



Received on 14 April, 2011; received in revised form 18 May, 2011; accepted 28 July, 2011

SPECTROFLUORIMETRIC DETERMINATION OF SOME H₁ RECEPTOR ANTAGONIST DRUGS IN PHARMACEUTICAL FORMULATIONS AND BIOLOGICAL FLUIDS

F. Ibrahim, M. K. Sharaf El- Din, M. Eid and M. E. K. Wahba*

Department of Analytical Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura, 35516, Egypt

Keywords:

Ebastine,
Cetirizine,
Fexofenadine,
2-cyanoacetamide,
Spectrofluorimetry

Correspondence to Author:

Mary Wahba

Lecturer Assistant, Department of Analytical Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura, 35516, Egypt

ABSTRACT

A validated simple, economic, selective, and stability indicating spectrofluorimetric method was developed for the determination of some antihistaminic H₁ receptor antagonist drugs namely ebastine (EBS), cetirizine dihydrochloride (CTZ), and fexofenadine hydrochloride (FXD). The method is based on the reaction of the cited drugs with 2-cyanoacetamide in alkaline medium to give highly fluorescent derivatives measured at 365 nm after excitation at 312 nm. The method was applied for the determination of the studied drugs in their dosage forms. Furthermore, the method was applied for the determination of the drugs in spiked human plasma, and used to reveal the pharmacokinetic characters in a healthy volunteer treated with oral administration of the different dosages of the drugs. The method was utilized to investigate the kinetics of the alkaline, acidic, oxidative, and ultraviolet degradation of the drugs. The apparent first order rate constants and half life times of the degradation products were calculated.

INTRODUCTION: Ebastine; (4'- *tert.*-butyl- 4- [4-(diphenylmethoxy)- piperidino] butyrophenone (Fig. 1), cetirizine dihydrochloride; (±)- [2- [4- [(4-chlorophenyl) phenylmethyl] - 1- piperazinyl] ethoxy]acetic acid (Fig. 2), and Fexofenadine hydrochloride; α, α - dimethyl-4- [1- hydroxy- 4- [4-(hydroxydiphenyl-methyl)-1 piperidiny]butyl]-benzene acetic acid (Fig. 3) are potent long acting antihistaminic H₁ receptor antagonist drugs¹.

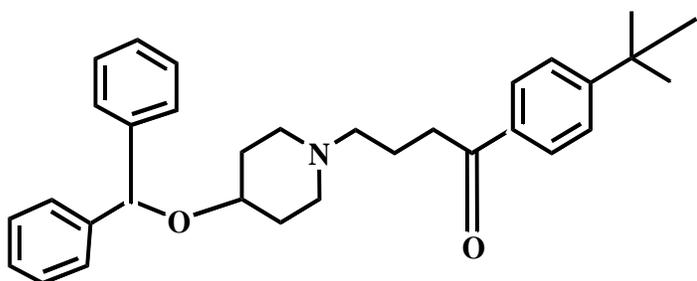


FIG. 1: STRUCTURAL FORMULA OF EBASTINE

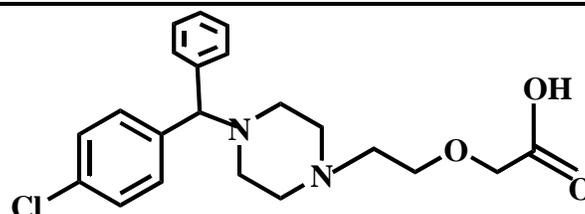


FIG. 2: STRUCTURAL FORMULA OF CETIRIZINE

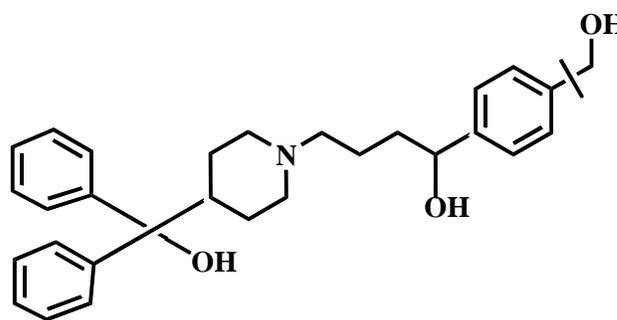


FIG. 3: STRUCTURAL FORMULA OF FEXOFENADINE

Literature survey reveals several methods for the determination of FXD in pharmaceutical preparations and biological fluids including: spectrophotometry²⁻⁴, spectrofluorimetry⁴, and HPLC⁵⁻⁹. On the other hand CTZ was reported to be determined by spectrophotometry^{3-4, 10}, spectrofluorimetry⁴, HPLC^{5, 10-13}, and capillary zone electrophoresis¹⁴. While HPLC was the only method of analysis used for the estimation of ebastine in presence of its metabolites¹⁵⁻¹⁶

2-cyanoacetamide has been used as a post column fluorogenic derivatizing agent for the bio-analysis and pharmacokinetics of chitosan ester in rabbit serum¹⁷, and for the HPLC separation of keratan-sulfate-derived disaccharides¹⁸ and capsular polysaccharide-K4- and defructosylated-K4-derived disaccharides¹⁹. It has also been used for direct determination of prenalterol hydrochloride²⁰, oxamniquine²¹, and for the micro-determination of chondroitin sulfate in normal human plasma by fluorophore-assisted carbohydrate electrophoresis²².

Up till now, fluorimetric methods of analysis have not been reported for the determination of ebastine and this encouraged us to perform the present work, which carries the advantage of being more sensitive than the reported method⁴ used for the determination of FXD and CTZ and hence is superior by virtue of its applicability to biological fluids.

Experimental:

Apparatus: The fluorescence spectra and measurements were recorded using a Perkin Elmer LS 45 Luminescence Spectrometer equipped with a 150 W Xenon arc lamp A 1 cm quartz cell was used.

Materials and reagents: All reagents and solvents were of Analytical Reagent grade.

- a) Ebastine (EBS); of purity 99.94% was kindly provided by Meivo Pharmaceutical Company, Cairo, Egypt.
- b) Cetirizine dihydrochloride (CTZ); of purity 99.87% was kindly provided by Pharco Pharmaceutical Company, Alexandria, Egypt.

c) Fexofenadine hydrochloride (FXD); of purity 99.68%; was kindly provided by El-Obour Modern Pharmaceutical Industries Company, Cairo, Egypt.

d) Pharmaceutical preparations:

- *Bastab[®] tablets (BN#112038), labeled to contain 20 mg ebastine/tablet, Meivo Pharmaceutical Company, Cairo, Egypt.
- *Evastine[®] syrup (BN# 94634), labeled to contain 5 mg ebastine/ 5ml, Marcyrl Pharmaceutical Industries, El Obour City, Egypt.
- *Zyrtec[®] oral solution (Batch # 092639A), labeled to contain 1mg/ ml Cetirizine dihydrochloride, and Zyrtec[®] oral drops (Batch # 093858A), labeled to contain 10mg/ml Cetirizine dihydrochloride, Glaxomithkline, El-Salam city, Cairo, Egypt-under license from UCB, Belgium.
- *Cetrak[®] syrup (Batch # 145), labeled to contain 5mg/ 5ml Cetirizine dihydrochloride, and Cetrak[®] tablet (Batch # 149), labeled to contain 10mg Cetirizine dihydrochloride /tablet, Pharco Pharmaceuticals, Alexandria, Egypt.
- *Clearest[®] capsules (Batch # 90380A), labeled to contain 5mg Cetirizine dihydrochloride and 120 mg pseudoephedrine hydrochloride / capsule, ChemiPharm Pharmaceutical Industries, S.A.E. 6th Ocober, Egypt.
- *Allercet cold[®] capsules (Batch # 820834), labeled to contain 10 mg Cetirizine dihydrochloride , 30 mg pseudoephedrine hydrochloride and 400 mg paracetamol / capsule, Global Napi Pharmaceuticals, 6th October city, Giza, Egypt.
- *Fastofen[®] tablets (Batch # 7065), labeled to contain 60 mg fexofenadine hydrochloride / tablet, El-Obour Modern Pharmaceutical Industries Company, Cairo, Egypt.
- *Fastofen[®] tablets (Batch # 109108), labeled to contain 120 mg fexofenadine hydrochloride / tablet, El-Obour Modern Pharmaceutical Industries Company, Cairo, Egypt.

- *Fexodine® capsules (Batch # 308134), labeled to contain 180 mg fexofenadine hydrochloride/capsule, Memphis Company for Pharmaceutical and Chemical Industries, Cairo, Egypt.

All were obtained from commercial sources in the local market.

- e) 2-cyanoacetamide (Aldrich); was prepared as 4.5% (w/v) aqueous solution
- f) Potassium persulfate (S.D Fine-Chem. LTD); was prepared as 4.5% (w/v) aqueous solution
- g) Ammonia (Cambrian-chemicals); (35% v/v).
- h) Sodium hydroxide (0.5 M solution), hydrochloric acid (0.5 M solution), hydrogen peroxide (3% v/v solution); (BDH, Poole,UK).
- i) Methanol (Aldrich)

Standard Solutions: Stock solutions were prepared by dissolving 100.0 mg of FXD or CTZ in 100 ml of distilled water or 100.0 mg of EBS in methanol and further diluted with the same solvent as appropriate. The standard solutions were stable for 7 days when kept in refrigerator.

General Procedure:

Procedure for the Pure Drug Materials: Aliquots of standard solutions covering the concentration ranges of 10–500, 40-400, and 60-600 ng/ml for EBS, CTZ, and FXD respectively were transferred into a series of 10 ml volumetric flasks; 3.5 ml (in case of EBS and FXD) or 2.5 ml (for CTZ) of 4.5% potassium persulfate solution was added, followed by 1.5 ml of 4.5% 2-cyanoacetamide and 3.5 ml (in case of EBS and CTZ) or 2.5 ml (for FXD) of 35% ammonia, the volume was completed to the mark with distilled water and mixed well.

The fluorescence intensity of the resulting solutions was measured at 365 nm after excitation at 312 nm against a blank experiment. The corrected relative fluorescence intensity was plotted *versus* the final concentration of the drug (ng/ml) to get the calibration graphs; alternatively, the corresponding regression equations were derived.

Procedure for Tablets and Capsules: Twenty tablets were weighed and pulverized. An accurately weighed quantity of the powdered tablets or the mixed capsular content equivalent to 100.0 mg of the studied drug was transferred into a small conical flask, extracted with distilled water or methanol on three successive times each with 30 ml (3x10). The extract was filtered into 100 ml volumetric flask. The conical flask was washed with few milliliters of the solvent and the washings were passed into the same volumetric flask and the volume was completed with the same solvent. Aliquots covering the working concentration range were transferred into 10 ml volumetric flasks and the "General Procedure" was followed. The nominal content of the tablets or capsules was determined either from the corresponding calibration graphs or regression equations.

Procedure for Syrup, Oral Solution and Oral Drops:

Aliquot volumes equivalent to 100 mg of the studied drug were quantitatively transferred into a 100 ml volumetric flasks, serial dilution was performed with distilled water or methanol to obtain the working concentration range and the steps described under "General Procedure" were followed. The nominal content of the dosage form was determined either from the corresponding calibration graphs or regression equations.

Procedure for preparation of Degradation Products:

For the kinetic study, aliquot volumes of EBS, CTZ, or FXD standard solutions were transferred into a series of 25 ml volumetric flasks to obtain a final concentration of 40 $\mu\text{g/ml}$, 0.5 mol dm^{-3} sodium hydroxide, 0.5 mol dm^{-3} hydrochloric acid, or 3% hydrogen peroxide were added. The solutions were left in a thermostatically controlled water bath at different temperature settings for a fixed time interval (10 minutes).

Regarding the UV degradation, the methanolic solutions of the studied drugs were exposed to Deuterium lamp in a cabinet distance of 15 cm at room temperature, and aliquots of the hydrolyzed solutions were analyzed every 10 minutes interval. Aliquot volumes of the degraded solutions were transferred to a series of 10 ml volumetric flasks and neutralized with 0.5 mol dm^{-3} hydrochloric acid or 0.5 mol dm^{-3} sodium

hydroxide for alkaline and acidic degradation respectively, and the steps were completed as described under "General procedure". The relative fluorescence intensity of the resulting degradation products was recorded at 365 nm after excitation at 312 nm, $\log a/a-x$ versus time (minutes) was plotted to get the reaction rate constant and the half life time $t_{1/2}$.

Procedure for Spiked Human Plasma: One ml aliquots of human plasma were transferred into a series of centrifugation tubes, spiked with increasing quantities of EBS, CTZ, or FXD to give a final concentration range of 10-100, 40-180, and 60-450 ng/ml respectively. Then 3 ml of acetonitrile (1 x 3) were added, centrifugation at 3000 rpm for 30 minutes was performed, the aqueous layer was transferred quantitatively to 10 ml volumetric flasks, and the steps described under "General Procedure" were performed.

Procedure for Pharmacokinetic study: The assay was applied to Phase-I pharmaceuticals study in a healthy adult female volunteer received oral administration. Concerning CTZ, after an overnight fast of 10 hours, the volunteer took Cetrak[®] tablet (10 mg CTZ) orally with 200 ml of water. Regular standardized low-fat meals were not provided until 4 hours after dose administration; water intake was allowed after 2 hours.

Following the drug administration, venous blood samples (5 ml) were collected into heparinized tubes according to the following schedule: immediately before administration and 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 10, 12, 16, 20, and 24 hours after dosing. Blood samples were centrifuged at 1500 g for 10 min to obtain the plasma. The plasma samples were labeled and kept frozen at -20°C until analysis. Pharmacokinetic parameters were determined from the plasma concentration-time data.

Similar procedures were performed to follow the pharmacokinetics of EBS and FXD, where Bastab[®] tablet (20 mg EBS), or Fastofen[®] tablet (60 mg FXD) were administered to the volunteer, after-which the steps described under "Procedure for spiked human plasma" were performed.

RESULTS AND DISCUSSION: The studied drugs form highly fluorescent products upon reaction with 2-cyanoacetamide in alkaline medium, this could be represented by the spectra in **Figure 4** showing the reaction product of EBS with 2-cyanoacetamide. The fluorophores were formed instantaneously and remained stable for more than 120 minutes. The proposed method was highly sensitive allowing its application to determine the concerned drugs in spiked human plasma, and follow their pharmacokinetic pattern in a healthy volunteer.

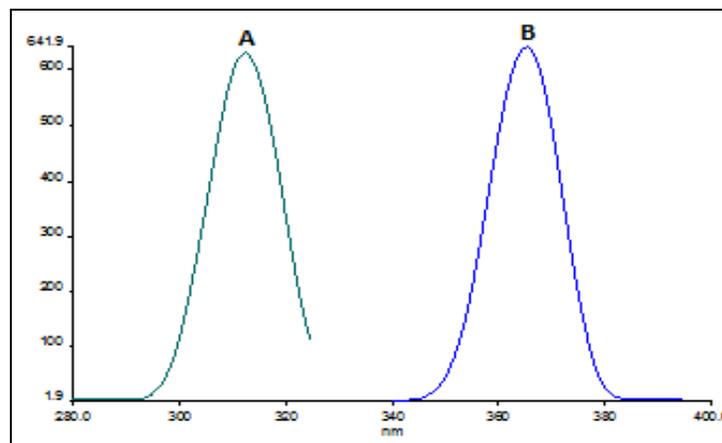


FIG. 4: FLUORESCENCE SPECTRA OF: A AND B ARE THE EXCITATION AND EMISSION SPECTRA RESPECTIVELY OF THE FORMED EBS-CYANOACETAMIDE REACTION PRODUCT

Optimization of the Reaction Conditions: The spectrofluorimetric properties of the formed fluorophores as well as the different experimental parameters affecting development and stability of the reaction products were carefully studied and optimized. Such factors were changed individually while the others were kept constant. These factors include: the concentration and volume of each of potassium persulfate and 2-cyanoacetamide, volume of ammonia, the effect of temperature, and different diluting solvent

Effect of addition order: The effect of addition order on the fluorescence intensity of the system was studied. The results showed that the addition order of drug- potassium persulfate - 2- cyanoacetamide - ammonia gave the best results.

Effect of different diluting solvents: Different diluting solvents were tested to choose the most suitable one for the formation of the reaction product, the investigated solvents included: water, methanol,

acetonitrile, dimethylsulfoxide, dimethylformamide, and acetone. The highest fluorescence intensities were achieved upon using water. Moreover, its choice adds another advantage to the method. The results are abridged in **Table 1**.

Table 1: Effect of diluting solvents on the RFI of the reaction products

Solvent	Relative Fluorescence Intensity		
	*EBS	**CTZ	***FXD
Water	817	600	428
Methanol	720	350	352
Acetonitrile	243	120	190
Dimethylsulfoxide	330	260	254
Dimethylformamide	440	378	380
Acetone	55	40	36

*EBS=500ng/ml, **CTZ=300ng/ml, ***FXD=300ng/ml

Effect of concentration of potassium persulfate: The effect of potassium persulfate concentrations on the values of the relative fluorescence intensity of the reaction product was investigated, keeping all the variables constant. It was found that concentrations lower than 2% were not sufficient to cause oxidation of the drugs, and hence their reaction with 2-cyanoacetamide did not proceed. Increasing the concentration resulted in a gradual increase in the fluorescence intensity of the formed fluorophore up to (4%) after which, it remained constant; therefore (4.5±0.5 %) was used throughout the study (**Fig. 5**).

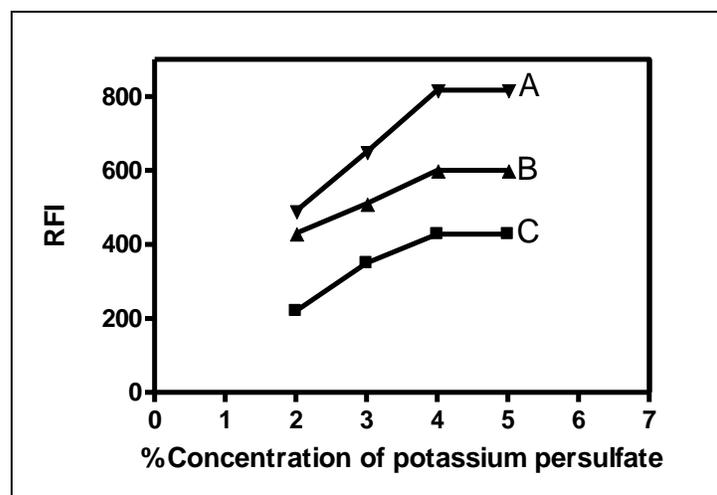


FIG. 5: EFFECT OF CONCENTRATION OF POTASSIUM PERSULFATE ON THE FLUORESCENCE INTENSITY OF THE FORMED REACTION PRODUCT WHERE: A: (EBS=500ng/ml), B: (CTZ=300 ng/ml), C: (FXD=300 ng/ml)

Effect of volume of potassium persulfate reagent: Keeping all the variables constant, it was found that increasing the volume of potassium persulfate (4.5%) resulted in a gradual increase in the relative fluorescence intensity of the reaction products up to 3 ml in case of EBS and FXD and 2 ml in case of CTZ, after which it remained constant, therefore, 3.5 ± 0.5 or 2.5 ± 0.5 ml were chosen respectively for the study (**Fig. 6**).

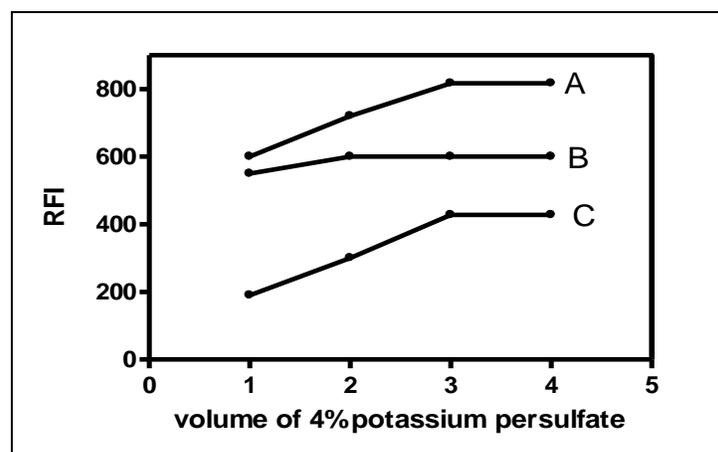


FIG. 6: EFFECT OF VOLUME OF POTASSIUM PERSULFATE (4.5%) ON THE FLUORESCENCE INTENSITY OF THE FORMED REACTION PRODUCT WHERE: A: (EBS=500ng/ml), B: (CTZ=300 ng/ml), C: (FXD=300 ng/ml)

Effect of concentration of 2-cyanoacetamide: The effect of 2-cyanoacetamide concentration was also studied keeping all the variables constant. Increasing the concentration resulted in an increase in the fluorescence intensity of the reaction products up to (4%) after which, it remained constant; hence (4.5±0.5 %) was used during the study (**Fig. 7**).

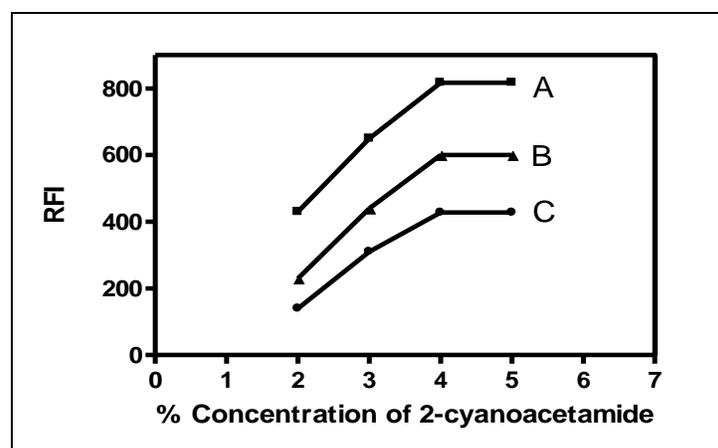


FIG. 7: EFFECT OF CONCENTRATION OF 2-CYANOACETAMIDE ON THE FLUORESCENCE INTENSITY OF THE FORMED REACTION PRODUCT WHERE: A: (EBS=500ng/ml), B: (CTZ=300 ng/ml), C: (FXD=300 ng/ml)

Effect of volume of 2-cyanoacetamide reagent: It was found that increasing the volume of the reagent (4.5%) resulted in a gradual increase in the relative fluorescence intensities up to 1 ml, after which it remained constant, therefore 1.5±0.5 ml of 4.5% 2-cyanoacetamide was used during this approach (Fig. 8).

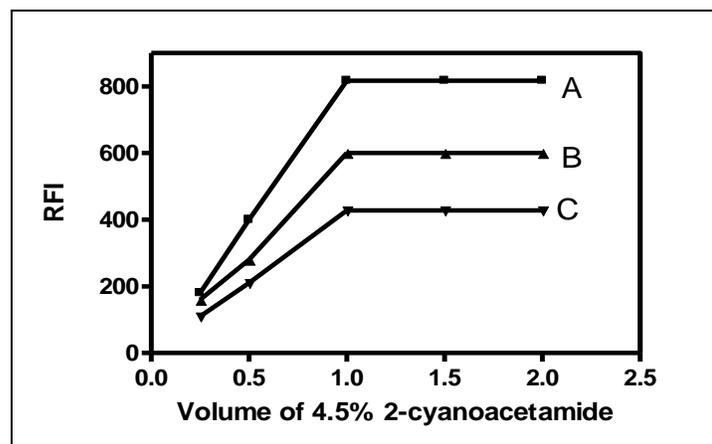


FIG. 8: EFFECT OF VOLUME OF 2-CYANOACETAMIDE (4.5 %) ON THE FLUORESCENCE INTENSITY OF THE FORMED REACTION PRODUCT WHERE: A: (EBS=500ng/ml), B: (CTZ=300 ng/ml), C: (FXD=300 ng/ml)

Effect of volume of ammonia: It was found that increasing the volume of ammonia (35%) increased the RFI gradually up to 3 ml in case of EBS and CTZ and 2

ml in case of FXD, after which it remained constant, therefore, 3.5 ± 0.5 or 2.5 ± 0.5 ml were used respectively throughout the study (Fig. 9).

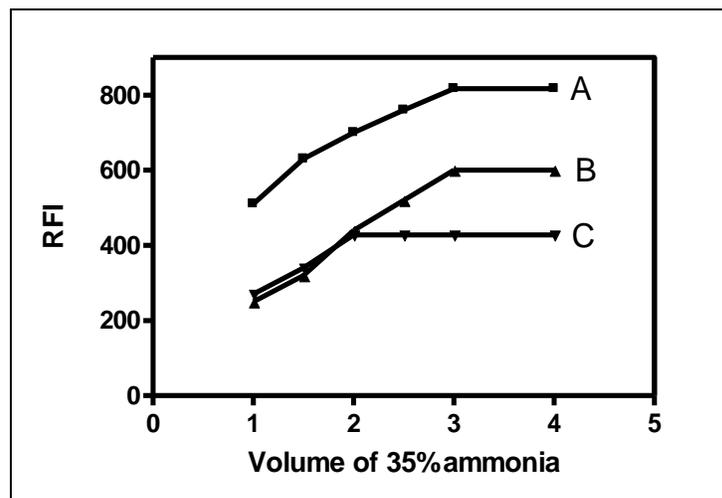


FIGURE 9: EFFECT OF VOLUME OF AMMONIA (35 %) ON THE FLUORESCENCE INTENSITY OF THE FORMED REACTION PRODUCT WHERE: A: (EBS=500ng/ml), B: (CTZ=300 ng/ml), C: (FXD=300 ng/ml)

Analytical performance and application: Using the above spectrofluorimetric method, linear regression equations were obtained. The relative fluorescence-concentration plots were found to be linear over the ranges mentioned in Table 2.

TABLE 2: PERFORMANCE DATA OF THE PROPOSED METHOD

Parameter	EBS	CTZ	FXD
Concentration range (ng/ml)	10-500	40-400	60-600
LOD(ng /ml)	1.6	2.7	5.1
LOQ(ng/ml)	4.8	8.3	15.6
Correlation coefficient (r)	0.9999	0.9996	0.9999
Slope	1.63	2.02	1.44
Intercept	1.96	3.56	2.78
$S_{y/x}$, Standard deviation of the residuals	2.89	5.79	3.82
S_a , Standard deviation of the intercept of the regression line	0.79	1.67	2.24
S_b , Standard deviation of the slope of the regression line	6.1×10^{-3}	1.7×10^{-2}	7.7×10^{-3}

Sensitivity: LOQ and LOD were calculated according to ICH recommendations²³. LOQ for EBS, CTZ, and FXD were found to be 4.8, 8.3, and 15.6 ng/ ml respectively. The corresponding LOD were calculated and found to be 1.6, 2.7, and 5.1 ng/ml. LOQ was calculated according to the following equation²³:

$$LOQ = 10\sigma/S$$

Where σ : the standard deviation of the intercept of the regression line; S: slope of the calibration curve. The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected and it was calculated according to the following equation²³:

$$LOD = 3.3 \sigma/S$$

Method Validation: The method was tested for linearity, selectivity, accuracy and precision. Using the above spectrofluorimetric method, linear regression equations were obtained. The regression plots showed that there was a linear dependence of the fluorescence intensity value on the concentration of the drug over the ranges cited in Table 2. The validity of the proposed method was evaluated by statistical analysis of the regression data regarding the standard deviation of the residual ($S_{y/x}$), the standard deviation of the intercept (S_a), and standard deviation of the slope (S_b)²⁴. The results are shown in Table 2.

The small values of the figures point to the low scattering of the points around the calibration graph and high precision of the proposed method.

Accuracy: The accuracy of the proposed method was evaluated by analyzing standard solutions of the drugs under investigation. The results obtained by the proposed method were favorably compared with those obtained by the comparison methods^{4, 16}. Statistical analysis²⁴ of the results obtained by the proposed and comparison methods using student's t-test and variance ratio F-test, showed no significant difference between the performances of the two methods (**Table 3**).

TABLE 3: APPLICATION OF THE PROPOSED METHOD TO THE DETERMINATION OF THE STUDIED DRUGS IN THEIR PURE FORM

Parameter	Taken (ng/ml)	Found (ng/ml)	% Found	**Comparison method
EBS	10	10.06	100.6	100.62 99.15 99.34
	50	49.95	99.90	
	100	100.35	100.35	
	200	199.65	99.83	
	300	302.21	100.74	
	400	398.45	99.61	
	500	504.12	100.82	
$\bar{X} \pm SD$ Student's t test F test			100.26±0.48 0.37 (1.94)* 2.71 (5.14)*	99.70±0.79
CTZ	40	39.69	99.23	100.32 99.51 99.31
	80	80.22	100.28	
	100	99.92	99.92	
	150	149.94	99.96	
	200	200.41	100.21	
	300	299.85	99.95	
	400	399.32	99.83	
$\bar{X} \pm SD$ Student's t test F test			99.91±0.34 0.51 (1.94)* 2.4 (5.14)*	99.71±0.53
FXD	60	60.21	100.35	100.25* 100.12 100.99
	100	100.41	100.41	
	200	199.66	99.83	
	300	299.72	99.91	
	400	403.12	100.78	
	500	499.59	99.92	
	600	599.35	99.89	
$\bar{X} \pm SD$ Student's t test F test			100.16± 0.36 0.62 (1.94)* 1.7 (5.14)*	100.40 ± 0.47

*Figures between parentheses are the tabulated t and F values respectively at $P = 0.05$ ²⁴. **¹⁶ for EBS, and ⁴ for FXD and CTZ

Precision:

Repeatability: The repeatability was evaluated through analysis of different concentrations of the studied drugs in pure or in dosage forms on 4 successive times. The mean percentage recoveries

listed in **Table 4** indicate the high precision of the proposed method.

Intermediate precision: It was performed through repeated analysis of variable concentrations of the drugs either *per se* or in dosage forms on four successive days. The results are abridged in **Table 4**.

TABLE 4: VALIDATION OF THE PROPOSED METHOD FOR THE DETERMINATION OF THE STUDIED DRUGS IN PURE AND DOSAGE FORMS

Preparation	Repeatability, % Found	Intermediate precision, %Found
Ebastine pure form	Ebastine (10 ng/ml)	Ebastine (200 ng/ml)
	99.65	100.32
	100.58	100.55
	100.32	99.45
	99.87	99.21
X⁻ ± SD	100.11±0.42	99.88±0.65
Bastab [®] tablets	Ebastine (300 ng/ml)	Ebastine (500 ng/ml)
	100.95	100.65
	100.58	100.84
	100.66	99.68
	99.86	99.75
X⁻ ± SD	100.51±0.46	100.23±0.61
Bastab [®] syrup	Ebastine (400 ng/ml)	Ebastine (100 ng/ml)
	99.32	100.11
	99.84	100.35
	100.58	100.06
	100.47	99.58
X⁻ ± SD	100.05±0.59	100.03±0.32
Cetirizine dihydrochloride pure form	CTZ (60 ng/ml)	CTZ (350 ng/ml)
	99.98	99.23
	100.58	99.45
	100.65	99.23
	100.78	100.05
X⁻ ± SD	100.49±0.35	99.49±0.39
Zyrtec [®] oral solution	CTZ (40 ng/ml)	CTZ (300 ng/ml)
	100.58	99.58
	100.77	99.68
	99.98	99.45
	99.89	100.06
X⁻ ± SD	100.31±0.44	99.69±0.26
Zyrtec [®] oral drops	CTZ (250 ng/ml)	CTZ (200 ng/ml)
	100.89	99.25
	100.57	99.78
	100.65	99.32
	100.24	99.44
X⁻ ± SD	100.59±0.27	99.45±0.24
Cetrak [®] syrup	CTZ (100 ng/ml)	CTZ (400 ng/ml)
	100.25	100.54
	100.58	99.25
	100.74	99.62
	100.61	100.75
X⁻ ± SD	100.55±0.21	100.04±0.72
Cetrak [®] tablets	CTZ (150 ng/ml)	CTZ (50 ng/ml)
	100.84	99.52
	100.49	99.78
	100.92	100.47
	100.15	100.24
X⁻ ± SD	100.61±0.35	100.01±0.43
Clearest [®] capsules	CT Z(350 ng/ml)	CTZ (150 ng/ml)
	100.14	99.45
	100.46	100.42
	100.76	100.24
	100.35	100.34
X⁻ ± SD	100.43±0.26	100.11±0.45

Allercet® cold capsules	CTZ (80 ng/ml)	CTZ (400 ng/ml)
	100.85	100.45
	100.74	100.24
	100.11	99.24
	100.34	99.56
$\bar{X} \pm SD$	100.51±0.35	99.87±0.57
FXD pure form	FXD (200 ng/ml)	FXD (600 ng/ml)
	100.25	99.62
	100.22	99.98
	100.65	100.47
	100.54	100.57
$\bar{X} \pm SD$	100.42±0.21	100.16±0.44
Fastofen® tablets (60 mg FXD / tablet)	FXD (60 ng /ml)	FXD (100 ng/ml)
	100.84	99.69
	100.76	100.58
	100.66	100.48
	99.58	99.96
$\bar{X} \pm SD$	100.46±0.59	100.18±0.42
Fastofen® tablets (120 mg FXD / tablet)	FXD (400 ng/ml)	FXD (300 ng/ml)
	99.62	99.58
	99.58	100.84
	100.84	100.16
	100.45	100.32
$\bar{X} \pm SD$	100.12±0.62	100.23±0.52
Fexodine® capsules	FXD (80 ng/ml)	FXD (500 ng/ml)
	100.12	100.45
	100.65	100.12
	99.89	100.06
	100.78	99.24
$\bar{X} \pm SD$	100.36±0.42	99.97±0.51

Robustness of the method: The robustness of the method adopted was demonstrated by the consistency of the relative fluorescence values with the deliberately minor changes in the experimental parameters such as, concentration of potassium persulfate ($4.5 \pm 0.5\%$), volume of potassium persulfate (3.5 ± 0.5 for EBS and FXD or 2.5 ± 0.5 ml for CTZ), 2-cyanoacetamide concentration ($4.5 \pm 0.5\%$), 2-cyanoacetamide volume (1.5 ± 0.5 ml) and volume of ammonia (3.5 ± 0.5 ml for EBS and CTZ or 2.5 ± 0.5 for

FXD) which did not greatly affect the fluorescence intensities of the reaction products.

Pharmaceutical Applications: The proposed method was successfully applied to the determination of the concerned drugs in their dosage forms.

The results are summarized in **Table 5**. No interference from the sample matrix was observed, the results were found to be in good agreement with the labeled amount.

TABLE 5: APPLICATION OF THE PROPOSED METHOD TO THE DETERMINATION OF THE STUDIED DRUGS IN THEIR DOSAGE FORMS

Parameter	Amount taken (ng/ml)	Amount found (ng/ml)	% Found	**Comparison method, % Found
Bastab® tablets	10	10.05	100.52	99.23 99.61 100.61
	50	49.61	99.21	
	100	99.93	99.93	
	200	200.24	100.12	
	300	301.65	100.55	
	400	401.36	100.34	
	500	497.25	99.45	
$\bar{X} \pm SD$			100.02±0.52	99.82±0.71
Student's t test			0.98 (1.94)*	
F test			1.86 (5.14)*	

Bastab® syrup	10	10.03	100.25	100.12 99.61 99.51
	50	50.06	100.12	
	100	99.58	99.58	
	200	198.90	99.45	
	300	301.95	100.65	
	400	403.36	100.84	
	500	497.70	99.54	
X̄ ± SD Student's t test F test			100.06±0.56 0.76 (1.94) 2.88 (5.14)	99.75±0.33
Zyrtec® oral solution	40	40.09	100.23	100.12 99.51 99.34
	80	79.40	99.25	
	100	99.62	99.62	
	150	150.87	100.58	
	200	201.72	100.86	
	300	299.67	99.89	
	400	396.60	99.15	
X̄ ± SD Student's t test F test			99.94±0.65 0.65 (1.94) 2.5 (5.14)	99.66±0.41
Zyrtec® oral drops	40	39.81	99.52	100.25 99.24 99.62
	80	79.94	99.92	
	100	100.45	100.45	
	150	150.98	100.65	
	200	200.84	100.42	
	300	298.14	99.38	
	400	397.80	99.45	
X̄ ± SD Student's t test F test			99.97±0.54 0.56 (1.94) 1.12 (5.14)	99.70±0.51
Cetrak® syrup	40	39.69	99.23	99.65 100.21 100.75
	80	80.42	100.52	
	100	100.92	100.92	
	150	150.23	100.15	
	200	199.30	99.65	
	300	298.35	99.45	
	400	402.28	100.57	
X̄ ± SD Student's t test F test			100.07±0.64 0.73 (1.94) 1.35 (5.14)	100.20±0.55
Cetrak® tablets	40	39.73	99.32	100.25 99.65 99.15
	80	79.86	99.82	
	100	100.15	100.15	
	150	150.86	100.57	
	200	201.56	100.78	
	300	301.53	100.51	
	400	397.80	99.45	
X̄ ± SD Student's t test F test			100.09±0.57 0.68 (1.94) 1.07 (5.14)	99.68±0.55
Clearest® capsules	40	39.85	99.63	100.32 100.95 99.51
	80	79.32	99.15	
	100	99.56	99.56	
	150	150.98	100.65	
	200	201.76	100.88	

	300	300.72	100.24	
	400	397.28	99.32	
X̄ ± SD			99.92±0.67	
Student's t test			0.89 (1.94)	100.26±0.72
F test			1.15 (5.14)	
Allercet® cold capsules	40	39.73	99.32	
	80	79.40	99.25	
	100	99.84	99.84	100.92
	150	150.84	100.56	99.65
	200	199.78	99.89	99.21
	300	302.67	100.89	
	400	401.05	100.25	
X̄ ± SD			100.02±0.61	
Student's t test			0.32 (1.94)	99.93±0.89
F test			2.13 (5.14)	
Fastofen® tablets (containing 60 mg FXD / tablet)	60	59.54	99.23	
	100	99.98	99.98	
	200	201.30	100.65	100.25
	300	301.68	100.56	99.34
	400	401.03	100.25	100.12
	500	498.20	99.64	
	600	604.62	100.77	
X̄ ± SD			100.15±0.57	
Student's t test			0.28 (1.94)	99.90± 0.49
F test			1.35 (5.14)	
Fastofen® tablets (containing 120 mg FXD / tablet)	60	60.39	100.65	
	100	100.32	100.32	
	200	199.24	99.62	100.32
	300	298.26	99.42	100.97
	400	401.04	100.26	100.05
	500	504.25	100.85	
	600	595.92	99.32	
X̄ ± SD			100.06±0.61	
Student's t test			0.93 (1.94)	100.45± 0.47
F test			1.68 (5.14)	
Fexodine® capsules (containing 180 mg FXD / capsule)	60	59.59	99.32	
	100	99.56	99.56	
	200	200.50	100.25	99.97
	300	301.68	100.56	99.21
	400	398.48	99.62	100.15
	500	499.10	99.82	
	600	604.50	100.75	
X̄ ± SD			99.98±0.54	
Student's t test			0.55 (1.94)	99.78± 0.49
F test			1.21 (5.14)	

*Figures between parentheses are the tabulated t and F values respectively at P = 0.05²⁴, **¹⁶ for EBS, and ⁴ for FXD and CTZ

Interferences: Drugs which are co-formulated with CTZ; pseudoephedrine hydrochloride and paracetamol, and others that are frequently co-administered with the investigated drugs such as ketoconazole and erythromycin were carefully tested using the proposed

method. All of the studied compounds showed no interference.

Stability study: The proposed method is based mainly on the reaction between 2-cyanoacetamide and the

active methylenic groups obtained after oxidation of the drugs with potassium persulfate. Therefore, degradation was attained upon induced alkaline degradation using sodium hydroxide (0.5 mol dm⁻³), acidic degradation using hydrochloric acid (0.5 mol dm⁻³), oxidative degradation using 3% hydrogen peroxide. Upon degradation of the drugs, the relative fluorescence readings of the reaction products decreased gradually with time, thus indicating that the proposed method is a stability indicating one. The degradation was found to be temperature dependant as represented by the oxidative degradation of CTZ (Fig. 10). The first order degradation rate constant and the half life time at each temperature were calculated (Table 6) according to the following equations:

$$\ln a/a-x = Kt$$

where, a is the initial concentration of the drug, x is the concentration of the resulting degraded solution

after time t, and K is the reaction rate constant, while the half life time could be calculated as follows: $t_{1/2}=0.693/K$.

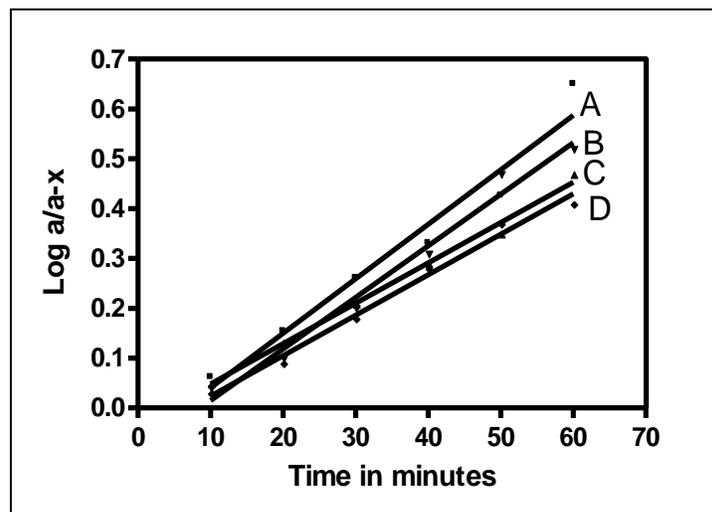


FIG. 10: SEMILOGARITHMIC PLOT OF CTZ (300ng/ml) VERSUS DIFFERENT HEATING TIMES WITH 3% H₂O₂ (A = 80°C, B = 70°C, C = 60°C, AND D = 50°C).

TABLE 6: KINETIC PARAMETERS FOR THE DEGRADATION OF THE STUDIED DRUGS APPLYING THE PROPOSED METHOD

Studied drug	Temperature (°C)	Alkaline degradation		Acidic degradation		Oxidative degradation	
		K (min. ⁻¹)	t _½ (min)	K (min. ⁻¹)	t _½ (min)	K (min. ⁻¹)	t _½ (min)
EBS	50	0.00958	72	0.00918	76	0.00941	74
	60	0.00898	77	0.00911	77	0.0088	79
	70	0.00885	78	0.00895	78	0.00814	85
	80	0.00861	80	0.00864	80	0.0077	90
	Ea (Joule mol ⁻¹)	3.2		3.5		5.3	
CTZ	50	0.01234	56	0.01182	59	0.01093	63
	60	0.01185	58	0.01163	60	0.01034	67
	70	0.01142	61	0.01102	63	0.008114	85
	80	0.011	63	0.01086	64	0.008097	86
	Ea (Joule mol ⁻¹)	3.1		2.4		8.8	
FXD	50	0.01408	49	0.01157	60	0.0112	62
	60	0.01323	52	0.01046	66	0.01097	63
	70	0.01245	56	0.00941	73	0.0100	69
	80	0.01179	59	0.008943	77	0.009429	73
	Ea (Joule mol ⁻¹)	4.6		6.9		4.6	

Through plotting log K_{obs} values versus 1/T, the Arrhenius plots were obtained and this could be exemplified by the degradation of FXD in Figure 11. The activation energy for each type of degradation was also calculated by the equation:

$$\ln K = -Ea/RT + \ln A$$

Where: Ea is the activation energy, K is first order reaction rate constant, R is the gas constant, and T is the temperature in Kelvin. Upon exposure of

methanolic solution of the concerned drugs to Deuterium lamp with a wavelength of 254 nm at a distance of 15 cm in a wooden cabinet for different time intervals, and then the studied method was applied, it was found that EBS, CTZ, and FXD decomposed by 28, 19, and 26 % respectively.

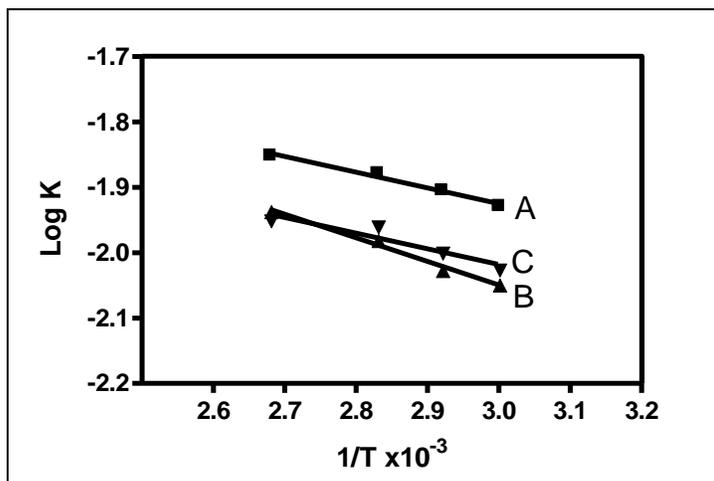


FIG. 11: ARRHENIUS PLOT FOR THE DEGRADATION OF FXD, WHERE: A: (ALKALINE DEGRADATION), B: (ACIDIC DEGRADATION), C: (OXIDATIVE DEGRADATION)

Application to Spiked and Real Human Plasma: It has been reported that ebastine undergoes virtually complete first pass bio-transformation to the pharmacologically active carboxylic acid metabolite carebastine (4-[4-(diphenylmethoxy)-1piperidinyl]-1-oxobutyl]- α , α dimethyl benzene- acetic acid (Fig. 12) in both experimental animals and human subjects¹⁶. The formed metabolite still carry the target chemical group that undergoes the fluorogenic reaction of concern, hence the pharmacokinetic study could be applied. Peak concentrations in plasma occur 3.61 ± 1.06 hours after oral dose and reach about 99.1 ± 28.6 ng/ml with elimination half-life of 10.3 ± 2.6 ²⁵. Analysis of serum samples following administration of a 10 mg oral dose of CTZ to a healthy subject provided maximum concentration of 167 ± 47 ng/ml reached within 0.7 ± 0.5 hours and having $t_{1/2}$ value of 6.76 ± 1.82 hours¹².

TABLE 7: APPLICATION OF THE PROPOSED METHOD FOR THE DETERMINATION OF THE STUDIED DRUGS IN SPIKED HUMAN PLASMA

Parameter	Taken (ng/ml)			Found (ng/ml)			%Recovery		
	EBS	CTZ	FXD	EBS	CTZ	FXD	EBS	CTZ	FXD
Intra-day precision	10	40	60	9.825	38.98	58.87	98.25	97.45	98.12
	20	60	80	19.42	59.75	77.86	97.12	99.58	97.32
	30	80	100	29.79	78.66	97.51	99.32	98.32	97.51
	40	100	150	39.65	100.81	148.55	99.12	100.81	99.03
	50	120	200	50.44	121.74	202.9	100.88	101.45	101.45
	60	130	250	60.59	132.54	252.4	100.98	101.95	100.96
	70	140	300	71.02	140.63	297.96	101.45	100.45	99.32
	80	160	350	81.32	157.84	345.87	101.65	98.65	98.82
	90	170	400	89.41	165.16	389.8	99.34	97.15	97.45
	100	180	450	98.45	181.71	453.51	98.45	100.95	100.78
						99.66 \pm 1.5	99.68 \pm 1.7	99.08 \pm 1.54	

Another study concerned with the pharmacokinetics of FXD mentioned that 734 ± 261 ng/ml is the maximum plasma concentration attained within 1.5 ± 0.6 hours, and half life time of 19.1 ± 7 hours⁹.

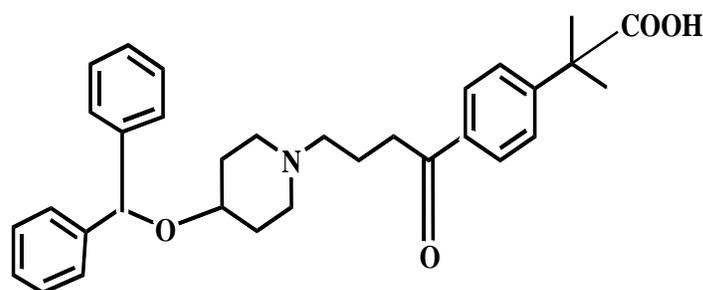


FIG. 12: STRUCTURAL FORMULA OF CAREBASTINE

These value lie within the working concentration range of the proposed spectrofluorimetric method, thus it could be successfully applied to the determination of the studied drugs in spiked human plasma over the working concentration range. The within- day precision was evaluated through replicate analysis of the drugs on three successive times, where the mean percentage recoveries based on the average of 4 separate determinations were 99.66 ± 1.5 , 99.68 ± 1.7 , and 99.08 ± 1.54 for EBS, CTZ, and FXD respectively.

The intra-day precision was evaluated through replicate analysis of plasma samples spiked with 50, 100, and 200 ng/ml of EBS, CTZ, and FXD respectively on three successive days. The mean percentage recoveries based on the average of 4 separate determinations were 97.56 ± 0.83 , 98.09 ± 0.96 , and 98.29 ± 0.84 for the three drugs respectively. The results are summarized in Table 7.

$\bar{X} \pm SD$	Inter- day precision								
	50	100	200	48.56	99.03	194.90	97.12	99.03	97.45
1 st day	50	100	200	48.56	99.03	194.90	97.12	99.03	97.45
2 nd day	50	100	200	49.26	98.12	196.64	98.52	98.12	98.32
3 rd day	50	100	200	48.53	97.12	198.24	97.05	97.12	99.12
$\bar{X} \pm SD$							97.56±0.83	98.09±0.96	98.29±0.84

The aforementioned reported pharmacokinetic data are in accordance with the results of the proposed method as illustrated from the concentration – time curve (Fig. 13), and the pharmacokinetic parameters in Table 8.

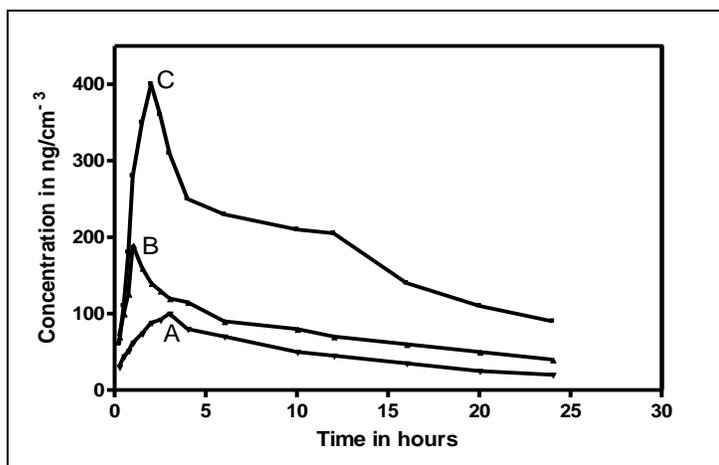


FIGURE 13: MEAN PLASMA CONCENTRATION-TIME CURVE FOR: A=EBS, B=CTZ, C=FXD

TABLE 8: PHARMACOKINETIC PARAMETERS OF THE STUDIED DRUGS APPLYING THE PROPOSED METHOD

Studied drug	Parameter			
	C_{max} (ng/ml)	T_{max} (hours)	$t_{1/2}$ (hours)	AUC
EBS	100	3	10	1148
CTZ	190	1	6	1854
FXD	400	2	12	4463

Mechanism of the reaction: The role of potassium persulfate in the reactions is to increase the polarity of EBS and CTZ causing keto-enol tautomerism that occurs in the highly alkaline medium provided by ammonia yielding active methylenic groups which are subjected to react with 2-cyanoacetamide²⁶ via condensation followed by cyclization reactions resulting in the formation of highly fluorescent products. In case of FXD, potassium persulfate causes oxidation of the secondary alcoholic group into ketone²⁶, which is then subjected to the same reaction pathway of the other two drugs.

The stoichiometry of the reaction between the studied drugs and 2-cyanoacetamide was studied using limiting logarithmic method²⁷. As shown in Figure 14, a plot of log RFI versus log [2-cyanoacetamide] and log [drug concentration] gave straight lines. The values of the slopes for EBS, CTZ, and FXD in the plots were (1.005, 1.007, 1.000) and (1.090, 0.9535, 0.9801) respectively; pointing out that the drugs react with the fluorogenic reagent in a ratio of 1:1. The reaction pathways are proposed to proceed as shown in the schemes 1-3.

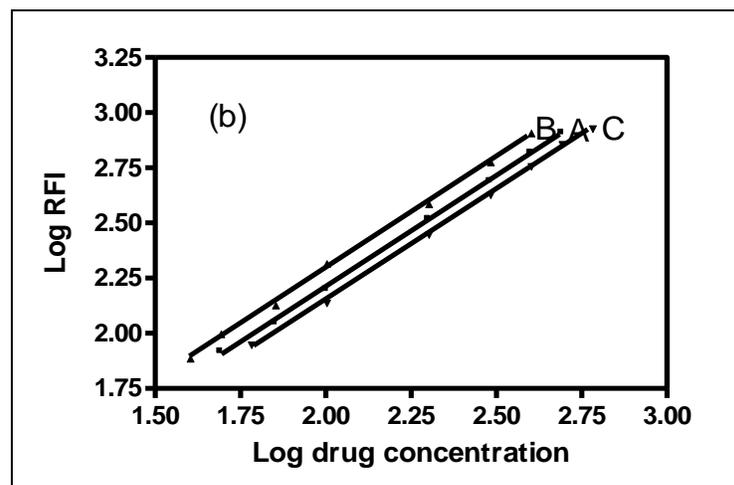
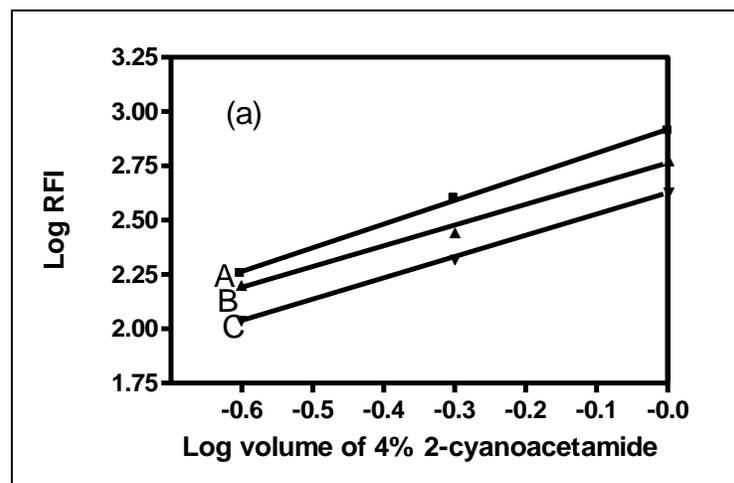
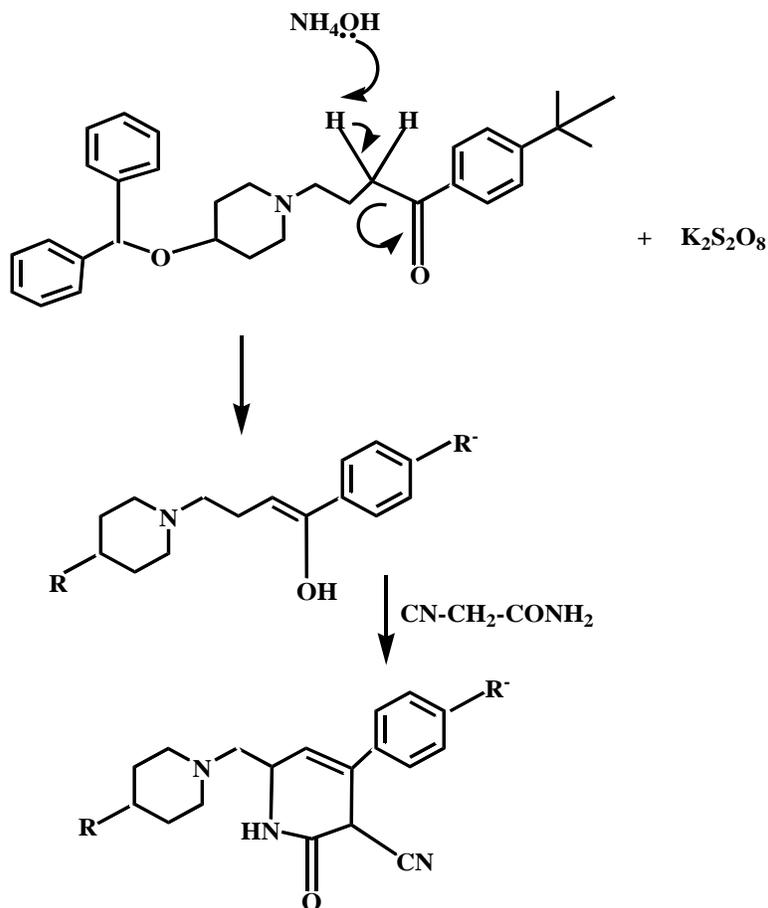
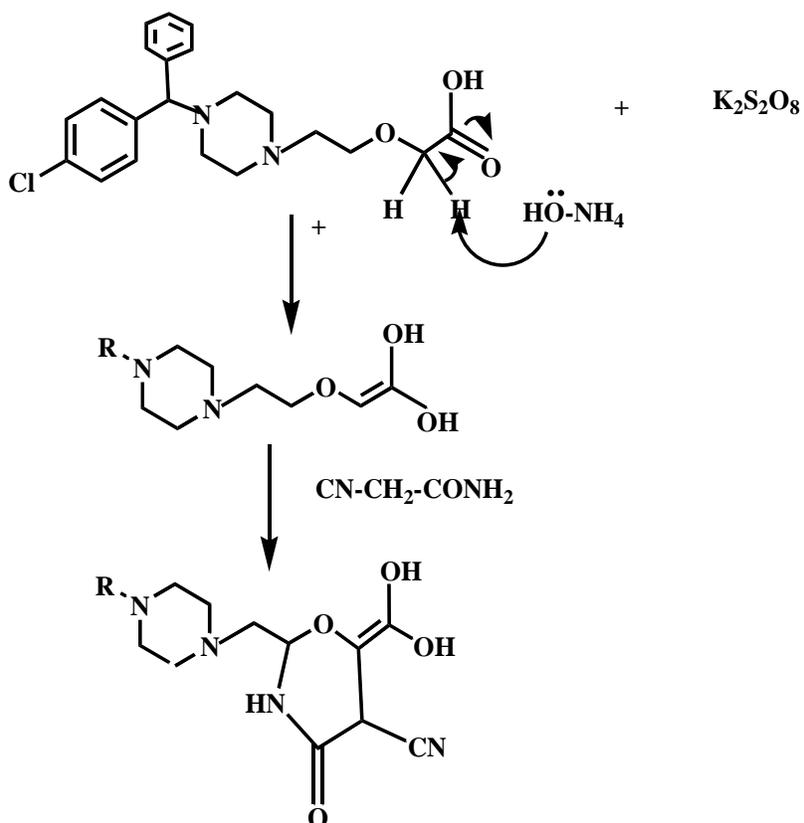


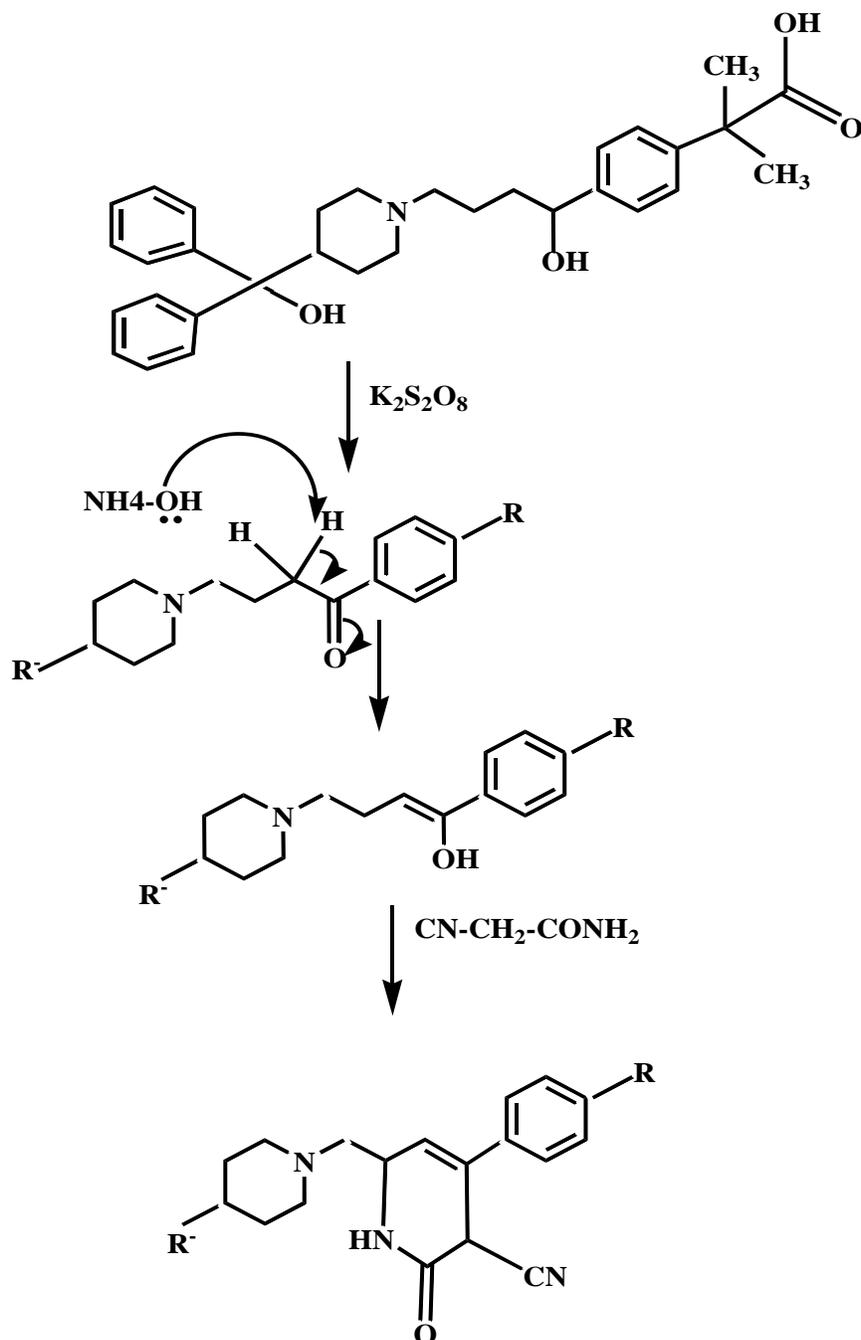
FIG. 14: LIMITING LOGARITHMIC PLOTS FOR THE MOLAR RATIO OF THE STUDIED DRUGS AND 2-CYANOACETAMIDE, WHERE: A=EBS, B=CTZ AND, C=FXD



SCHEME 1: PROPOSAL OF THE REACTION PATHWAY BETWEEN EBASTINE AND 2-CYANOACETAMIDE



SCHEME 2: PROPOSAL OF THE REACTION PATHWAY BETWEEN CETIRIZINE AND 2-CYANOACETAMIDE



SCHEME 3: PROPOSAL OF THE REACTION PATHWAY BETWEEN FEXOFENADINE AND 2-CYANOACETAMIDE

CONCLUSION: The drugs of concern were determined either in their pure or dosage forms using the proposed method which carries many advantages as for example being sensitive, rapid, cheap, and selective. The method was subjected to validation and to extensive stability study. The sensitivity of the present work allows its application to biological fluids and to follow the pharmacokinetic pattern of the studied drugs. These criteria support the use of this work in routine quality control laboratories.

ACKNOWLEDGEMENT: We would like to thank the analytical chemistry department, faculty of pharmacy, Mansoura University, for providing the instruments and materials required for the experimental procedures.

REFERENCES:

1. Sweetman S (Ed.) Martindale: The complete drug reference, Pharmaceutical press. Electronic version: London, 2006.
2. Saleh H M, EL-Henawee M M, Ragab G H, Abd El-Hay S S: Utility of NBD-Cl for the spectrophotometric determination of some

- skeletal muscle relaxant and antihistaminic drugs. *Spec.chim. Acta* 2007; 67: 1284-1289.
3. Gazy A A, Mahgoub H, El-Yazbi F A, El-Sayed M A, Youssef R M: Spectrophotometric determination of binary mixtures of pseudoephedrine with some histamine H₁-receptor antagonists using derivative ratio spectrum method. *J. Pharm. Biomed. Anal.* 2003; 31: 801-809.
 4. Gazy A A, Mahgoub H, El-Yazbi F A, El-Sayed M A, Youssef R M: Determination of some histamine H₁-receptor antagonists in dosage forms. *J. Pharm. Biomed. Anal.* 2002; 30: 859-867
 5. Karakuş S, Küçükgülzel İ, Küçükgülzel Ş G: Development and validation of a rapid RP-HPLC method for the determination of cetirizine or fexofenadine with pseudoephedrine in binary pharmaceutical dosage forms. *J. Pharm. Biomed. Anal.* 2008; 46: 295-302.
 6. Pathak S M, Kumar A R, Musmade P, Udupa N: A simple and rapid high performance liquid chromatographic method with fluorescence detection for the estimation of fexofenadine in rat plasma—Application to preclinical pharmacokinetics. *Talanta* 2008; 76: 338-346.
 7. Miura M, Uno T, Tateishi T, Suzuki T: Determination of fexofenadine enantiomers in human plasma with high-performance liquid chromatography. *J. Pharm. Biomed. Anal.* 2007; 43: 741-745.
 8. Uno T, Yasui-Furukori N, Takahata T, Sugawara K, Tateishi T: Liquid chromatographic determination of fexofenadine in human plasma with fluorescence detection. *J. Pharm. Biomed. Anal.* 2004; 35: 937-942.
 9. Radhakrishna T, Om Reddy G: Simultaneous determination of fexofenadine and its related compounds by HPLC. *J. Pharm. Biomed. Anal.* 2002; 29: 681-690.
 10. El Walily A F M, Korany M A, El Gindy A, Bedair M F: Spectrophotometric and high performance liquid chromatographic determination of cetirizine dihydrochloride in pharmaceutical tablets. *J. Pharm. Biomed. Anal.* 1998; 17: 435-442.
 11. Hadad G M, Emara S, Mahmoud W M M: Development and validation of a stability-indicating RP-HPLC method for the determination of paracetamol with dantrolene or/and cetirizine and pseudoephedrine in two pharmaceutical dosage forms. *Talanta* 2009; 79: 1360-1367.
 12. Ma M, Feng F, Sheng Y, Cui S, Liu H: Development and evaluation of an efficient HPLC/MS/MS method for the simultaneous determination of pseudoephedrine and cetirizine in human plasma: Application to Phase-I pharmacokinetic study. *J. Chromatog. B* 2007; 846: 105-111
 13. Jaber A M Y, Al Sherife H A, Al Omari M M, Badwan A A: Determination of cetirizine dihydrochloride, related impurities and preservatives in oral solution and tablet dosage forms using HPLC. *J. Pharm. Biomed. Anal.* 2004; 36: 341-350
 14. Azhagvuel S, Sekar R: Method development and validation for the simultaneous determination of cetirizine dihydrochloride, paracetamol, and phenylpropanolamine hydrochloride in tablets by capillary zone electrophoresis. *J. Pharm. Biomed. Anal.* 2007; 43: 873-878.
 15. Kang W, Liu K-H, Ryu J-Y, Shin J-G: Simultaneous determination of ebastine and its three metabolites in plasma using liquid chromatography-tandem mass spectrometry. *J. Chromatog. B* 2004; 813: 75-80.
 16. Matsuda M, Mizuki Y, Terauchi Y: Simultaneous determination of the histamine H₁-receptor antagonist ebastine and its two metabolites, carebastine and hydroxyebastine, in human plasma using high-performance liquid chromatography. *J. Chromatog. B* 2001; 757: 173-179
 17. Han Y, Lv Z, Jiang T, Wang Y: Bioanalysis and pharmacokinetics of chitosan ester in rabbit serum by HPLC with post column fluorescence derivatization. *J. Chromatog. B* 2007; 845: 138-142
 18. Volpi N, Maccari F, Ferrari S, Luca M, Pellegrini G: Separation of keratan-sulfate-derived disaccharides by high-performance liquid chromatography and post column derivatization with 2-cyanoacetamide and fluorimetric detection. *Anal. Bioch.* 2005; 342: 200-205
 19. Volpi N: Separation of capsular polysaccharide-K4- and defructosylated-K4-derived disaccharides by high-performance liquid chromatography and post column derivatization with 2-cyanoacetamide and fluorimetric detection. *Anal. Bioch.* 2004; 330: 359-361
 20. Aly F A: Spectrofluorimetric determination of prenalterol hydrochloride in pharmaceutical preparations and biological fluids. *J. Pharm. Biomed. Anal.* 1999; 18: 993-997
 21. Rizk M, Belal F, Ibrahim F, Ahmed S M, El-Enany N: Fluorimetric determination of oxamniquine in biological fluids. *Il Farmaco* 1999; 54: 47-50
 22. Volpi N, Maccari F: Microdetermination of chondroitin sulfate in normal human plasma by fluorophore-assisted carbohydrate electrophoresis (FACE). *Clinica Chimica Acta* 2005; 356: 125-133
 23. Guidance for industry; Q2B of analytical procedure: Methodology; International Conference on Harmonization (ICH), 2005. <http://www.fda.gov/eder/guidance/1320fnl>.
 24. Miller J C, Miller J-N: *Statistics for analytical chemistry*; Wiley; New York, 2005; 256.
 25. Vincent J, Liminana R, Meredith P A, Reld J L: The pharmacokinetics, antihistamine and concentration-effect relationship of ebastine in healthy subjects. *Br. J. clin. Pharmacol.* 1988; 26: 497-501
 26. Fieser L F, Fieser M, *Reagents for organic synthesis*, Wiley; London 1976; 889.
 27. Skoog D A, S R Crouch, Holler F J, "Principles of Instrumental Analysis". Brooks & Cole, New York, 6th edition, chapter 14, 2006, 329
