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UTILITY OF CERTAIN SPECTROFLUORIMETRIC METHODS FOR ANALYSIS OF TWO PHARMACEUTICAL BINARY MIXTURES

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تم فى هذا البحث استخدام طرق لصفية لتعيين اثنين من المخاليط الثنائية. يتكون الأول من الادرينالين والبروكايين هيدروكلوريد والثانى من كبريتات السالبيوتامول والجوايفينسين. فى المخلوط الأول تم تعيين الأدرينالين بمفرده فى وجود البروكايين اعتمادا على التزاوج بينه وبين ملح ديازوترايزول حمض الكربوكسيل (د. ت. ك. ا.) فى وسط قاعدى وقياس شدة الوميض الناتج عند طول موجى قدرة ٣٤٠ نانوميتر (عند إثارة قدرها ٢٤٥ نانوميتر) بينما لم يعط البروكايين اى وميض. فى المخلوط الثانى تم تعيين كبريتات السالبيوتامول من خلال تفاعله مع استيل اسيتواسيتات لكى يكون احد مشتقات الكيومارين والتى لها وميض يمكن قياسة عند طول موجى قدرة ٣٢٠ نانوميتر (عند طول موجى للإثارة قدره ٢٨٠ نانوميتر). بينما تم تعيين الجوايفينسين عن طريق قياس شدة الوميض الذاتى لة فى الميثانول عند طول موجى قدرة ٣١٠ نانوميتر (عند طول موجى للإثارة قدرة ٢٣٠ نانوميتر). وقد تمت دراسة جميع المتغيرات التى توثر على التفاعلات واختيار انسبها. وتم تطبيق الطرق المقترحة بنجاح لتحليل العقاقير تحت الدراسة سواء فى صورتها النقيه اوفى مستحضراتها الصيدلية مع مقارنة نتائجها بنتائج الطرق المنشورة وقد وجد ان هذه النتائج متطابقة احصائيا مما يدل على دقة واحكام الطرق المقترحة.

*Simple and very sensitive spectrofluorimetric methods were developed for determination of adrenaline (**I**)–procaine hydrochloride (**II**) mixture and salbutamol sulfate (**III**) – guaifenesin (**IV**) mixture. Adrenaline (**I**) in the first mixture was determined by coupling with 5-diazo-1,2,4-triazolo-3-carboxylic acid (DTCA) reagent in alkaline medium forming fluorigenic product which can be measured at 340 nm (λex. 245 nm), while procaine hydrochloride (**II**) gave no fluorescence. Salbutamol sulfate (**III**) was analyzed by reaction with ethyl acetoacetate (EAA) forming coumarin derivative, which can be measured at 320 nm (λex. 280 nm). Guaifenesin (**IV**), the second drug in mixture has*

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*a considerable native fluorescence in methanol was measured at 310 nm (λex. 230 nm). All variables affecting reaction conditions were optimized. Linear correlations were obtained over the range of 19-100, 37-400 and 22-150 ng/ml for (**I**), (**III**) and (**IV**), respectively. The proposed methods were successfully applied for the analysis of the studied drugs in their pure and commercial dosage forms and the obtained results were in good agreement with those obtained from the reported methods; no significant difference in the accuracy and precision as revealed by the accepted values of t- and F-tests, respectively.*

INTRODUCTION

Adrenaline, is mainly used as a constrictor with procaine hydrochloride, which is a local anesthetic drug, to enhance the activity, retard diffusion, and limit absorption and to prolong their duration of action[1]. Guaifenesin is used as an expectorant[2] with salbutamol sulfate, which is a selective β_2-adrenergic agonist, in treatment of airway obstruction as in asthma and is used for treatment of cough[1].

Several analytical methods have been reported for the determination of the studied drugs either simultaneously or for the determination of one drug in the presence of the others. The studied drugs were determined spectrophotometrically[3-9], fluorimetrically[10-13], voltammetrically[14&15] and by high performance liquid chromatography[16-26]. A DTCA reagent, which was previously used as a chromogenic reagent for many drug classes[27&28] to form colored products in alkaline media, now it is used as fluorogenic reagent for determination of adrenaline.

EXPERIMENTAL

Apparatus

An RF-5301 PC (Shimadzu, Tokyo, Japan) Spectrofluorometer was used for fluorimetric measurements, the slit width of both excitation and emission monochromators were set at 5 nm.

Materials and reagents

All chemicals and solvents used throughout this work were of analytical grade. Adrenaline, procaine hydrochloride and guaifenesin (CID, Cairo, Egypt), salbutamol sulphate (Pharco, Alexandria, Egypt) were used as working standards. 5-Diazo-1,2,4-triazol-3-carboxylic acid (DTCA) was synthesized according to reported method[29&30]. Sodium hydroxide and sulfuric acid (El-Nasr Pharmaceutical Chemical Co., Abo-Zaabal, Egypt).

Adrenaline-procaine hydrochloride ampoule and Bronchovent syrup (Misr Co., Cairo, Egypt), Adrenaline ampoules (Memphis Co., Cairo, Egypt) and Ventoline tablets and syrup (Glaxo smithkline Co., Cairo,

Egypt) were purchased from local market.

Preparation of standard solutions

An accurately weighed amount (50 mg) of each of the studied drugs were transferred into a 50-mL standard flask containing about 30 mL of methanol or ethanol (in case of salbutamol sulfate) and completed to the mark with the same solvent to provide a stock standard solution containing 1 mg/mL of each drug. Serial dilutions with the same solvent were made to obtain the suitable concentrations.

DTCA solutions

Aqueous solution of 1 mg/mL of DTCA was prepared and protected from light. Serial dilutions were made to obtain the suitable concentrations (0.02-0.16 mg/mL).

Sodium hydroxide

One molar solution of sodium hydroxide was prepared in previously boiled and cooled distilled water. Several dilutions were made to obtain the suitable concentrations (1- 9 mM).

Ethyl acetoacetate (EAA) solutions

EAA solution (4% v/v) was prepared by diluting 2.0 mL of EAA in 50-mL absolute ethanol. Further dilutions with ethanol were made to obtain the desired concentrations.

Dosage forms

Tablets

Twenty tablets were accurately weighed, finely powdered and mixed thoroughly. An accurately weighed quantity of the powdered tablets equivalent to 5.0 mg of the studied drug was transferred into a 50-mL standard flask containing about 30 mL of ethanol. The contents of the flask were shaken well for 5 minutes, completed to the mark with ethanol and sonicated for 10 minutes. The solution was filtered, and the first portion of the filtrate was rejected. The obtained filtrate was used as a stock sample solution for application of the general procedures.

Ampoules

Procaine-adrenaline ampoules

The contents of ten ampoules were mixed well, in case adrenaline an accurately measured volume equivalent to 0.05 mg of adrenaline (5 mL) was transferred into a 50-mL standard flask. The procedure was completed as mentioned under tablets starting from "containing about 30 mL of methanol …… "without filtration.

Adrenaline ampoules

The contents of ten ampoules were mixed well and an accurately measured volume equivalent to about 5 mg was transferred into a 50-ml standard flask. The procedure was completed as mentioned under tablets starting from "containing about 30 mL of methanol…." without filtration.

Syrup

An accurately measured volume of the syrup equivalent to 5.0 mg of the drug was transferred to a 30-mL standard flask containing about 30

mL of methanol (in case of guaifenesin) or ethanol (in case of salbutamol sulfate). The contents of the flask were shaken well for 5 minutes, completed to the mark with the same solvent and sonicated for 5 minutes. The obtained solution was used as a stock sample solution for application of the general procedures.

General assay procedures

I- Determination of adrenaline in presence of procaine hydrochloride

One milliliter of the working standard or sample solution in the concentration range (190-1000 ng/mL) was transferred into a 10-mL standard flask. One milliliter of DTCA reagent (0.1 mg/mL) and one milliliter of 6 mM sodium hydroxide were added. The contents of the flask were diluted with methanol. The relative fluorescence intensity (RFI) of the resulting solution was measured at $\lambda_{em.}$340 nm ($\lambda_{ex.}$ 245 nm).

II- Binary mixture of salbutamol sulfate and guaifenesin

a) For salbutamol sulfate

One milliliter of the working standard or sample solution in the concentration rang (370-4000 ng/mL) was transferred into a 10-mL standard flask. One milliliter of 1.2% v/v EAA in ethanol and 0.4 mL of conc. sulfuric acid were added. The contents of the flask were allowed to stand at room temperature (25 ± 5°C) for 10 minutes and then diluted with ethanol. RFI of the resulting solution was measured at $\lambda_{em.}$320 nm ($\lambda_{ex.}$ 280 nm).

(b) For guaifenesin

One milliliter of the working standard or sample solution in the concentration rang (220-1500 ng/mL) was transferred into a 10-mL standard flask. The content of the flask was diluted with methanol. RFI of the resulting solution was measured at $\lambda_{em.}$310 nm ($\lambda_{ex.}$230 nm).

RESULTS AND DISCUSSION

I- Determination of adrenaline in presence of procaine hydrochloride

The spectrofluorimetric method is based on the coupling reaction of adrenaline with DTCA reagent. The relative fluorescence intensity of the fluorigenic product was measured at $\lambda_{em.}$ 340 nm ($\lambda_{ex.}$ 245 nm). (Fig. 1) shows the excitation and emission spectra of the fluorigenic product. While procaine hydrochloride has no fluorescence under the same conditions.

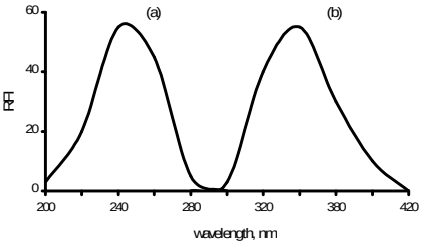


Fig. 1: (a) and (b) excitation and emission spectra of the reaction product of DTCA with adrenaline (50 ng/ml).

Optimization of Reaction Variables in fluorimetric method

Various parameters such as concentration of the diazonium salt (DTCA), type and concentration of alkali, diluting solvent, reaction and stability time were studied for their effect on the intensity and stability of the developed fluorigenic product.

(1) Concentration of diazonium salt (DTCA)

Different concentrations of DTCA reagent (0.02-0.16 mg/mL) were tested during this study. Fluorescence intensity reached its maximum value when the reagent concentration was between 0.08 and 0.12 mg/mL. A gradual decrease in fluorescence intensity was observed with further increase in reagent concentration. Therefore, 0.1 mg/mL was selected to be used (Fig. 2).

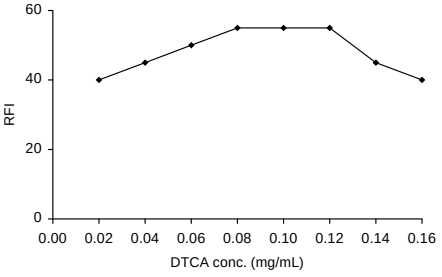


Fig. 2: Effect of DTCA concentration on relative fluorescence intensity of the reaction product of adrenaline (50 ng/ml).

(2) Type and concentration of alkali

Different types of alkali were tested for the reaction of the diazonium salt with the studied drug e.g.; sodium hydroxide, sodium carbonate, sodium bicarbonate and sodium acetate. It was found that sodium hydroxide gave the best intensity reading, so it was selected as alkali medium. Different concentrations of sodium hydroxide were studied from 1-10 mM. Fluorescence intensity reached its maximum value when the sodium hydroxide concentration was between 5 and 7 mM. Therefore, 6 mM was selected to be used as shown in (Fig. 3).

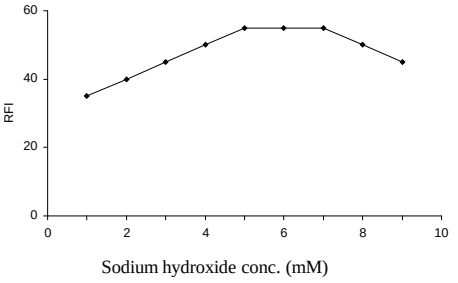


Fig. 3: Effect of sodium hydroxide concentration on relative fluorescence intensity of the reaction product of adrenaline (50 ng/ml) with DTCA reagent.

(3) Reaction time at room temperature

The effect of time on the relative fluorescence intensity of the fluorigenic products of adrenaline

with DTCA was studied at room temperature (25 ± 5 ºC). It was found that constant fluorescence values were obtained at once and remain stable for further 30 minutes.

(4) Diluting solvent

Different solvents such as distilled water, methanol, ethanol, acetonitrile, dimethyl formamide (DMF) and dimethyl sulphoxide (DMSO) were tested as a diluting solvent. Methanol gave the highest intensities, so it was used as a diluting solvent (Table 1).

(5) Stability time

The fluorigenic product of the adrenaline with DTCA remains stable for about 20 minutes after dilution, and then gradual decrease in the intensities was observed. So measurement must be done within these 20 minutes.

II- Binary mixture of salbutamol sulfate and guaifenesin

a- For salbutamol sulfate

Spectrofluorimetric determination of salbutamol sulfate was based on coumarin reaction. Salbutamol sulfate has phenolic hydroxyl group, which involved in a condensation reaction with EAA in ethanolic sulfuric acid media (Fig. 4) shows the excitation and emission spectra of the fluorigenic product obtained from coumarin condensation reaction. Guaifenesin has no fluorescence under this condition.

b- For guaifenesin

Guaifenesin has a native fluorescence in methanol and the relative fluorescence intensity was measured at λ_{em} = 310 nm (λ_{ex} = 230 nm) as shown in (Fig. 5). Salbutamol sulfate has no fluorescence under the same condition.

Table 1: Effect of diluting solvents on the RFI of the fluorogenic product of adrenaline, salbutamol sulphate and guaifenesin

Solvent	Adrenaline		Salbutamol sulfate		Guaifenesin	
	Δλ	RFI	Δλ	RFI	Δλ	RFI
Methanol	94	52.2	38.0	30.1	78.6	65.0
Ethanol	98	45.5	42.0	73.0	78.0	19.6
Acetonitrile	85	42 .0	35.0	24.9	33.0	58.4
DMF	67	28.0	41.5	53.4	96.0	15.9
DMSO	72	25.3	39.5	55.0	55.0	21.3
Distilled water	50	8.0	43.7	52.1	79.8	60.6

Δλ : Stoke's shift.

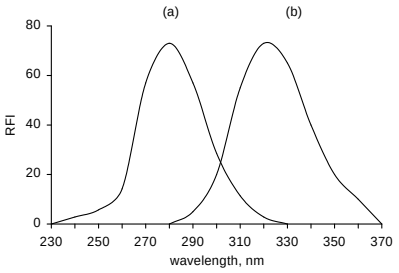


Fig. 4: (a) and (b) excitation and emission spectra of the reaction product of EAA with salbutamol sulfate (200 ng/ml).

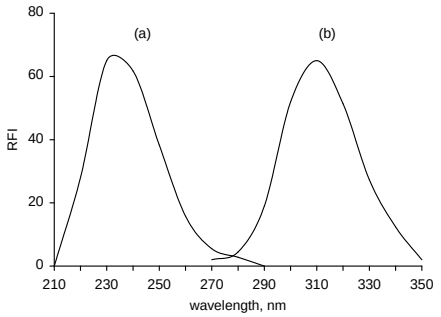


Fig. 5: (a) and (b) excitation and emission spectra of guaifenesin (75 ng/ml) in methanol.

Optimization of Reaction Variables

Various parameters such as concentration of the EAA reagent, volume of sulfuric acid, diluting solvent, reaction and stability time were studied for their effect on the intensity and stability of the developed fluorigenic product.

(1) Concentration of EAA reagent

Different concentrations of EAA reagent were tested during this study. Fluorescence intensity reached its maximum value when the reagent concentration was between 1.0 and 2.2% v/v. A gradual decrease in fluorescence intensity was observed with further increase in reagent concentration. Therefore, 1.2% v/v was selected to be used in the subsequent work (Fig. 6).

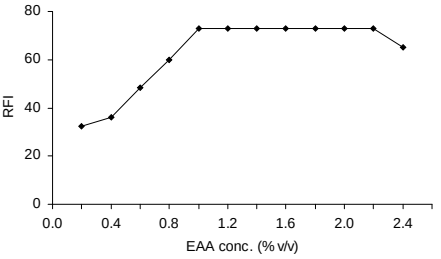


Fig. 6: Effect of EAA concentration on fluorescence intensity of its reaction product with salbutamol sulfate (200 ng/ml).

(2) Volume of sulfuric acid

Figure 7 shows that there is gradual increase in the fluorescence intensity with the increase of sulfuric acid volume until it reaches constant RFI values between 0.3 and 0.6 mL, therefore 0.4 mL of sulfuric acid was selected to be used in the reaction.

(3) Reaction time at room temperature

The effect of time on the RFI of the fluorigenic product at room temperature (25±5ºC) was studied. It was found that constant fluorescence values were obtained after 10 minutes and remain stable for further 10 minutes.

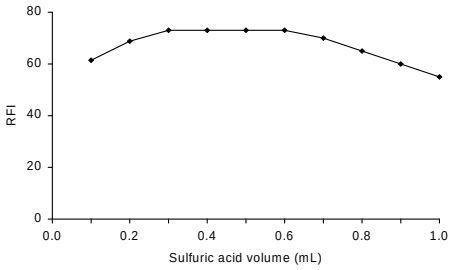


Fig. 7: Effect of volume of concentrated sulfuric acid on fluorescence intensity of the reaction product salbutamol sulfate (200 ng/ml) with EAA.

(4) Diluting solvent

Different solvents such as distilled water, methanol, ethanol, acetonitrile, dimethyl formamide (DMF) and dimethyl sulphoxide (DMSO) were tested as a diluting solvent. Ethanol was selected as a diluting solvent for salbutamol sulfate and methanol was used for guaifenesin, because they gave the highest intensities (Table 1).

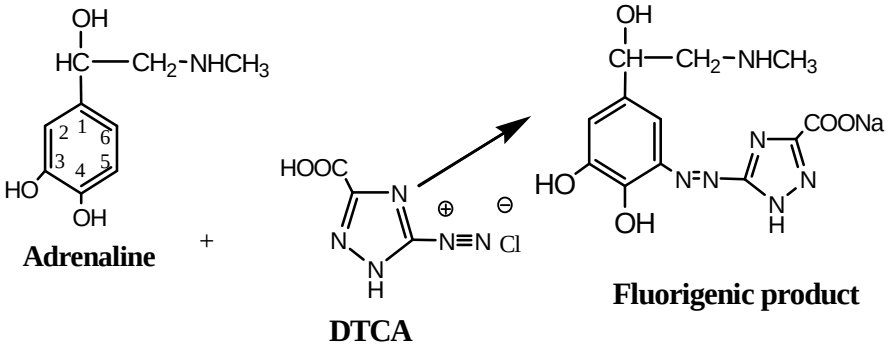
(5) Stability time

The Fluorigenic product of salbutamol sulfate with EAA reagent (coumarin derivative) remains stable for about 20 minutes after dilution, and then gradual decrease in the RFI was observed. So measurements must be done during 10 minutes.

Suggested mechanisms

For adrenaline

Adrenaline was found to couple with DTCA in alkaline medium to form fluorigenic product which was measured at $\lambda_{em.}$340 nm ($\lambda_{ex.}$245 nm). Coupling reaction may occur at para or ortho position[31]. The para position is occupied, so coupling takes place at ortho position at C_5 not at C_2 or C_6 due to steric hinderance (Scheme 1).



Scheme 1

For salbutamol

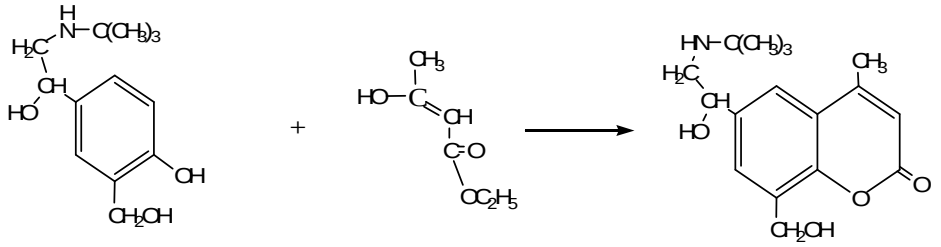
Salbutamol sulfate was analyzed by reaction with ethyl acetoacetate using sulfuric acid as dehydrating agent. This method depends on the condensation between ethyl acetoacetate and the phenolic compound salbutamol sulfate to form coumarin derivative[32]. The fluorigenic product was measured at 320 nm with excitation wavelength at 280 nm (Scheme 2).

Linearity, detection and quantitation limits[33]

Under the above mentioned optimal reaction conditions, the relationship between the relative fluorescence intensity and concentration of each of the studied drug was quite linear in the concentration range 19–100, 37-400, 22-150 ng/ml for adrenaline, salbutamol sulfate, and guaifenesin, respectively. The regression equations were derived using the least square method. The limit of detection (LOD) and limit of quantitation (LOQ) values were determined using the formula:

$$LOD \text{ or } LOQ = k.\delta/S$$ [34]

Where k = 3.3 for LOD and 10 for LOQ, δ is the standard deviation of the response and S is the slope. The results are represented in Table 2, indicating higher sensitivity of the proposed procedures as compared to the reported methods (3, 6 and 9).



Scheme 2

Table 2: Comparative summary of some statistical data for intact drugs using the proposed methods

Drug	Calibration Range (ng/ml)	Intercept ± SD (a)	Slope ± SD (b)	Correlation Coefficient (r)	Determination Coefficient (r^2)	LOD* (ng/ml)	LOQ** (ng/ml)
Adrenaline	19-100	0.805 ± 1.948	1.027± 0.027	0.9983	0.9966	5.69	18.9
Salbutamol sulfate	37 – 400	3.425 ± 1.268	0.349 ± 0.005	0.9994	0.9988	10.9	36.3
Guaifenesin	22-150	-6.630 ± 2.053	0.953 ± 0.021	0.9985	0.9970	6.5	21.5

*Limit of Detection **Limit of Quantitation

Precision

The precision of the developed procedures was confirmed by analyzing six replicate samples at three concentration levels for all the studied drugs by the two suggested methods. The relative standard deviations by the proposed methods were found to be ranged 0.90-1.31, 0.90-1.11 and 1.10-1.50 for I, II and III respectively. As we see, the values of RSD were less than 2 and this level of precision is adequate for the routine analysis in quality control laboratories.

Accuracy and analysis of pharmaceutical formulations

The commercially available pharmaceutical formulations of the studied drugs were subjected to analysis by the proposed and reported methods (3, 6 and 9). The obtained results were then statistically compared with each other. The mean percentages label claim, relative to the labeled amounts, obtained by the proposed method ranged from 97.9-101.2 ± 0.37– 0.90 (Table 3). With respect to t- and F-tests, no significant differences were found between the calculated and theoretical values of the proposed and the reported methods at 95% confidence level this indicated similar accuracy and precision in the analysis by the proposed and reported methods.

Table 3: Determination of studied drugs in some pharmaceutical preparations using proposed and reported methods.

Pharmaceutical Dosage forms	Ingredient (mg)	% Recovery ± SD (n=6)*		F-value	t-value
		Proposed method	Reported method		
Adrenaline (ampoules)	Adrenaline (1/ml)	100.1 ± 0.66	99.5 ± 0.40 [3]	2.067	1.711
ProcaineHCl-adrenaline ampoule	Procaine HCl (10/ml)	----------	--------	--------	--------
	Adrenaline (1/ml)	97.9 ± 0.90	99.6 ± 0.83 [3]	2.156	1.564
Bronchovent (syrup)	Salbutamol sulfate (2/5ml)	98.9 ± 0.53	99.5 ± 0.46 [6]	1.299	2.213
	guaifenesin (50/5ml)	101.2 ± 0.37	100.8 ± 0.50 [9]	2.011	1.586
Ventoline (syrup)	Salbutamol sulfate (2/5ml)	100.1 ± 0.66	99.5 ± 0.47 [6]	2.054	1.821
Ventoline (tablets)	Salbutamol sulfate (2 mg)	99.7 ± 0.47	99.9 ± 0.42 [6]	1.191	0.659

*Theoretical values of F and t at 95% confidence limit are 5.050 and 2.228.

Standard addition method

To confirm the accuracy of the proposed method, recovery studies were performed by using standard addition method. This depends upon the addition of different amounts of each drug from their corresponding pharmaceutical dosage forms to a known fixed amount of the standard drug. The resulting solution was analyzed by the proposed method. The difference in RFI of standard and sample plus standard was used to calculate the concentration of sample. Results presented in Table 4 indicates good recoveries and confirm the absence of interference due to common excipients and, hence accuracy of the proposed method.

Table 4: Standard addition method for the assay of the studied drugs in their pharmaceutical dosage forms by fluorimetric methods.

Pharmaceutical dosage form	Claimed (ng)	% Recovery ± SD*				
Adrenaline (ampoules)		a	b	c	d	e
Adrenaline	30	99.4 ± 0.5	98.3 ± 0.7	101.4 ± 1.2	101.3 ± 0.3	98.7 ± 0.1
Bronchovent (syrup)						
Salbutamol sulfate	50	97.1 ± 1.0	101.0 ± 0.4	98.4 ± 0.1	100.9 ± 0.5	98.0 ± 0.7
Guaifenesine	40	101.4 ± 1.1	99.1 ± 0.4	99.7 ± 0.3	101.6 ± 0.9	97.7 ± 1.1
Ventoline (syrup)						
Salbutamol sulfate	50	100.3 ± 0.6	99.3 ± 0.5	98.1 ± 0.2	100.1 ± 0.9	98.3 ± 0.9
Ventoline (tablets)						
Salbutamol sulfate	50	97.0 ± 0.7	99.3 ± 0.1	101.6 ± 0.8	99.4 ± 0.1	100.1 ± 0.6

*Average of three determinations

The added amount of adrenaline a= 20, b= 30, c= 40, d= 50, e= 70 ng

The added amount of salbutamol a= 40, b= 80, c= 150, d= 200, e= 350 ng.

The added amount of guaifensin a= 30, b= 50, c= 70, d= 90, e= 110 ng.

Conclusion

The present study developed simple and accurate spectrofluorometric methods for the analysis of adrenaline in presence of procaine hydrochloride and analysis of salbutamol sulphate with guaifenesin. Adrenaline was determined by coupling with DTCA reagent in alkaline medium. Salbutamol sulfate was analyzed by reaction with ethyl acetoacetate (EAA) forming coumarin derivative, while guaifenesin has a considerable native fluorescence in methanol. The methods are reliable for the accurate determination of the studied drugs in bulk and dosage forms without interference from the common additives in dosage forms. The proposed methods are superior to the previously reported methods (3, 6 and 9) in terms of simplicity and sensitivity. Therefore, these methods can be recommended for the routine analysis of the studied drugs in quality control laboratories.

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