation constant (K_f) of the complex was calculated using the following expression:

$$K_f = \frac{(F_{obs}/F_{extp})\bar{C}}{\left[C_{IM} - 2\left(\frac{F_{obs}}{F_{extp}}\right)\bar{C}\right]^2 [C_E - (F_{obs}/F_{extp})\bar{C}]} \quad \textbf{Eq.1}$$

Where $F_{obs.}$ and $F_{extp.}$ are observed and extrapolated fluorescence emission intensities of the complex. C_{IM}, C_E and $\bar{C}$ are initial concentration of imipramine, eosin Y and limiting concentration ($=C_E$) in mol L^{-1}, respectively. The K_f of the complex was found to be 1.437×10^{14}. The apparent Gibbs free energy (ΔG^o) was calculated using $\Delta G^o = -2.303$ RT log K_f and found to be -80.783 KJ mol^{-1}, confirming the feasibility of the reaction.

Figure 3 showed the IR spectra of (a) free diphenhydramine (b) Eosin Y and (c) diphenhydramine-eosin Y complex. The FTIR spectra of free diphenhydramine exhibited peaks at 3032.10 and 2893.22 cm^{-1} for C-H stretching vibrations [23], [24]. Other FTIR peaks of free diphenhydramine were exhibited at 713.66, 756.10, 991.41, 1018.41, 1103.28 and 1180.44 cm^{-1}. Eosin exhibited most important band at 3398 cm^{-1} for O-H stretching vibration and 1604 and 1554 cm^{-1} for C=O stretching vibrations. On comparing IR spectra, it was noticed that in diphenhydramine-eosin complex the peak at 3398 cm^{-1} (which was in Eosin Y) disappeared and showed a decrease in the intensity over 2449.60-2565.33 cm^{-1} [24], [25] due to overlap of γ-NH^+ of the diphenhydramine and γ -C=O of eosin Y. The reaction sequence is shown in Figure 4.

Method Optimization

The experimental variables such as reaction time, concentration of eosin Y, buffer solutions of different pH, solvents and shaking time for extraction of complex were optimized for the proposed spectrofluorimetric method.

The effect of the reaction time on the fluorescence emission intensity of ion-pair complex was studied from 1 to 70 min and found to be stable for 1 h at 25 ± 1˚C.

The influence of the volumes of 2.89×10^{-4} M eosin Y was studied at 20.0 µg mL^{-1} diphenhydramine hydrochloride on the fluorescence emission intensity of ion pair complex. The maximum fluorescence emission intensity was achieved at 1.0 mL of 2.89×10^{-4} M eosin Y and above this volume up to 1.6 mL, the fluorescence emission intensity remained constant. 1.3 mL of 2.89×10^{-4} M eosin Y was used as the optimum volume for the determination of diphenhydramine hydrochloride.

The influence of pH on the fluorescence emission intensity of diphenhydramine-eosin Y complex was investigated using 2.0 mL of Na_2HPO_4-citric acid buffer solution of different pH in the range of 3.0-6.6

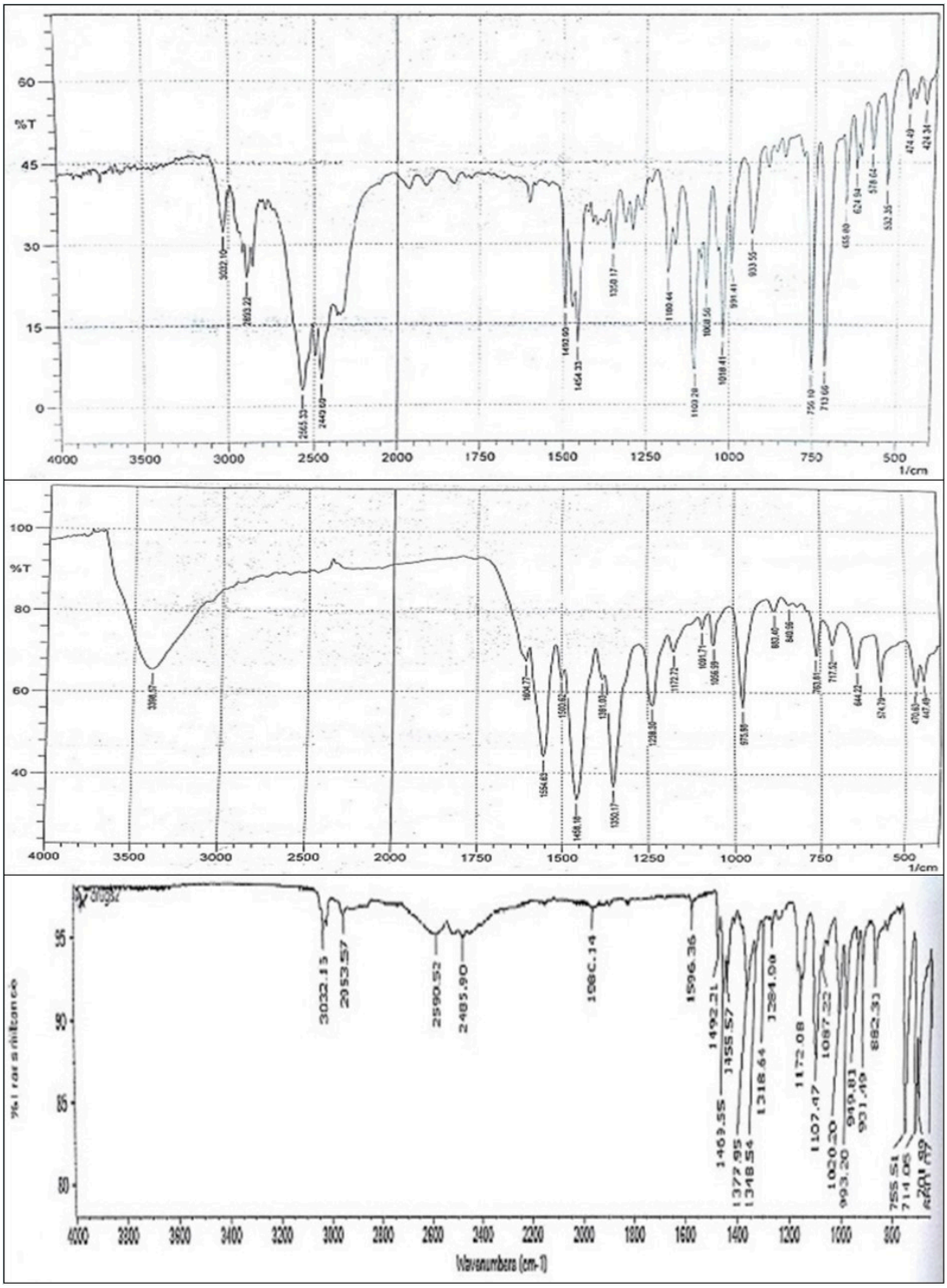


Figure 3. Infrared spectra of (a) pure diphenhydramine (b) free eosin Y and (c) diphenhydramine-eosin Y complex in KBr (2 mg sample/200 mg KBr).

with 20.0 µg mL^{-1} diphenhydramine and 1.3 mL of 2.89×10^{-4} M eosin Y. The fluorescence emission intensity was recorded for each determination and the maximum fluorescence intensity of ion-pair complex was achieved at pH 4.2 and constant up to pH 5.8. Above pH 5.8, the fluorescence emission intensity decreased. Therefore, 2.0 mL of Na_2HPO_4-citric acid buffer solution of pH 5.0 was selected as the optimum pH for determination of diphenhydramine hydrochloride.

The effect of the shaking time for the extraction of the ion pair complex was tested in the range of 0.5-3.0 min. The maximum fluorescence emission intensity of the complex was obtained at 1.5 min.

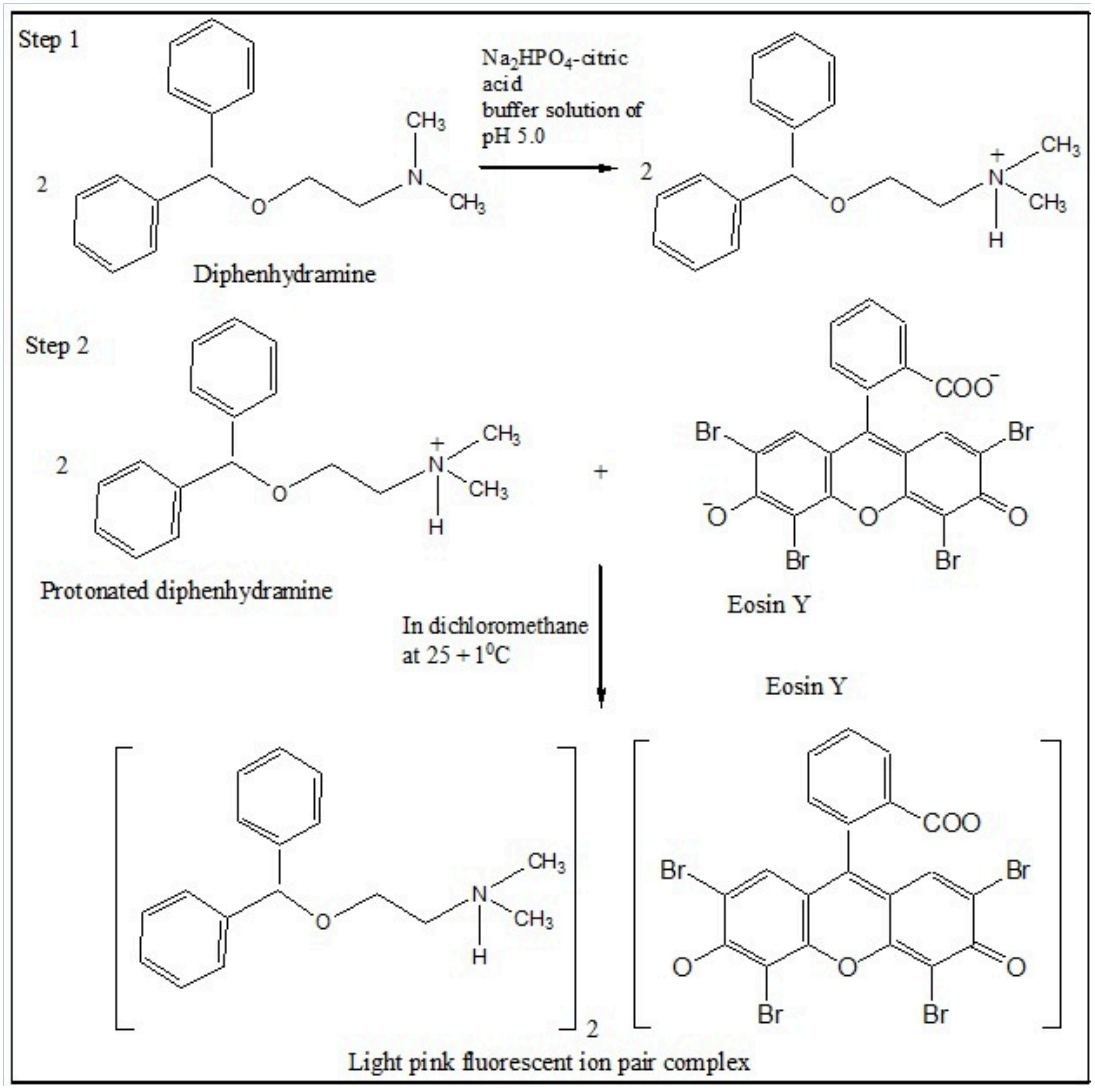


Figure 4. Reaction sequence of diphenhydramine-eosin Y ion pair complex.

After this time, the intensity remained constant up to 2.5 min. Therefore, the shaking time of 2.0 min was adequate for the extraction of ion-pair complex into dichloromethane.

The fluorescence emission intensity of the ion-pair complex was recorded in different solvents of benzene, hexane, chloroform, carbon tetrachloride, dichloromethane, dichloroethane, and ethyl acetate and found to be maximum in dichloromethane. There was no fluorescence emission intensity recorded in benzene and hexane. The fluorescence emission intensity in the blank solution was also recorded and found to be negligible in the solvent. Therefore, dichloromethane was selected as the best solvent for extraction of the complex.

Validation

For the proposed method, a series of nine different concentrations of diphenhydramine were prepared and the fluorescence emission intensity of the complex was recorded at an emission wavelength of 554 nm. The calibration graph was constructed by plotting fluorescence emission intensity against initial concentration of diphenhydramine in µg mL^{-1}. The linear relationship between the measured fluorescence emission intensity and initial concentration of diphenhydramine HCl was found to be in the concentration range 2-22 µg mL^{-1}. The calibration data were utilized to get slope, intercept, coefficient of correlation, detection limit and quantitation limit, standard deviation of intercept and slope, variance, standard deviation of the calibration line of proposed and reference methods using OriginPro 6.1 software. The results are summarized in Table 1. The linearity of the regression line was excellent.

Table 1. Optical and analytical characteristics of the proposed and reference methods

Parameters	Proposed method	Reference method [21]
Absorption wavelength (nm)	259	285
Maximum emission wavelength (nm)	554	207
Linear dynamic range (µg mL^{-1})	2-22	1-5
Linear regression equation	FI = 0.361 + 13.675 C	FI = 0.410 + 39.155 C
Standard deviation of intercept, S_a	0.329	0.384
Confidence limit of the intercept	0.778	1.222
Standard deviation of slope, S_b	0.022	0.116
Confidence limit of the slope	0.052	0.369
Correlation coefficient (r)	0.999	0.999
Standard deviation of calibration line (S_o)	0.426	0.367
Variance (S_o^2)	0.181	0.135
Limit of detection, LOD (µg mL^{-1})	0.103	0.031
Limit of quantification, LOQ (µg mL^{-1})	0.310	0.094

This fact was explored due to the high value of correlation coefficient (0.9999). The significance of intercept was studied by calculating t-value using the following equation [26]:

$$t=a/S_a \quad \textbf{Eq. 2}$$

The value of t was found to be 1.095 which is less than the tabulated t-value (2.365, ν=7) at 95% confidence level [27]. Therefore, it is concluded that a significant relationship between the experimental fluorescence emission intensity and concentration existed and the proposed method was error free. Limits of detection (LOD) and quantitation (LOQ) were calculated according to the International conference on Harmonization guidelines. The low value of LOD (0.103 µg mL^{-1}) indicated good sensitivity of the proposed method.

The precision of the proposed method was evaluated in terms of intra-day and inter-day precisions. The intra-day precision was tested using test samples for five times per day at 3 concentration levels i.e. 4, 12 and 20 µg mL^{-1}. Inter-day precision was evaluated by investigating the test samples for five times once daily for five consecutive days. The results are summarized in Table 2. The RSD values were in the ranges of 0.07-0.82 % for intraday and inter day precisions. The low % RSD values at different concentrations showed that the proposed method is precise and can be used to analyze diphenhydramine HCl in pharmaceutical preparations.

The statistics was applied to establish the confidence limit [28] at 95% confidence level and n-2 degrees of freedom for the quantitation of an unknown concentration of diphenhydramine using the following formula:

$$C_i \pm \frac{tS_0}{b}\left[1+\frac{1}{n}+\frac{(F-\bar{F})^2}{b^2\sum(C-\bar{C})^2}\right]^{\frac{1}{2}} = C_i \pm \Delta C \quad \textbf{Eq. 3}$$

Where $\bar{C}$ and $\bar{F}$ are average concentration (diphenhydramine) and fluorescence emission intensity values, respectively, for n standard solutions. Figure 5 provided % uncertainty plot in concentration (ΔC/

Table 2. Intra and Inter day precisions of the proposed method

Actual concentration (µg mL^{-1})	Intra day assay: Found concentration ± SD (µg mL^{-1}); RSD (%)[a]	Inter day assay: Found concentration ± SD (µg mL^{-1}); RSD (%)[a]
4.0	3.979± 0.010; 0.28	4.026 ±0.033; 0.82
12.0	11.974±0.022; 0.19	12.004 ± 0.068; 0.57
20.0	20.014±0.013; 0.07	20.002 ± 0.048; 0.24

[a] Mean for five independent analysis.

C_i×100) at 95% confidence level versus initial concentration of diphenhydramine. Thus, the relative uncertainty can be estimated directly on the concentration level tested.

In order to assure the applicability of the proposed method, a systematic quantitative study was performed in order to find out the tolerated amount of common excipients (ammonium chloride, sodium citrate, glucose, fructose, lactose, sodium benzoate, starch, povidone, methyl cellulose and

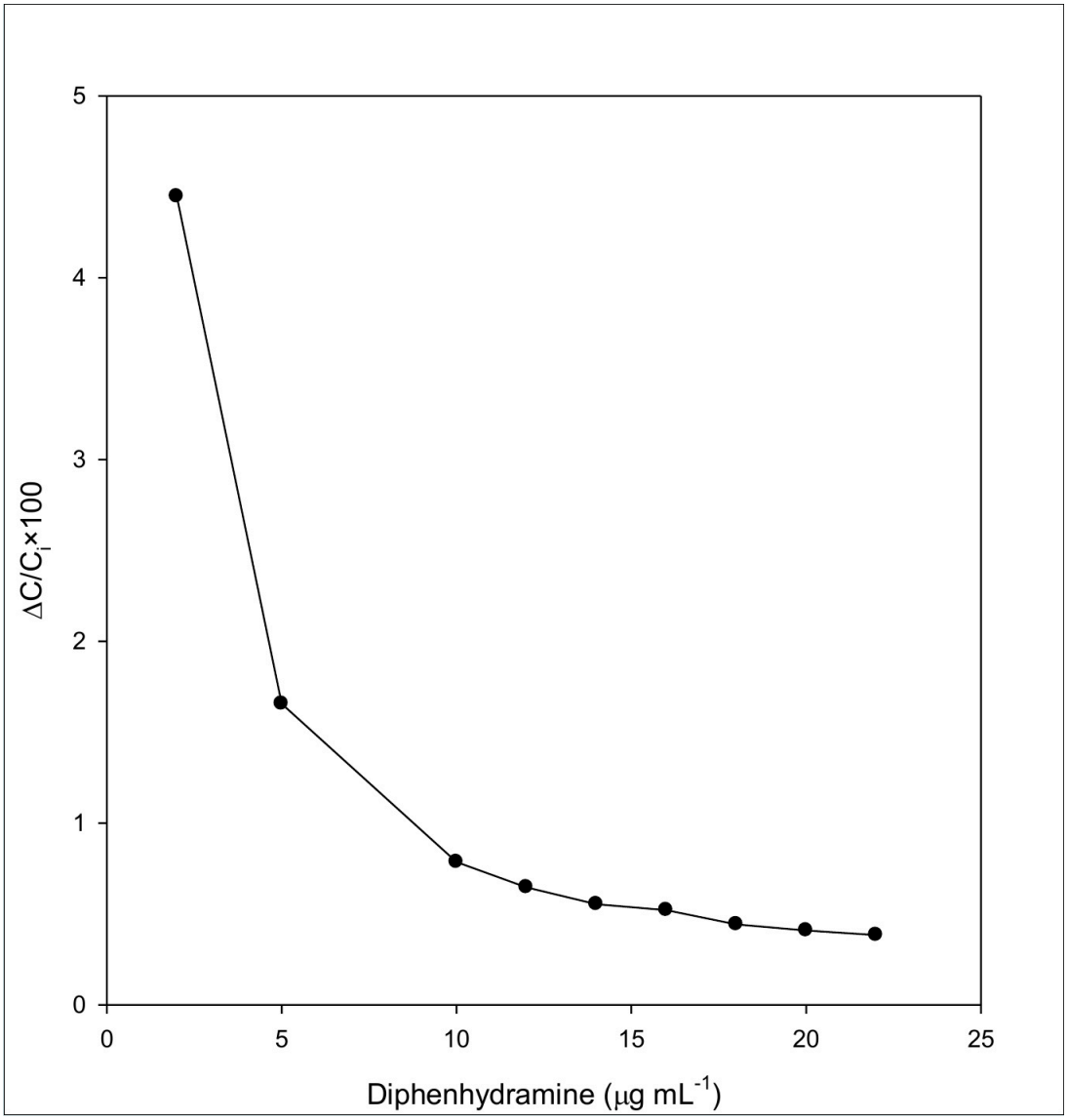


Figure 5. % uncertainty plot in concentration at 95% confidence level.

Table 3. Specificity and selectivity of the proposed method: Tolerated amount of excipients at 20 µg mL^{-1} diphenhydramine

Excipients	Tolerance amount (µg mL^{-1})
Glucose	5.504
Fructose	5.504
Sodium benzoate	1.502
NH_4Cl	1.002
Sodium citrate	1.502
Lactose	9.557
Starch	0. 152
Povidone	0. 152

micro crystalline cellulose) in drug formulations. The tolerated amount of excipients at 20 µg mL^{-1} diphenhydramine HCl was calculated using the following expression:

$$\text{Mass/Volume(mg/L)}=C\times MW\times 1000 \quad \textbf{Eq.4}$$

where C and MW are concentration and molecular weight of excipients, respectively.

The results are summarized in Table 3. It is evident from the table that the proposed method tolerated larger amount of excipients and hence indicated that the proposed method was more specific and selective. Therefore, the proposed method is suitable to determine active diphenhydramine in pharmaceutical preparations.

The accuracy of the proposed method was investigated by standard addition technique. In this method, the known amount of pure diphenhydramine was spiked with a constant amount of Stopkof syrup solution and the fluorescence emission intensity of the complex was recorded. Standard addition plot was constructed using fluorescence emission intensity at y-axis and initial concentration of drug at x-axis (Figure 6). The regression line was generated with intercept of 137.33 and slope of 13.61, respectively. The amount of diphenhydramine in syrup solution was estimated and found to be 10.09 µg mL^{-1}. The amount of drug in syrup solution is subjected to standard deviation, S_{xE}, and can be calculated by the following expression [28]:

$$S_{x_E} = \frac{S_{y/x}}{b}\sqrt{\frac{1}{n}+\frac{\bar{y}^2}{b^2\sum_i(x_i-\bar{x})^2}} \quad \textbf{Eq.5}$$

The value of S_{xE} was found to be 0.04 µg mL^{-1}. The confidence limit for the concentration of diphenhydramine in syrup was calculated using $x_E \pm tS_{xE}$ at n - 2 degrees of freedom and found to be 10.09 ± 0.14 µg mL^{-1}.

The applicability of the proposed method for the determination of active diphenhydramine in Stopkof, Histalix and Amydramine has been tested. The results of the proposed method were statistically compared with those of the reference method using point and interval hypothesis tests. t- and F-values at 95% confidence level were calculated using point hypothesis test. The results are summarized in Table 4. It is clear from the table that the calculated t- and F values are less than the theoretical ones at 95% confidence level, indicating no significant difference between the methods compared.

Interval hypothesis test was also utilized to calculate bias within the acceptable range of ±2% [29] using the following quadratic equation.

$$\theta^2\left(x_1^2 - S_p^2 t_{tab}^2/n_1\right) + \theta \times -2x_1x_2 + \left(x_2^2 - S_p^2 t_{tab}^2/n_2\right) \quad \textbf{Eq. 6}$$

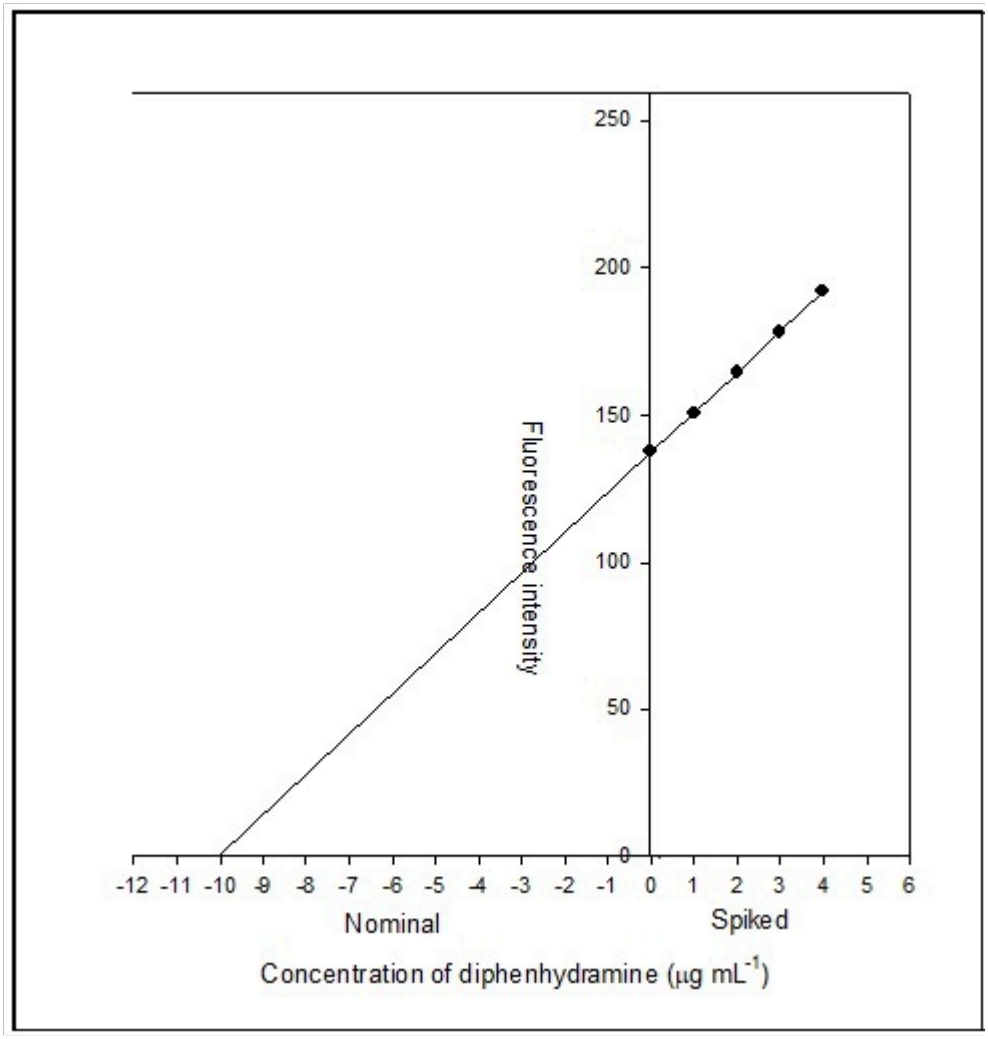


Figure 6. Standard addition plot: 0.5 mL of 0.02% diphenhydramine Stopkof syrup solution was spiked with 0, 0.05, 0.1, 0.15 and 0.2 mL standard solution of 0.02% pure diphenhydramine

Lower limit (θ_L) and upper limit (θ_U) were calculated and found to be in the range of 0.98-1.02 indicating the compliance of regulatory guidelines [30].

Table 4. Point and interval hypothesis tests for the determination of diphenhydramine in pharmaceutical preparations at 95% confidence level.

Formulations	Proposed method		Reference method		Paired t-value[c]	F-value[c]	θ_L[d]	θ_U[d]
	Recovery (%)	RSD[b] (%)	Recovery (%)	RSD[b] (%)				
Stopkof[a]	99.88	0.371	100.10	0.291	1.564	2.83	0.996	1.008
Histalix[a]	99.81	0.311	100.05	0.235	1.366	1.75	0.995	1.007
Amydramine[a]	99.42	0.449	99.92	0.206	1.357	4.67	0.998	1.011

[a] Stopkof, Histalix and Amydramine excipients are NH_4Cl, sodium citrate and menthol.
[b] Mean for 5 independent analyses.
[c] Theoretical *t* (V = 10) and *F*-values (V = 5, 5) at 95% confidence level are 2.306 and 6.39, respectively.
[d] A bias, based on recovery experiments, of ± 2% is acceptable.

Conclusions

The proposed method based on spectrofluorimetry is very useful because many of the excipients are non-fluorescent in nature. The method was successfully applied for the determination of active diphenhydramine in stopkof, histalix and amydramine in the presence of excipients. The proposed method is easy to operate, utilizing less analysis time and economical with a commonly available solvent. The proposed method can be utilized as an alternate method for routine quality control analysis of active diphenhydramine in research laboratories, hospitals and pharmaceutical industries.

Competing Interests

The authors have declared that no competing interests exist.

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