Validated Sensitive Spectrophotometric methods for Determination of Carvedilol and Nebivolol HCl in dosage forms.

Fawzia Ibrahim, Nahed M El-Enany, Shereen Shalan, Rasha Abo Shabana[*]

Department of Analytical Chemistry, Faculty of Pharmacy,

University of Mansoura, 35516, Mansoura, Egypt.

ABSTRACT

A simple, sensitive and rapid spectrophotometric methods were developed and validated for the determination of two antihypertensive drugs namely, carvedilol and nebivolol hydrochloride in pure form and pharmaceutical formulations. Method (I) is based on the formation of a binary complex between the studied drugs and eosin Y in presence of tween 80 at (pH 3.0).The formed complex exhibited maximum absorption at 545 nm for carvedilol and 543 nm for nebivolol. The concentration plots were rectilinear over concentration range of 0.5-5 and 1-7 µg/mL with lower detection limits of 0.09 and 0.11µg/mL and lower quantitation limits of 0.28 and 0 .34 µg/mL for carvedilol and nebivolol respectively. Method (II) is based on the reaction of studied drugs through their secondary amino groups with 2, 4-dinitrofluorobenzene (DNFB) at pH 8 to form yellow colored reaction products peaking at 383 nm and 390 nm for carvedilol and nebivolol, respectively. The absorbance-concentration plots were rectilinear over the concentration ranges of 5-30 and 4-28 µg/ mLwith the lower detection limits of 0.48 and 0.51 µg/mL and the lower quantitation limits of 1.45 and 1.54 µg/mL for cavredilol and nebivolol respectively. The different experimental parameters affecting the development and stability of the formed complex and reaction products were carefully studied and optimized for both methods. Both methods were successfully applied for determination of the studied drugs in their dosage forms.

KEYWORDS: Carvedilol; Nebivolol HCl; spectrophotometry; Eosin Y; dinitrofluorobenzene.

[*]Corresponding author

Fax: +20 502247496   Tel.: +20 502247496

E-mail address: rashaaboshabana@yahoo.com.

1. INTRODUCTION

Nebivolol•HCl is (1RS,1'RS)-1,1'-[(2RS,2'SR)-bis(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)]-2,2'-iminodiethanol hydrochloride(Fig.1a). Nebivolol HCl (NEB) is approximately 3.5 times more β1-adrenoceptor-selective than other β1-adrenergicblockers in human myocardium and thus might be the most β1-adrenoceptor-selective antagonist available for clinical practice at the moment. It has a combined vasodilating β1-blocker activity with a vasodilatin effect mediated by the endothelial L-arginine nitric oxide pathway .Several analytical methods were developed for the assay of nebivolol [1]. These methods include; spectrophotometry [4-12], spectrofluorimetry [13] HPLC [15]. Carvédilol,1-Carbazol-4-yloxy-3-[2-(2-methoxyphenoxy)ethyl amino] propan-2-ol. (Fig.1b). Carvedilol (CAR) is a non-cardio selective beta blocker. It has vasodilating properties, which are attributed mainly to its blocking activity at alpha1 receptors at higher doses calcium-channel blocking activity may contribute. It also has antioxidant properties. Carvedilol is reported to have no intrinsic sympathomimetic activity and only weak membrane-stabilising activity. Carvedilol is used in the management of hypertension and angina pectoris and as an adjunct to standard therapy in symptomatic heart failure. It is also used to reduce mortality in patients with left ventricular dysfunction after myocardial infarction [1, 2].
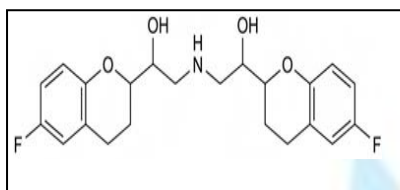
(a) Nebivolol
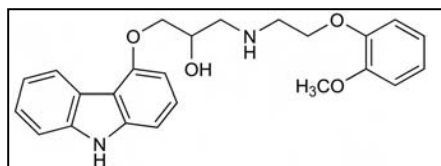
(b) Carvedilol

Fig 1: Structures Formula of studied drugs.

Several analytical methods were developed for the assay of carvedilol. These methods include; nonaqueous titration with 0.01M perchloric acid and 1% violet crystal as the indicator method and spectrophotometry [16-21], spectrofluorimetry [21,22] and HPLC [23-27].

The formation of complexes between eosin Y as an ion pairing agent and many pharmaceutical compounds for their spectrophotometric or spectrofluorimetic analysis with or without metal ions has been frequently investigated [28-33]. 2,4-Dinitrofluorobenzene have been used as chromogenic reagents for the analysis of many pharmaceuticals [35,36].

The main goal of the study is to develop accurate, simple, non-expensive spectrophotometric and more sensitive methods 2:10 times than reported ones from for the determination of NEB and CAR in pure form and in pharmaceutical preparations.

EXPERIMENTAL

2.1. Instruments

- A Shimadzu UV-Visible 1601 PC Spectrophotometer (Kyoto, Japan) was used for spectrophotometric measurements (P/N 206-67001). The recording range was 0-1.0.

- A Consort NV P901 digital pH Meter (Belgium) calibrated with standard buffers was used for checking the pH of the buffer solutions.

2.2. Reagents and materials

All the reagents used were of Analytical Reagent grade and distilled water was used throughout the work.

- Nebivolol hydrochloride was kindly provided by Marcyrl pharmaceutical industries. (El Obour City Cairo, Egypt) and was used as received without further purification. The purity percentage of NEB 101.2%.

- Carvedilol was kindly provided by Global NAPI Pharmaceuticals, October City- Egypt and was used as received without further purification the purity percentage of CAR 99.99%.

- Pharmaceutical preparations containing NEB and CAR were obtained from commercial sources in the local market:

- Nevilob tablets; batch # 308521, labeled to contain 2.5 mg NEB.

- Nevilob tablet; batch # 310031, labeled to contain 5 mg NEB.

- Carvid tablets; batch # 530213, labeled to contain 6.25 mg CAR.

- Carvipress tablets; batch # 110624, labeled to contain 12. 5 mg CAR.

- Dilatrol tablets; batch # 121131, labeled to contain 25 mg CAR. Nevilob 5mg &2.5 mg tablets are manufactured by Marcyrl pharmaceutical industries (El Obour City Cairo, Egypt). Carvid tablets manufactured by Multi-Apex for Pharmaceutical Industries S.A.E –Bader City. Carvipress tablets manufactured by Global NAPI Pharmaceuticals,October City- Egypt. Dilatrol tablets manufactured by Chemi Pharm. Egypt.

- 2,4-Dinitrofluorobenzene (Fluka Chemie, Germany) solution was freshly prepared as 0.2% (v/v) in methanol.

- Eosin Y (Merck, Darmstadt, Germany), 2×10^{-3} M aqueous solution was prepared in distilled water.

- Acetic acid and anhydrous sodium acetate (Merck, Darmstadt, Germany).

- Acetate buffer, 0.2M was prepared by mixing various volumes of 0.2M acetic acid and 0.2M sodium   acetate solutions to obtain the required pH value (37).

- Methanol, hydrochloric acid, sodium hydroxide and boric acid (BDH, UK).

- Borate buffer solutions (0.2 M) were prepared by mixing appropriate volumes of 0. 2 M boric acid with 0.2 M sodium hydroxide and adjusting the pH using pH Meter.

- Surfactant (Tween 80, Citrimide, methyle cellulose, sodium dodecyle sulphate (SDS) (El-Nasr Pharmaceutical Chemicals Company (ADWIC), Abu Zaabal, Egypt).

2.3. Standard solutions

Stock solution of NEB and CAR were prepared by dissolving 10.0 mg of the studied drugs in 100 mL of methanol for method I or II, respectively. These solutions were further diluted with the same solvent as appropriate to obtain the working concentration range. The stock solutions are stable for 7 days when kept in the refrigerator.

2.4. GENERAL RECOMMENDED PROCEDURES

2.4.1. Procedures for calibration graphs

2.4.1. 1. Method I

Aliquots of the stock solutions in the concentration range shown in table (1) were transfer into a series of 10mL volumetric flasks. 1.2 mL of 0.1% tween 80 for (NEB) and 1mLfor (CAR) then 1.4 mL and1.0 mL of 2×10^{-3} M of eosin Y solution were added for NEB and CAR respectively, and the solutions were mixed well before the addition of 1.0 mL of 0.2 M acetate buffer (pH 3.0). For (NEB) only the solution was allow to stand for 15 minute and then the solutions completed with distilled water to the volume. The absorbance was measured at 545 or 543nm for (CAR) or (NEB) respectively against an appropriate reagent blank prepared simultaneously. To get the standard calibration graphs, the values of the absorbance were plotted against the final concentration µg/mL, alternatively the corresponding regression equations were derived.

Parameter	Method I		Method II	
Compounds	NEB	CAR	NEB	CAR
Linearity range (µg/mL)	1.0-7.0	0.5 – 5.0	4.0-28.0	5.0-30.0
Intercept (*a*)	8.66×10^{-2}	1.20×10^{-2}	1.49×10^{-2}	1.51×10^{-2}
Slope (*b*)	1.21×10^{-2}	1.77×10^{-2}	3.12×10^{-2}	2.85×10^{-2}
Correlation coefficient (*r*)	0.9999	0.9998	0.9999	0.9999
S.D. of residuals ($S_{y/x}$)	4.10×10^{-3}	6.70×10^{-3}	5.70×10^{-3}	4.40×10^{-3}
S.D. of intercept (S_a)	4.10×10^{-3}	8.63×10^{-3}	4.80×10^{-3}	4.10×10^{-3}
S.D. of slope (S_b)	9.00×10^{-4}	1.82×10^{-3}	3.00×10^{-4}	2.00×10^{-4}
Percentage relative standard deviation, % RSD	0.61	1.22	1.08	0.87
Percentage relative error, % Error	0.25	0.38	0.44	0.36
Limit of detection, LOD (µg/mL)	0.11	0.09	0.51	0.48
Limit of quantitation, LOQ (µg/mL)	0.34	0.28	1.54	1.45
Molar absorptivity, ε (L/mol./cm.)	53334	71951	12655	11551

Table 1. Analytical performance data for the determination of the studied drugs by the proposed method

2.4.1.1. Method II

To a set of 10 mL volumetric flasks, appropriate aliquots of the standard working solutions were quantitavely transferred, to obtain final concentrations range shown in table (1). To each flask, 0.4 mL and 0.5 mL of borate buffer solution (pH 8.0) followed by 2.4 and 2.2 ± 0.2 mL of DNFB solution (0.2% v/v) were added for NEB and CAR respectively and mixed well. The solutions were heated in thermostatically controlled water bath at 80 ± 5°C for 20 and 30 min for NEB and CAR respectivly. The reaction was stopped by cooling under tap water, and then 0.2 mL of conc HCl was added and the solutions were made up to volume with methanol. The absorbance was measured at 390 nm and 383 nm for (NEB) or (CAR) respectively against an appropriate reagent blank prepared simultaneously. The absorbance was plotted *versus* the final concentration of the drugs to obtain the calibration graphs. Alternatively, the corresponding regression equations were derived**.**

2.4.2. Assay procedure for tablets:

Ten tablets (Nevilob 2.5 mg or 5 mg or Carvid, Carvipress, Dilatrol tablets) were accurately weighed, finely pulverized, and thoroughly mixed well. An accurately weighed amount of powdered tablets equivalent to 10.0 mg of NEB and CAR were transferred into small conical flask and extracted with 3 x 30 mL of methanol for method I or method II. The extract was filtered into 100 mL volumetric flask. The conical flask was washed with few mLs of methanol. The washings were passed into the same volumetric flask and completed to the volume with the same solvent. Aliquots of these solutions were transferred into a series of 10 mL volumetric flasks and the procedures described under **"Calibration Graphs"** were then performed for both methods. The nominal content of the tablets was determined either from the previously plotted calibration graphs or using the corresponding regression equations.

3. RESULTS AND DISCUSSION

3.1. Method I

Eosin has been utilized for the determination of many pharmaceutical compounds of interest either through spectrophotometric measurment such as, antibacterials ,gliclazide (28), fluoroquinolone (29), carbinoxamine maleate (30), ramipril and enalapril (31) .

The purpose of the present study was to develop simple and sensitive spectrophotometric methods for the determination of NEB and CAR in there pharmaceutical formulations without prior extraction. In the present study the studied drugs were found to form an ion pair red complex with eosin at pH 3.0 with maximum absorbance at 543 nm and 545 nm for NEB and CAR respectively (Figures. 2 &3). The formed complex is mainly due to the electrostatic interaction between the studied drug and anionic functional group of eosin under acidic pH.
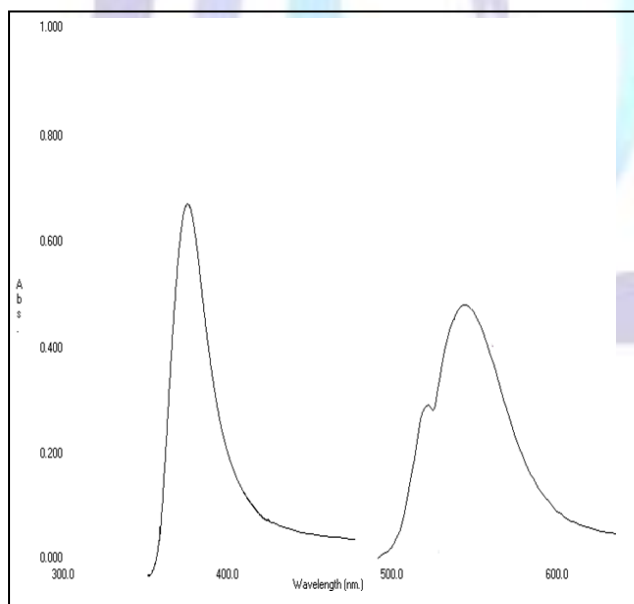

Fig 2: Absorption spectrum of the reaction products of nebivolol HCl (5.0 µg/mL) with (2 × 10^{-4}) M eosin at pH 3.0 and nebivolol HCl (20.0 µg/mL) with 0.2% v/v DNFB at PH 8.0
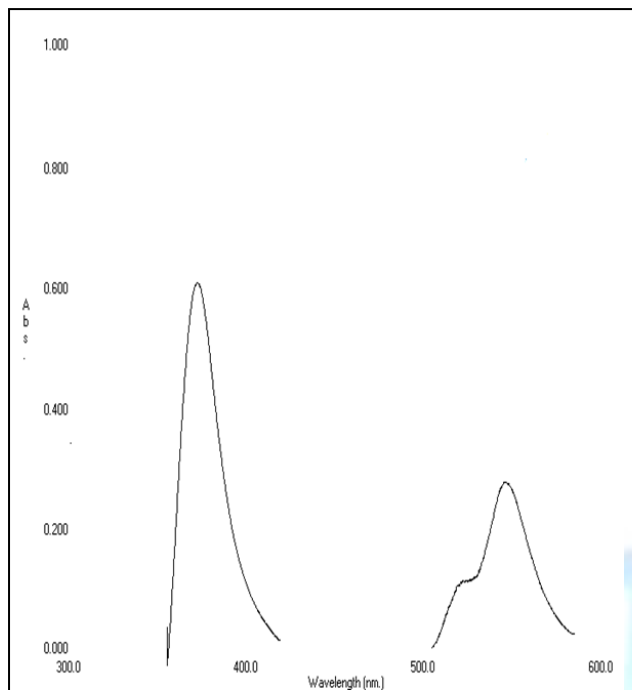
Fig. 3: Absorption spectrum of the reaction products of carvedilol (2.0 μg/mL) with (2 × 10-4) M eosin at pH 3.0 and carvedilol (25.0 μg/mL) with 0.2% v/v DNFB at PH 8.0.

3.2. Method II

In the present study, NEB and CAR were found to react with DNFB in presence of borate buffer producing a yellow color peaking at 383 and 390 nm. (Figures. 2, 3)

The analytical applications of DNFB in the assay and characterization of amines have been established by Conner (34).

To remove the interference of excess DNFB reagent on absorbance measurements of the reaction products, the excess reagent was acid hydrolyzed to colorless 2, 4- dinitrophenol by adding 0.2 mL of HCl.

3.3. STUDY OF EXPERIMENTAL PARAMETERS

The experimental conditions were optimized by varying each in turn while keeping all others constant. These variables include; effect of pH and volume of buffer, effect of the concentration of reagents, effect of heating temperature and heating times, effect of diluting solvent and effect of time on stability of the reactions products.

3.3.1. Effect of pH and volume of buffer:

For method I:

The influence of pH on the absorbance value of the binary complexes was studied over the pH range 2.5-5.0. The optimum absorbance values were obtained at pH 3.0 ± 0.2 for both drugs as shown in (Fig 4.a). One milliliters of 0.2M acetate buffer were sufficient to bring the optimum pH value for CAR Fig (5.a) and NEB . For the highest color intensity and maximum precision, the buffer solution should be added after mixing the drug-dye solution at neutral pH.
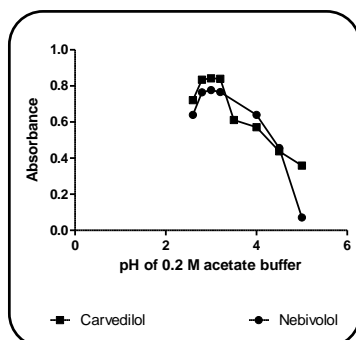

. **Fig 4.a: Effect of pH of 0.2M acetate buffer on the absorbance of the reaction products of 5.0 μg/mL of studied drugs with eosin Y (2×10-3 M).**

For method I I:

According to the literature, the reaction of amines with DNFB was carried out in alkaline medium. So, the influence of pH on the formation of the reactions products was studied over the range of 5.0-12.0 using 0.2 M borate buffer solution. Maximum and constant absorbance intensities were achieved at pH 8.0 ± 0.2 for the studied drugs. At pH higher than 10 precipitations occurred. Therefore, pH 8.0 were chosen as the optimum pH values for studied drugs , respectively(Fig .4b)
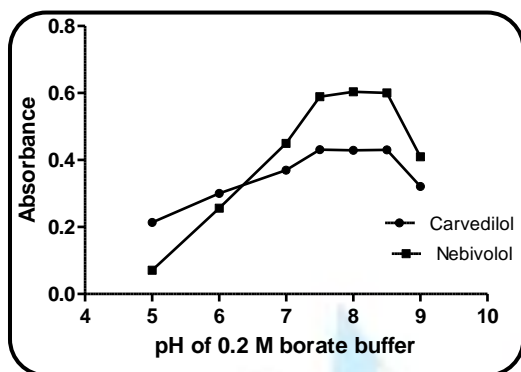

Fig 4.b: Effect of pH of 0.2M borate buffer
on the absorbance of the reactions products of
15.0 μg/mL CAR and 20.0 μg/mL NEB with DNFB.

Maximum absorbance intensities were achieved using 0.4 ± 0.2 mL or 0.5 ± 0.3 mL of borate buffer solutions for NEB and CAR , respectively (Fig5.a&b). So that, 0.4 and 0.5 mL of borate buffer solution of pH 8.0 were chosen as the optimum buffer volumes for method II.
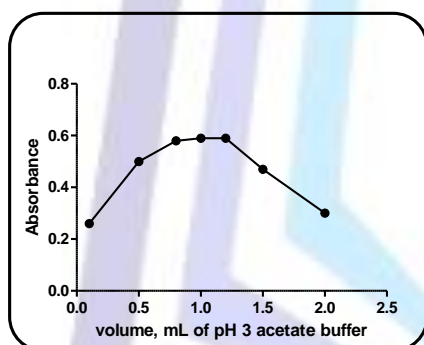

Fig 5.a: Effect of volume of 0.2M acetate buffer
on the absorbance of the reaction product
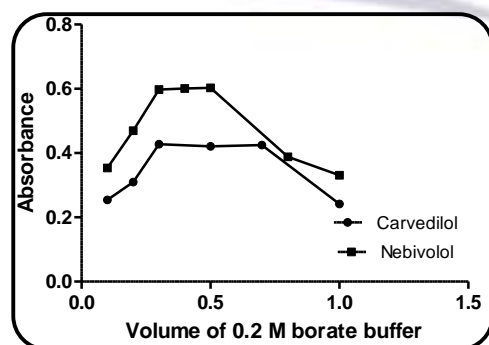**of 5.0 μg/mL CAR with eosin Y (2×10-3 M).**

Fig .5.b: Effect of volume of 0.2 M borate buffer
on the absorbance of the reactions products
of 15.0 μg/mL CAR and 20.0 μg/mL NEB with 0.2% .DNFB

3.3.2. Effect of the concentration of reagents solutions

The influence of the reagents concentrations was studied using different volumes of either 2×10^{-3} M eosin Y (method I) or 0.2% v/v solution of DNFB (method II). It was found that, increasing volumes of the reagents produced a proportional increase in the absorbance values. Maximum and constant absorbance intensities were achieved using volumes of the reagents ranged from 0.5 -2.5 mL of 2×10^{-3} M eosin Y and 0.5 -2.5 mL of 0.2% v/v solution of DNFB. Further increase of the reagents concentrations produced gradual decrease in the absorbance intensities. Therefore, 1.0 and 1.4 ± 0.2 mL of 2×10^{-3} M eosin Y solution (Fig.6.a) For method I and 2.2 and 2.4 ± 0.2 mL of 0.2% v/v DNFB solution were chosen as the optimal volumes of the reagents for method II (Fig.6.b). In method II, addition of 0.2 mL of conc HCL is essential to remove excess DNFB reagent which interferes with the measurement of reaction product.
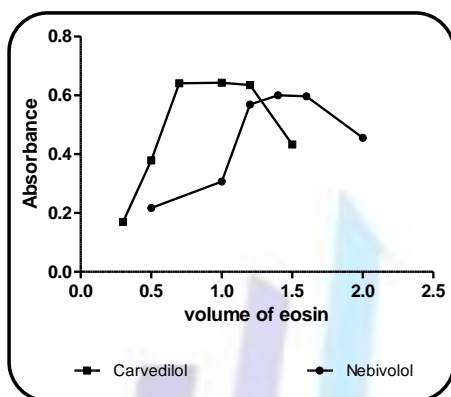

Fig 6.a: Effect of volume of eosin Y (2×10-3 M).
on the absorbance of the reaction products
of 5.0 µg/mL CAR and 4.0 µg/mL NEB.

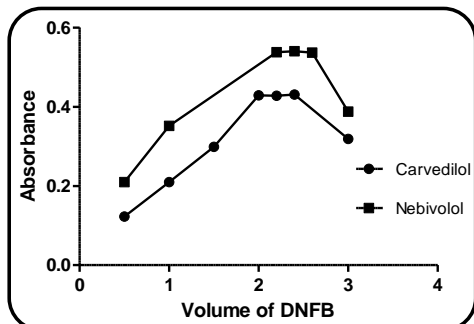**Fig 6.b: Effect of volume of the reagents on**
 the absorbance of the reactions products
of 15.0 µg/mL CAR and 20.0 µg/mL NEB with DNFB.

3.3.4. Effect of type and volume of surfactant

Due to the slight solubility of complexes formed with eosin Y in aqueous acidic solutions, it was difficult for the produced color to be accurately and precisely measured. Therefore, several trials for solving this problem were conducted, via extraction with organic solvent or addition of different surfactant such as methyl cellulose, tween 80, citrimide and SDS to solubilize and stabilize the formed complex were attempted

Methyl cellulose and tween 80 were attempted to prevent complex precipitation, however the tween 80 give good reproducibility.

The influence of the surfactant concentrations was studied using different volumes of 0.1% tween 80 Maximum absorbance intensities were achieved using 1.2 ± 0.2 mL or 1.0 ± 0.2 mL of 0.1% tween 80 for NEB and CAR, respectively (Fig.7).
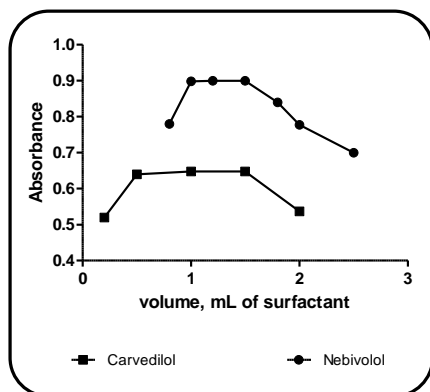
Fig 7: Effect of volume of surfactant
on the absorbance of the reaction product
of 5.0 µg/mL CAR and
4.0 µg/mL NEB with Eosin Y (2×10-3 M).

3.3.3. Effect of temperature and heating time

For method I the intensity of the colored product was maximum at room temperature for both drugs; increasing the temperature resulted in formation of a precipitate which may be due to coagulation of the formed complex. The formation of the complex was instantaneous and the development of the color was complete within few seconds for CAR but for NEB the development of the color was achieved within 15 minute (Fig 8). The intensity of the final color was stable for 2 hours with no precipitation of the complex.
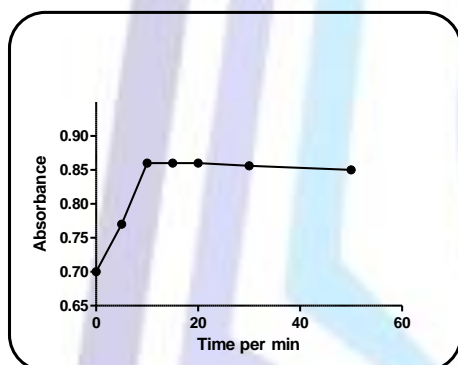

Fig 8: Effect of time on the absorbance of
the reaction product of nebivolol with eosin Y (2×10-3 M).

For method II studies showed that the reaction rates were very slow at room temperature so, the reaction was performed in a thermostatically controlled water bath at different temperature settings ranging from (25–100 °C) for various time intervals. For both drugs, the results revealed that increasing the temperature resulted in an increase in the absorbance values of the reactions products. The maximum absorbance values were attained at 75-85 °C within 20 min and 30 min for NEB and CAR, respectively (Fig.9.a&b). At higher temperatures, precipitation was observed. The decrease in the absorbance was probably attributed to the instability of the drugs derivatives at higher temperatures. Therefore, the studies were carried out at 80 ± 5.0°C for NEB and CAR, respectively.
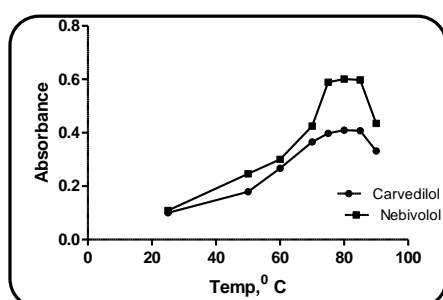

Fig 9.a: Effect of heating temperature on
the absorbance of the reactions products
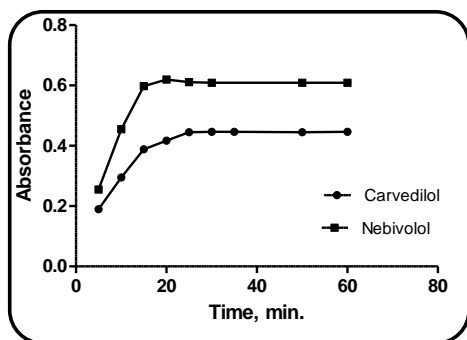**of 15.0 µg/mL CAR and 20.0 µg/mL NEB with DNFB 0.2% .**

Fig 9.b: Effect of heating time on the absorbance of the reactions products of 15.0 µg/mL CAR and 20.0 µg/mL NEB with DNFB 0.2% .

3.3.5. Effect of diluting solvent

The effect of diluting solvent on the absorbance intensities of the reaction products was tested using different solvents *viz* water, methanol, ethanol, acetone, acetonitrile and dimethylformamide. Using water and methanol as diluting solvents gave the highest absorbance values and more reproducible results for methods I and II, respectively. So, they were selected as the best solvents.

3.3.6. Effect of time on stability of the reactions products

Regarding the stability of the produced derivatives, both were found to be stable at room temperature for approximately 1 hour.

3.2. VALIDATION OF THE PROPOSED METHODS

The validity of the proposed methods was tested regarding linearity, specificity, accuracy, repeatability and intermediate precision according to ICH Q2 (R1) recommendations [38].

3.2.1. Linearity and range

The calibration graphs obtained by plotting the values of the absorbance *versus* the final concentrations of the drug (µg/ml) were found to be rectilinear over the concentration ranges cited in Table 1.

Method I:

$\qquad$ A= 0.015+ 0.029C $\qquad$ (r= 0.9999) For (CAR)

$\qquad$ A= 0.015+ 0.312C $\qquad$ (r= 0.9999) For (NEB)

Method II:

$\qquad$ A= 0.012+ 0.178C $\qquad$ (r= 0.9998) For (CAR)

$\qquad$ A= 0.087+ 0.121C $\qquad$ (r= 0.9999) For (NEB)

Where (A) is the absorbance, (C) is the concentration in µg/ml and (r) is the correlation coefficient

The validity of the methods was proved by statistical evaluation of the regression data, regarding the standard deviation of the residuals ($S_{y/x}$), the standard deviation of the intercept (S_a) and standard deviation of the slope (S_b) (Table 1). The small values of the figures indicate low scattering of the points around the calibration line and high precision.

3.2.2. Limit of Quantitation and Limit of Detection

Limit of Quantitation (LOQ) and Limit of Detection (LOD) were calculated according to ICH Q2 (R1) recommendation using the following equations [38]:

LOQ = 10 S_a/b

LOD = 3.3 S_a/b

Where S_a is the standard deviation of the intercept of regression line, and b is the slope of the regression line. The values of LOD and LOQ for both methods are abridged in Table 1.

3.2.3. Accuracy

To test the validity of the proposed methods, they were applied to the determination of pure sample of NEB and CAR over the concentration ranges cited in Table 1. The results obtained were in good agreement with those obtained

using the comparison method [3] for NEB and official method [2] for CAR. Statistical analysis of the results obtained using Student t-test and the variance ratio F-test [39] revealed no significance differences between the proposed and comparison methods regarding the accuracy and precision, respectively (Tables 2&3). The comparison method is based on measuring the absorption spectrum of nebivolol hydrochloride dissolved in methanol in the range of 200-400 nm against the blank similarly prepared. The standard solution show maximum absorbance at 281 nm [3]. The official method based on determination of carvedilol by HPLC using sodium dihydrogen phosphate and acetonitrile at pH 3.0 as mobile phase at wavelength 240 nm [2].

Table 2. Assay results for the determination of the Nebivolol in pure form by the proposed and comparison methods

Parameter	Method I			Method II			Comparison method [3]
	Conc. taken (µg/mL)	Conc. found (µg/mL)	% Found [*]	Conc. taken (µg/mL)	Conc. found (µg/mL)	% Found [*]	% Found
	1.0	0.993	99.34	4.0	4.0096	100.24	97.76
	3.0	2.998	99.94	8.0	8.0801	101.00	100.00
	4.0	3.980	99.51	12.0	12.1506	101.26	101.00
	5.0	5.036	100.73	16.0	15.7083	98.18	98.88
	6.0	6.035	100.58	24.0	24.0737	100.31	
	7.0	6.959	99.41	28.0	28.1122	100.40	
$\bar{x}$ ± S.D.		99.92 ±0.61			100.23 ± 1.09		99.41 ± 1.40
t		0.801 (2.306)[**]			1.049 (2.306)[**]		
F		5.123 (5.409)[**]			1.663 (5.409)[**]		

Table 3. Assay results for the determination of the Carvidilol in pure form by the proposed and comparison methods

Parameter	Method I			Method II			Comparison method [2]
	Conc. taken (µg/mL)	Conc. found (µg/mL)	% Found [*]	Conc. taken (µg/mL)	Conc. found (µg/mL)	% Found [*]	% Found
	0.5	0.495	98.94	5.0	5.056	101.12	98.00
	1.5	1.529	101.93	10.0	10.037	100.39	100.74
	3.0	2.990	99.68	15.0	14.916	99.44	101.48
	3.5	3.496	99.89	20.0	19.723	98.61	100.32
	4.0	3.946	98.65	25.0	25.091	100.36	
	5.0	5.042	100.84	30.0	30.004	100.01	
$\bar{x}$ ± S.D.		99.99 ±1.22			99.99 ± 0.87		100.14 ± 1.50
t		0.170 (2.306)[**]			0.198 (2.306)[**]		
F		1.505 (5.409)[**]			2.983 (5.409)[**]		

N.B. [*] Each result is the average of three separate determinations.

[*] *The figures between parentheses are the tabulated t and F values at P = 0.05 (25)

3.2.4. PRECISION

3.2.4.i. Repeatability (intra-day):

The repeatability was performed over the specific concentration ranges through replicate analysis of three concentrations of studied drugs in pure form on three successive occasions. The results are presented in Table (4, 5).

3.2.4.ii. Intermediate precision (inter-day):

Intermediate precision was tested by repeated analysis of NEB and CAR in pure form using the concentrations shown in Tables (4, 5) for a period of 3 successive days. High % recovery and low % RSD indicate high accuracy and precision of the proposed methods, respectively.

Table 4. Precision data for the determination of NEB by the proposed methods.

Amount taken (µg/mL)	% Found	% RSD	% Error
Method I			
Intraday			
1.0	99.09 ± 1.87	1.89	1.09
3.0	99.61 ± 0.68	0.68	0.39
5.0	98.91 ± 0.51	0.52	0.30
Interday			
1.0	99.38 ± 0.88	0.89	0.51
3.0	102.03 ± 0.05	0.05	0.03
5.0	100.47 ± 2.33	2.32	1.34
Method II			
Intraday			
8.0	99.41 ± 0.70	0.70	0.40
12.0	99.38 ± 0.84	0.85	0.49
16.0	99.44 ± 1.93	1.94	1.12
Interday			
8.0	99.48 ± 0.32	1.33	0.77
12.0	100.15 ± 1.32	1.32	0.76
16.0	100.6 ± 0.57	0.57	0.33

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N. B. Each result is the average of three separate determinations.

Table 5. Precision data for the determination of CAR by the proposed methods.

Amount taken (µg/mL)	% Found	% RSD	% Error
Method I			
Intraday			
3.0	99.78 ± 0.74	0.74	0.43
4.0	100.06 ± 0.53	0.53	0.31
5.0	99.09 ± 1.87	1.89	1.09
Interday			
3.0	100.15 ± 0.74	0.74	0.43
4.0	102.03 ± 0.05	0.05	0.03
5.0	100.06 ± 2.33	2.32	1.34
Method II			
Intraday			
10.0	99.53 ± 0.91	0.91	0.53
15.0	99.83 ± 1.10	1.10	0.49
20.0	99.44 ± 1.93	1.94	1.12
Interday			
10.0	99.41 ± 0.70	0.70	0.40
15.0	100.15 ± 1.32	1.32	0.76
20.0	100.33 ± 1.87	1.87	0.67

N. B. Each result is the average of three separate determinations.

3.2.5. Robustness

The robustness of the procedures adopted in the two proposed methods was demonstrated by the constancy of the absorbance value with the deliberated minor changes in the experimental parameters. For method I, the changes included the pH of acetate buffer solution, 3.0 ± 0.2, the change in the volume of acetate buffer solution, 1.0 ± 0.2 mL, the change in the volume of $2{\times}10^{-3}$ M eosin Y 1.0 ± 0.2 for CAR and 1.4± 0.2 for NEB. Meanwhile, for method II these changes included the pH of borate buffer solution, 8.0 ± 0.5, the change in the volume of the buffer solution, 0.4 and 0.5 ± 0.2 mL, the change in the volume of DNFB (0.2% v/v), 2.2 and 2.4 ± 0.2 mL, the change in the heating temperature, 80 ± 5°C and the change in the heating time, 20 and 30 ± 5 min. These minor changes that may take place during the experimental operation didn't affect the absorbance value of the reaction products.

3.4.6. Selectivity

The selectivity of the methods was investigated by observing any interference encountered from the common tablets excipients such as starch, lactose and magnesium stearate. It was found that, these excipients didn't interfere with the results of the proposed methods (Tables 6, 7).

Table 6. Application of proposed methods to the determination of the CAR in their dosage forms.

Pharmaceutical preparation	Method I			Method II			Comparison method [2]
	Conc.taken (µg/mL)	Conc. found (µg/mL)	% Found	Conc.taken (µg/mL)	Conc. found (µg/mL)	% Found	% Found
1- **Carvid tablet**	0.5	0.504	100.74	10.0	9.901	99.00	98.94
(6.25 mg CAR /tablet)	1.5	1.518	101.21	15.0	15.279	101.86	99.23
	3.5	3.448	98.51	20.0	19.741	98.71	101.32
	5.0	5.031	100.62	25.0	25.080	100.32	99.78
Mean ± S.D.	100.27 ± 1.20			99.97 ± 1.44			99.82 ± 1.06
t	0.564 (2.447)*			0.283 (2.446)*			
F	1.281 (9.276)*			1.147 (9.276)*			
2- **Carvipress tablets**	0.5	0.512	102.42	10.0	10.045	100.45	99.00
(12.5 mg CAR/tablet)	1.5	1.491	99.41	15.0	14.889	99.26	100.50
	3.5	3.486	99.59	20.0	19.976	99.88	102.00
	5.0	5.012	100.24	25.0	24.993	99.97	99.50
Mean ± S.D	100.42 ± 1.38			99.89 ± 0.49			100.25 ± 1.32
t	0.172 (2.447)*			0.511 (2.447)*			
F	1.093 (9.276)*			7.322 (9.276)*			
3- **Dilatrol tablets**	0.5	0.502	100.44	10.0	9.848	98.48	102.00
(25 mg CAR/tablet)	1.5	1.524	101.57	15.0	15.174	101.16	101.00
	3.5	3.437	98.20	20.0	19.993	99.96	99.50
	5.0	5.037	100.73	25.0	24.884	99.54	101.80
Mean ± S.D	100.24 ± 1.44			99.79 ± 1.11			101.08 ± 1.14
t	0.916 (2.447)*			1.626 (2.447)*			
F	1.605 (9.276)*			1.049 (9.276)*			

**Table7. Application of proposed methods to the determination of the NEB in their dosage forms.**

Pharmaceutical preparation	Method I			Method II			Comparison method [3]
	Conc.taken (µg/mL)	Conc. found (µg/mL)	% Found	Conc.taken (µg/mL)	Conc. found (µg/mL)	% Found	% Found
1- **Nevilob tablets**	1.0	1.014	101.44	8.0	7.968	99.60	100.55
(2.5 mg NEB /tablet)	3.0	3.029	100.96	16.0	15.925	99.53	100.85
	5.0	4.899	97.99	24.0	24.276	101.15	98.70
	7.0	7.058	100.82	28.0	27.753	99.12	100.35
Mean ± S.D.	100.30 ± 1.56			99.85 ± 0.85			100.22 ± 1.17
t	0.207 (2.447)*			0.399 (2.446)*			
F	2.634 (9.276)*			1.167 (9.276)*			
2- **Nevilob tablets**	1.0	1.005	100.50	8.0	8.062	100.78	99.00
(5 mg NEB/tablet)	3.0	3.026	100.88	16.0	15.905	99.41	100.50
	5.0	4.924	98.48	24.0	24.075	100.31	102.00
	7.0	7.036	100.52	28.0	28.029	100.11	99.50
Mean ± S.D	100.10 ± 1.09			100.15 ± 0.57			100.25 ± 1.32
t	0.181 (2.447)*			0.135 (2.447)*			
F	1.471 (9.276)*			5.403 (9.276)*			

3.4. PHARMACEUTICAL APPLICATIONS

The proposed methods were successfully applied to the determination of the studied drugs in their pharmaceutical preparations. The results obtained were statistically compared to those of the comparison method [3] for NEB and official method [2] for CAR using Student's *t*-test for accuracy and the variance ratio F-test for precision, respectively (Tables 6, 7). The results obtained indicate no significant difference between the proposed methods and the comparison methods regarding accuracy and precision.

3.5. MOLAR RATIO AND MECHANISM OF THE REACTION

The stoichiometry of the reactions in the two methods was studied adopting the limiting logarithmic method [40]. Plots of log absorbance *versus* log [reagent] and log [drug] gave straight lines. The slopes of which were 0.97 and 0.8 for eosin and 0.8 and1.06 for CAR and NEB in method I, respectively (Fig. 10). It is concluded that the complex formation proceed in the ratio of 1:1, confirming that one molecule of the drugs with one molecule of eosin in method I, respectively. Based on the absorbed molar ratios, proposed reaction pathways are given in scheme 1(a, b).
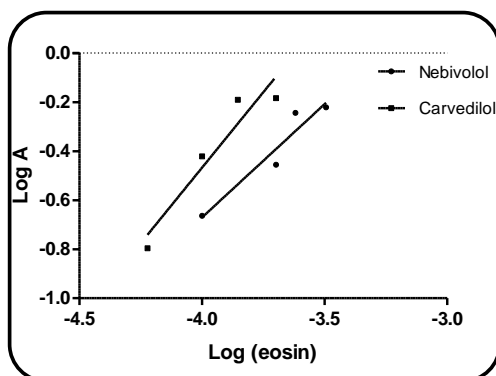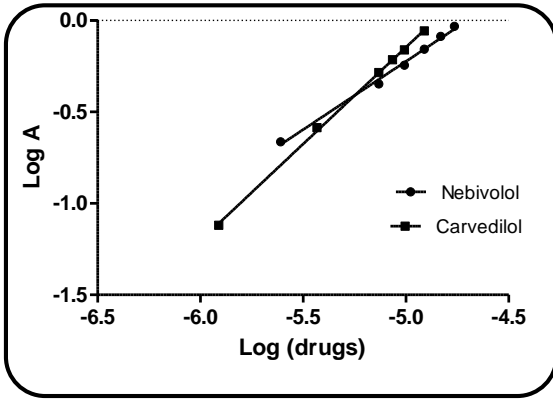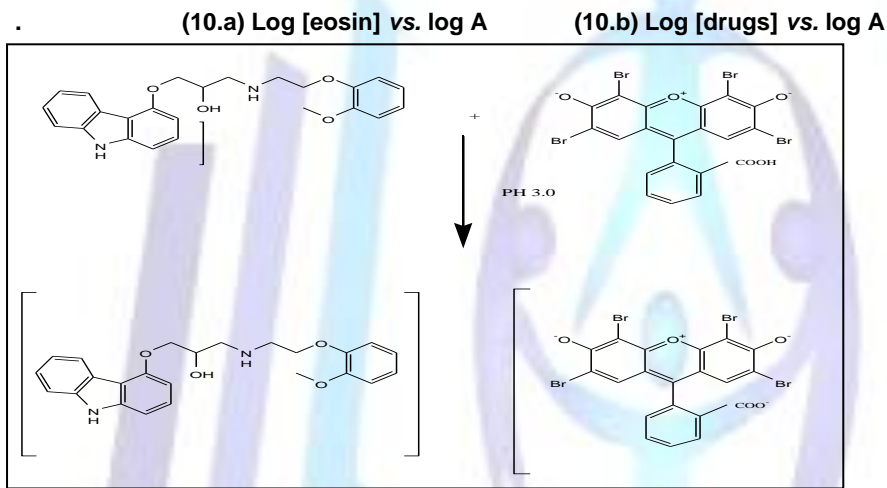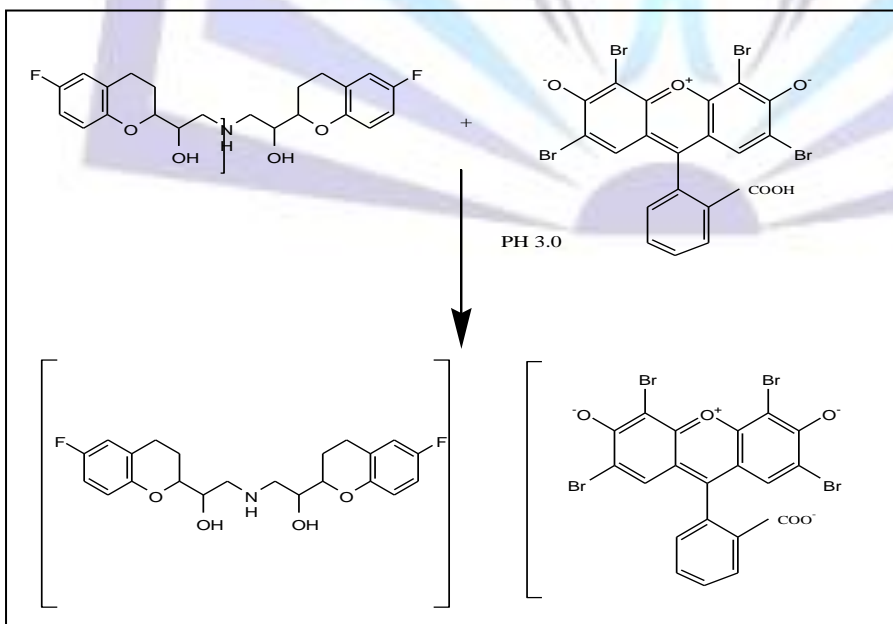
Fig 10: Stoichiometry of the reaction between studied drugs and eosin (2×10^{-3} M) adopting limiting logarithmic method.

.   **(10.a) Log [eosin]** *vs.* **log A**   **(10.b) Log [drugs]** *vs.* **log A**

Scheme 1. a: The proposal mechanism for the reaction between carvedilol and Eosin Y.

Scheme 1.b: The proposal mechanism for the reaction between NEB and Eosin Y.

The absorbance of the reaction products were alternatively measured in the presence of excess of either DNFB or drug. A plot of log Absorbance versus log [DNFB] and log [drug] gave straight lines, the values of the slopes are 0.99 and 1.02 respectively for CAR and 1.05 and 1.00 for NEB (Fig. 11). Hence, it is concluded that, the molar reactivity of the reaction is 0.99 / 1.02 for CAR and 1.05 / 1.00 for NEB, i.e. the reaction proceeds in the ratio of 1: 1 in both drugs. It is confirmed that one molecule of the drug reacts with one molecule of DNFB in alkaline medium through the secondary amino group of any of the two drugs to give the final reaction product. A schematic proposal of the reaction pathway is given in Scheme 2 (a.b).
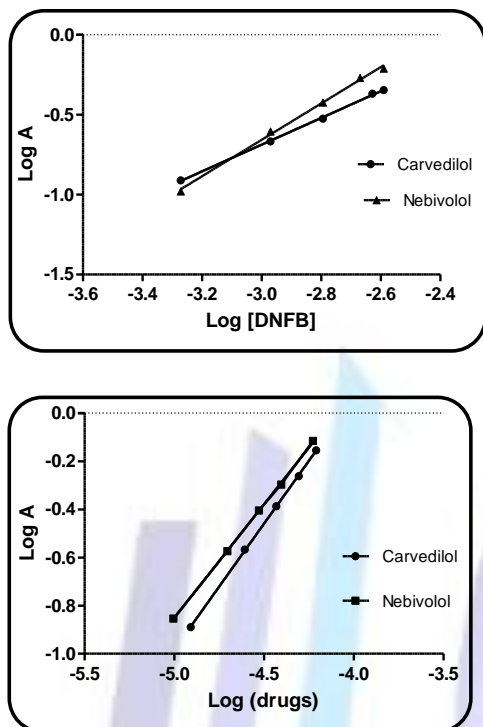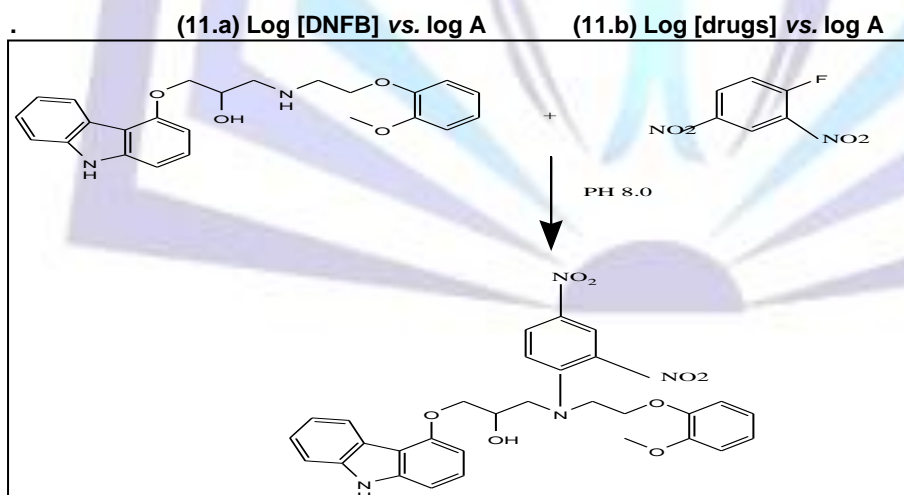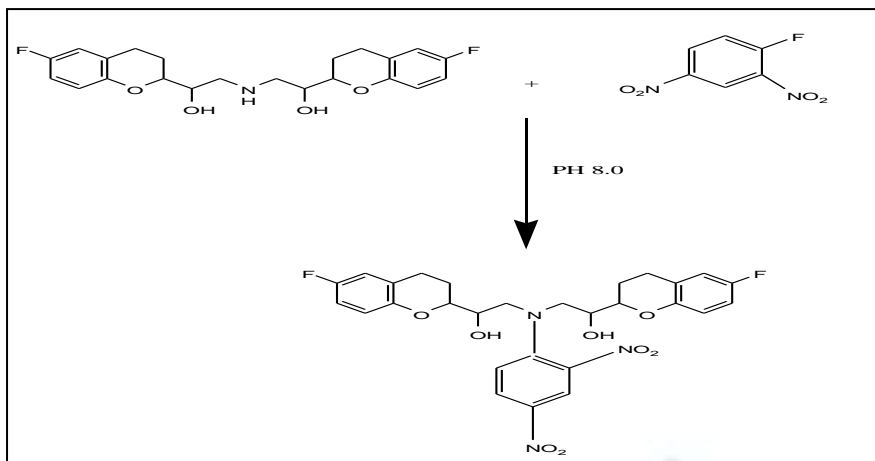

Fig 11: Stoichiometry of the reaction between

studied drugs and 0.2% of DNFB

Adopting limiting logarithmic method.

. **(11.a) Log [DNFB] *vs.* log A** **(11.b) Log [drugs] *vs.* log A**

Scheme 2. a: Proposed reaction pathway between carvedilol and 2, 4-Dinitrofluorobenzene

under the described reaction condition.

Scheme 2. b: Proposed reaction pathway between nebivolol and 2, 4-Dinitrofluorobenzene under the described reaction condition.

4. CONCLUSION

New simple and sensitive spectrophotometric methods for the determination of CAR and NEB have been successfully developed and validated. The method involved simple reaction of CAR and NEB with Eosin Y and DNFB reagents and subsequent measuring of the absorbance of the reaction products. The proposed methods are specific, accurate, reproducible, and highly sensitive to be applied on the analysis of studied drugs in pure form and pharmaceutical dosage forms. They were found to be sensitive, accurate and don't need expensive sophisticated instrument. Moreover, the reproducibility as well as convenience makes the two proposed methods suitable for routine analysis in quality control laboratories.

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