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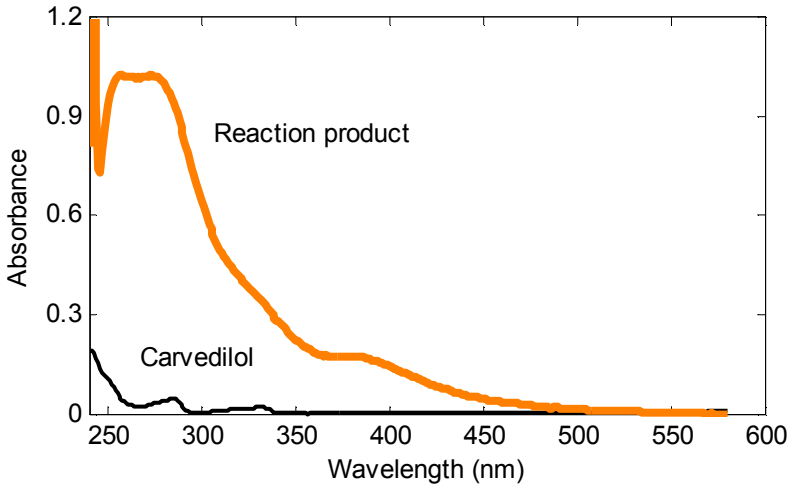

A simple, sensitive, selective, accurate and cost-effective spectrophotometric method for determination of CAR in pharmaceutical and urine samples was developed.

Title Page

Development of a highly sensitive and selective spectrophotometric method for determination of carvedilol in pharmaceutical and urine samples

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Abstract

A simple, sensitive, selective, accurate and cost-effective spectrophotometric method for determination of CAR in pharmaceutical and urine samples was developed. The method is based on the reaction between carvedilol and nitrite. Factors affecting the reaction were concentration of the reagents including hydrochloric acid, sodium nitrite and sodium hydroxide. The factors were optimized using central composite design. In the optimum conditions, two calibration curves at 250 and 278 nm were constructed with linear ranges of 0.05-0.20 and 1.5-3.5 mg L^{-1} and detection limits of 1.8×10^{-4} and 8.1×10^{-4} mg L^{-1}, respectively. In application of the proposed method, the results were precise and accurate. In complex urine samples the method was free from the interferences. Comparison with the reported methods for carvedilol determination using statistical characteristics revealed that the proposed method is superior over all.

Keywords: Carvedilol, Central composite design, Urine samples, Nitrite.

Introduction

Carvedilol (CAR), 1-(4-carbazolyloxy)-3-[2-(2-methoxy) ethylamino]-2-propanol (Fig. 1), is a non-selective β-adrenergic receptor antagonist and a α_1-adrenoceptor blocker. The β_1-blockade produces a decrease in heart rate and in the force of contraction of the cardiac muscle.[1-3] The FDA first approved CAR in 1995.[4] It is an official drug in British and European Pharmacopoeias. Lately, it was recognized as an effective agent for the treatment of congestive heart failure.[5,6]

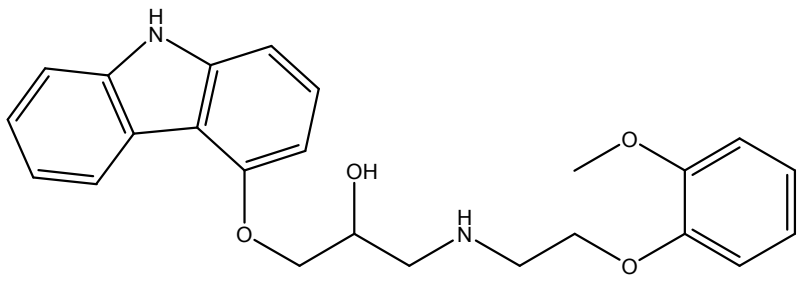


Fig. 1. Chemical structure of carvedilol.

Analytical methods such as spectrophotometry,[7,8] HPLC,[9-12] capillary electrophoresis,[13] flow injection spectrofluorimetry,[14] differential pulse voltammetry,[15] GC-MS,[16] liquid chromatographic,[17] chemiluminometry[18] and spectrofluorimetry[19,20] have been used for the determination of CAR.

It has been observed that in some of the applications of HPLC,[11,12] reproducibility and recovery are not satisfactory. Excessive extraction steps and using organic solvents can also be mentioned as their limitations. Capillary electrophoresis and tandem mass spectrometry for determination of CAR have been reported, but procedures were inconvenient.[21,22] A differential pulse voltametric procedure using a glassy carbon electrode was developed for the analysis of CAR in tablets.[13] However, this method has various limitations including time-consuming sample clean-up, laborious extraction steps, low sensitivity and long run times. Therefore, it is less suitable for routine analysis.

Spectrophotometric methods have been reported for determination of CAR in the UV-Vis region.[7,8] Using charge transfer complex formation systems for analytical determinations[7] needs extensive use of toxic organic solvents. In the UV-Vis region, interferents are unavoidable

especially in complex samples like urine. Moreover, the methods cannot be used for analyze of complex samples such as urine. An extractive spectrophotometric method has also been used to determine CAR in pharmaceuticals.[23] In this procedure, extraction by chloroform as a toxic organic solvent has been proposed.

In the view of the importance of determination of CAR, we developed a sensitive and selective UV-Vis spectrophotometric method for determination of CAR in pharmaceutical and biological samples. For optimization of the reaction conditions, central composite design (CCD) was employed. It was originally developed by Box and Wilson[24] and improved by Box and Hunter.[25] The proposed method when compared with the reported spectrophotometric methods takes the advantage over the UV spectrophotometric method in terms of selectivity and sensitivity.

Experimental

Apparatus and software

Recording of the absorption spectra in the spectral range of 200-600 nm was performed by an Agilent 8453 UV-Vis spectrophotometer with diode array detector equipped with 1 cm path length quartz cells. Design and analysis of the central composite experiments were carried out by MINITAB (Minitab Inc. Release 16.0) statistical package.

Reagents and Solutions

All chemicals were of analytical grade (>99%) and used as received without any further purification. These were sodium nitrite, sodium hydroxide and hydrochloric acid (Merck KGaA, Darmstadt, Germany).

A 1000.0 mgL^{-1} standard stock solution of CAR was prepared in absolute ethanol. Accurately, about 10.00 mg of CAR was weighed and transferred to 100 mL volumetric flask, ethanol added to dissolve drug then volume was completed to the mark with ethanol. The stock solution was diluted appropriately to get the working concentrations. Moreover, solutions of sodium nitrite (3.0% (w/v) in water), sodium hydroxide (15.0% (w/v) solution in water) and hydrochloric acid (6.0 M) were prepared for experiments.

Procedure

Two calibration curves were constructed in different concentration ranges of CAR. Accurately, measured volumes of standard stock solution of CAR (1.25-7.50 µL and 37.5-87.5 µL) were transferred to separate series of 25 mL volumetric flasks. Then, to each of these 25.0 mL volumetric flasks, volumes of the stock solutions of hydrochloric acid, sodium nitrite and sodium hydroxide were added such that the final concentrations of these reagents reached 0.432 M, 0.234% and 11.04%, respectively. Then, volume of the flasks was completed to the mark with doubly distilled water, the contents were shaken well and the absorbance of the solutions was measured at the wavelengths 250 nm and 278 nm, respectively. Calibration curves were constructed for CAR by plotting absorbance versus concentrations at wavelengths 250 and 278 nm.

Pharmaceutical preparation

Of each type of the tablets (Carvedilol 12.5 and Carvedilol 6.25 from Farabi Pharmaceutical Company) ten tablets were pulverized. An accurately weighed quantity of the mixed powder equivalent to one tenth of the weight of a tablet was transferred into 10 mL volumetric flask, and

made up to the mark with ethanol. The content was shaken for 30 min, filtered and transferred quantitatively into 10 mL volumetric flask. The solution was completed to the mark with ethanol. A 0.4 mL aliquot of this solution was transferred into 25 mL volumetric flasks. Then, the reagents were added based on the manner explained in "Procedure" section. The volume was made up to 25 mL with doubly distilled water and the absorbance was measured at 250 and 278 nm.

Human urine samples

5.0 mL aliquots of urine from healthy informed volunteers were spiked with different concentrations of CAR. In 25 mL volumetric flasks, 1.0 mL of the resulting urine solution was added followed by adding the reagents based on the manner explained in "Procedure" section. The volume was completed to the mark with doubly distilled water and the solution was stand for 90 min. The absorbance of each solution was measured and the nominal concentration of the drug in urine was determined based on the equation of the calibration curve.

Results and discussion

Central composite experimental design and optimization of factors

Experimental design methodology involves changing all factors from one experiment to the next, simultaneously. The reason for this is that factors can influence each other, and the optimum value for one of them might be related to the values of the others.

In CCD, it is assumed that the central point for each factor is 0 and the design is symmetrical around it.[26] Factors influencing the studied system and their considered levels for

design are shown in Table 1. For a system with three factors (n = 3), CCD consists of 18 experiments. Values of the factors in these 18 experiments and obtained responses are shown in Table 2.

Table 1

Experimental factors and their levels investigated in the reaction between CAR (2.0 mgL^{-1}) and nitrite.

Factor	Level		
	-1	0	1
	Parameter values		
X_1 (Concentration of hydrochloric acid; M)	0.12	0.30	0.48
X_2 (Concentration of nitrite; w/v%)	0.12	0.24	0.36
X_3 (Concentration of sodium hydroxide; w/v%)	3.0	6.0	9.0

Table 2

Experiments based on the central composite design with three factors (responses at 278 nm).

Experiment No.	Concentration of hydrochloric acid (M)	Concentration of sodium nitrite (w/v%)	Concentration of sodium hydroxide (w/v%)	Response
1	0.30	0.24	11.04	0.985
2	0.30	0.24	0.95	0.000
3	0.30	0.24	6.00	0.673
4	0.30	0.44	6.00	0.211
5	0.30	0.04	6.00	0.327
6	0.48	0.12	9.00	0.524
7	0.12	0.12	9.00	0.309
8	0.30	0.24	6.00	0.739

9	0.48	0.12	3.00	0.362
10	0.12	0.12	3.00	0.371
11	0.00	0.24	6.00	0.139
12	0.12	0.36	3.00	0.214
13	0.30	0.24	6.00	0.672
14	0.12	0.36	9.00	0.135
15	0.48	0.36	9.00	0.498
16	0.30	0.24	6.00	0.466
17	0.48	0.36	3.00	0.151
18	0.60	0.24	6.00	–

Analysis of variance (ANOVA) of the experiments performed (Table 2) is given in Table 3. The response of experiment no. 18 in Table 2 was higher than the scale of the spectrophotometer and noisy and it was not possible to assign an exact response for this experiment. Therefore, the response of this experiment was not considered in ANOVA.

Among the linear terms, concentration of hydrochloric acid (X_1) is significant and among the squared ones, X_2X_2 (X_2 is concentration of sodium nitrite) is significant (see Table 3). None of the interaction terms is significant at the 95% confidence level.

Table 3

ANOVA table for the factors and different interaction terms (coded units).

Term	Coefficient	t^a	p^b
Constant	0.600	6.439	0.000
X_1	0.209	2.459	0.044
X_2	-0.100	-1.064	0.323
X_3	0.204	2.157	0.068
X_1X_1	-0.247	-2.017	0.083
X_2X_2	-0.366	-2.360	0.050

X_3X_3	-0.142	-0.916	0.390
X_1X_2	0.026	0.175	0.866
X_1X_3	0.182	1.209	0.266
X_2X_3	0.059	0.312	0.764

[a]. *t* statistics.

In order to gain insight about the effect of each factor, response surfaces were constructed. These surfaces which indicate the variations of response with two of the factors are shown in Fig. 2. For obtaining each surface, the third factor is at its central level. From the sign of the coefficients in Table 3, it is clear that in higher values of factors X_1 and X_3, the response should be larger. This can also be observed from different panels of Fig. 2. Moreover, from Fig. 2, it can be concluded that in the intermediate amounts of factor X_2 (concentration of nitrite) the response is higher. Curvatures in the surfaces when factors X_1 and X_2 (concentration of hydrochloric acid and concentration of sodium nitrite, respectively) vary (Fig. 2) indicate some self-interaction (squared term) for these factors. For squared terms of these factors, *p* values are very low and close to the critical value of 0.05 (see Table 3). It can be observed from Fig. 2b that some interaction exists between factors X_1 and X_3 (concentration of hydrochloric acid and concentration of sodium hydroxide, respectively). From Table 3 it can be found that the *p* value of the interaction X_1X_3 is lower than the corresponding value for X_1X_2 and X_2X_3 interactions.

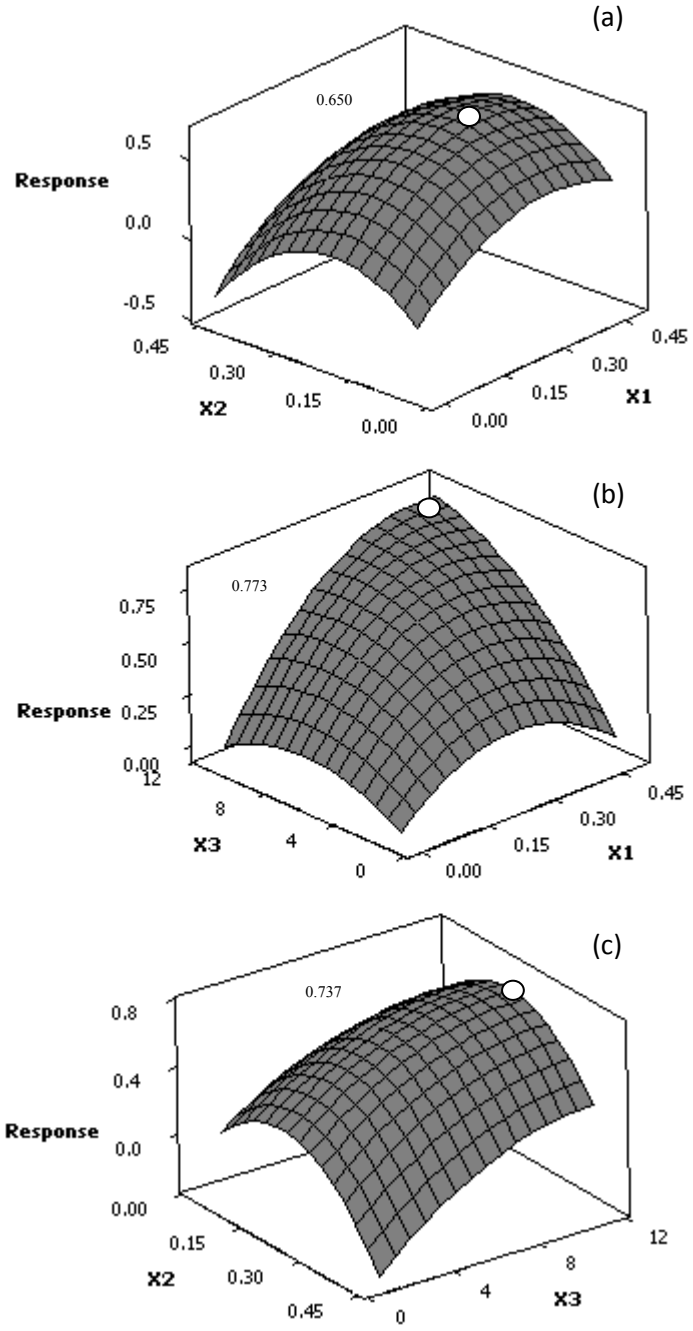


Fig. 2. Variation of response with (a) concentration of nitrite (X_2) and concentration of hydrochloric acid (X_1), (b) concentration of sodium hydroxide (X_3) and concentration of hydrochloric acid (X_1) and (c) concentration of nitrite (X_2) and concentration of sodium hydroxide (X_3). Maximum of each surface and its value has been shown.

The response surface plots in Fig. 2 show that all of the response surfaces have the maximum points. Therefore, response surface optimization could be used. Results of the response optimization showed that the response should be maximum with 0.432 M hydrochloric acid, 0.234 %(w/v) sodium nitrite and 11.04%(w/v) sodium hydroxide. Therefore, a high concentration of hydrochloric acid and concentrations near the center of the design for sodium nitrite are suitable for the studied reaction.

Absorption spectra

The spectrum of CAR in doubly distilled water (pH = 7.0) and spectrum of its reaction product with nitrite in the optimum conditions are shown in Fig. 3.

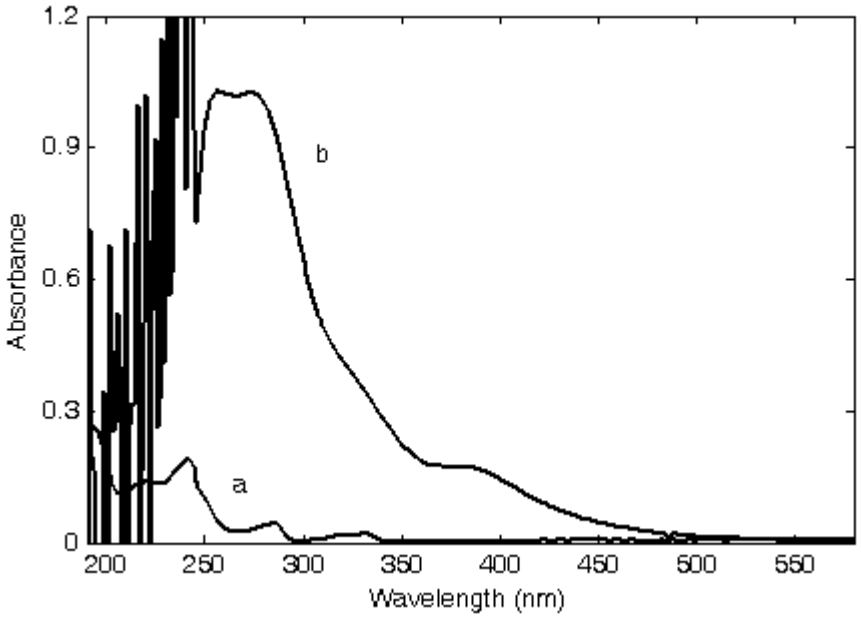
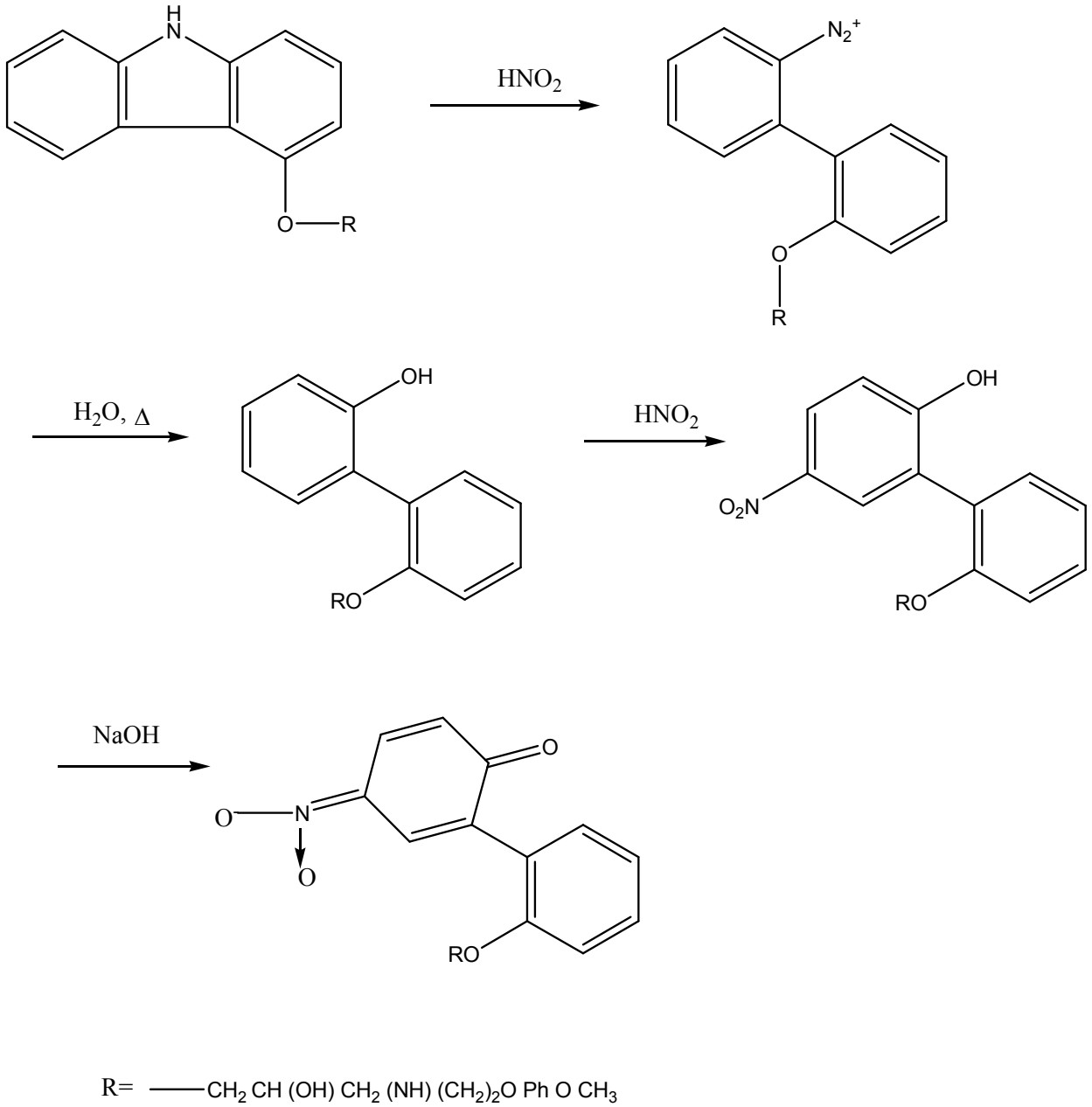


Fig. 3. Absorption spectra of CAR with concentration of 2.0 mg L^{-1} (a) in water pH = 7.0 and (b) after reaction with nitrite in optimum conditions.

As can be seen from Fig. 3, CAR shows weak absorption in the range of 190-350 nm with a maximum absorption at 240 nm. These low absorbances cause very low sensitivity in CAR determination. However, after reaction with nitrite[27,28] in optimum conditions, a product is formed which shows an intense spectrum which extends to about 500 nm. The maximum of the spectrum is located at 250-280 nm (b in Fig. 3) and corresponding shoulder is located at about 370 nm. This indicates the high sensitivity of the proposed method for determination of the studied drug.

Based on the reaction between indole derivatives and nitrite in the same conditions reported in,[27,28] the possible reaction between CAR and nitrite can be shown in Scheme 1.



Scheme 1. The possible reaction between CAR and nitrite in the optimum conditions.

In the first step, CAR with nitrous acid (HNO_2) undergoes diazotization. In the next step, the unstable diazonium group is replaced by hydroxyl group in aqueous medium. Now, the formed hydroxyl compound has a free *para*-position which can react with nitrous acid HNO_2 and give a

compound which after dissociation of the hydroxyl group in alkaline medium is responsible for the yellow color formation.

Analytical data

Under the optimized experimental conditions, adherence to Beer's law was studied by measuring the absorbance values of the solutions with varying drug concentration prepared as discussed in Section 2.4. Two calibration curves were constructed. The calibration curves were linear at concentration ranges of 0.05-0.20 and 1.5-3.5 mg L^{-1} at the wavelengths 250 and 278 nm, respectively. Calibration curves have been shown in Fig. 4. Statistical parameters of the calibration curves were calculated and reported in Table 4. The optical characteristics such as molar absorptivities of the products are also given in Table 4. The high values of molar absorptivity and slope of the calibration curves and low values of DL indicate the high sensitivity of the proposed method. However, statistical data in Table 4 imply that calibration curve at 250 nm is about four times more sensitive than the one at 278 nm. Standard errors of the parameters of the calibrations are also very low. The linearity of the calibration curve is validated by the high value of the correlation coefficient (which were close to unity) of the calibration curve.

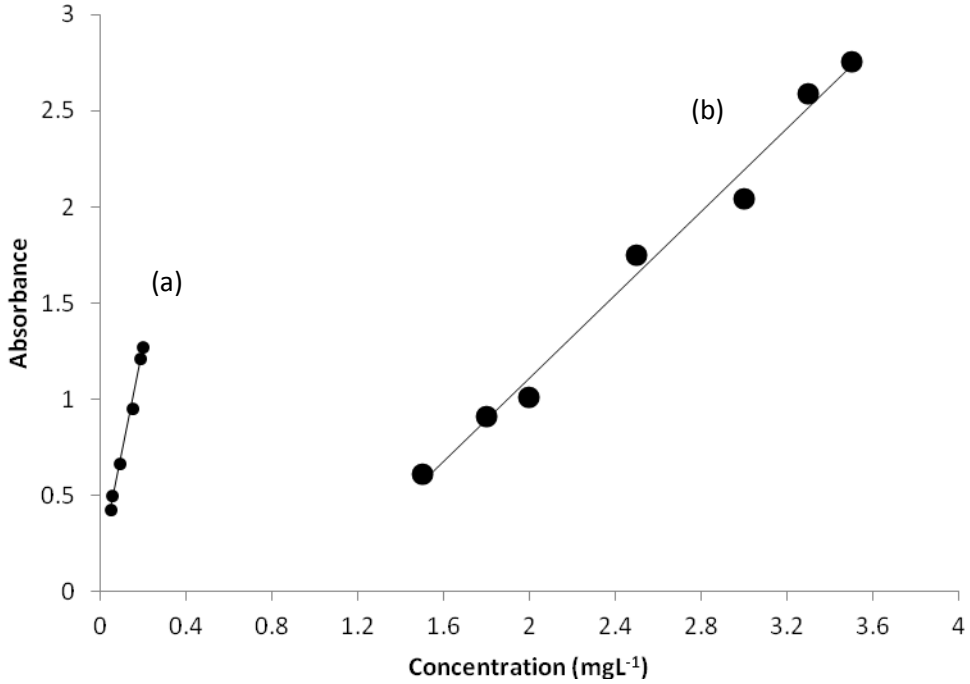


Fig. 4. Calibration curves constructed with the absorbances at 250 nm (a) and 278 nm (b) in optimum conditions.

Table 4

Statistical results of the calibration of CAR by the proposed method.

Parameters	Calibration curve 1	Calibration curve 2
λ max (nm)	250	278
Molar absorptivity of the product ($L\ mol^{-1}cm^{-1}$)	3.41×10^{6}	3.19×10^{5}
Linear range (mgL^{-1})	0.05-0.20	1.5-3.5
Intercept of calibration curve	0.159	1.051
Slope of calibration curve	5.423	1.081
Standard error of intercept	0.028	0.141

t statistics of intercept	21.18	7.45
Standard error of slope	0.237	0.054
Standard error of regression	0.027	0.103
t statistics of slope	22.88	20.02
Correlation coefficient	0.998	0.994
Detection Limit (DL)[a]	1.76×10^{-4}	8.12×10^{-4} mgL^{-1}

[a]. Calculated as DL = $y_B + 3s_B$, where y_B is the signal of the blank (intercept of the calibration curve) and s_B is the standard deviation of the blank.[29]

Application of the proposed method for pharmaceutical preparation

The constructed calibration curves were examined for determination of CAR in tablets and biological samples.

Under the optimized experimental conditions, the proposed method was used to determine CAR in commercial tablets. In this case, since the solution resulted by dissolving commercial tablets have higher concentrations of CAR, the second calibration curve was used for prediction of concentration. Results of determination of CAR in two commercial tablets have been collected in Table 5. The percentage of relative standard deviation values (RSD%) are acceptable (below 6%). Accuracy was evaluated as percentage relative error in prediction (RE%). As the RE% values in Table 5 suggest, the proposed method is highly accurate with values about 1% and lower.

Table 5

Determination of CAR in two different pharmaceutical preparations.

Tablet	Solution concentration (mgL^{-1})	Predicted concentration (mgL^{-1})[a]	RE%	RSD%
Carvedilol (12.5 *mg* per tablet)	2.00	1.98	-1.0	3.1
Carvedilol (6.25 *mg* per tablet)	2.00	1.99	-0.5	5.8

[a]. Mean of four determinations.

Application of the proposed method for urine samples

The high selectivity and sensitivity of the proposed method can allow determination of CAR in human urine samples. The results of the analysis of the urine samples have been summarized in Table 6. The data obtained by the method indicated that the percent relative error (RE%) and percentage relative standard deviation (RSD%) values are satisfactory. The results are especially excellent for higher concentrations where the first calibration curve is employed. Very low percent relative errors indicate that the method is selective. It is known that urine is a complex sample with matrix effect and unknown spectral interferences. However, in this application, the results indicate that the method is highly selective.

Table 6

Results of determination of CAR in urine samples.

Urine	Added concentration (mgL^{-1})	Predicted concentration (mgL^{-1})[a]	RE%	RSD%
Sample 1[b]	2.000	2.004	0.2	3.9
Sample 2[c]	0.100	0.106	6.0	6.3

a. Mean of four determinations.

b. Using the first calibration curve in Table 4.

c. Using the second calibration curve in Table 4.

Interference Study

In the application of the proposed method to pharmaceutical and especially biological samples, it is important to test the selectivity towards different potential interferents. Several species that can occur in the real samples together with drug were investigated. The tolerance limit of the potentially interfering species was taken as its maximum amount causing an error of ≥±5% during determination of the drug. The anions were used as sodium and potassium salts and the cations as chlorides. The results are shown in Table 7.

Table 7

Effect of interfering ions and drugs on the determination of CAR (2.0 mgL^{-1}).

Foreign ions	Added as	Tolerance limit (mg L^{-1})
K^+	KCl	13
Ca^{2+}	$CaCl_2.2H_2O$	20
Mg^{2+}	$MgCl_2.4H_2O$	4.5
NO_3^-	$NaNO_3$	138
SO_4^{2-}	Na_2SO_4	152
Paracetamol	-	45
Cephalexin	-	10
Diphenhydramine	-	25

Nitrate and sulphate as typical anions do not interfere with determination of CAR. As typical drugs, paracetamol, cephalexin and diphenhydramine were tested and it was observed that in determination of CAR, they do not interfere. Potassium and calcium do not interfere seriously. The only interferent was magnesium ion which can be masked along with other cations by EDTA.

Comparison with the reported methods

In Table 8, the main characteristics of the reported methodologies for determination of CAR have been collected. Detection limit of the proposed method is the lowest one compared with the spectrophotometric methods reported in Table 8. The high values of molar absorptivity and low

values of DL indicate the high sensitivity of the proposed method. The lowest DL reported for CAR determination has been obtained by gas chromatography with mass spectrometric detection (GC-MS). However, the proposed method in this work for determination of CAR is about two times more sensitive than GC-MS. The values of RSD% and RE% of the proposed method are low and the linear ranges of the method presented here are satisfactory and relatively wide. In this method, we broadened the linear ranges of the calibration. Moreover, the spectrophotometric methods developed have potential to apply to only simple samples like pharmaceuticals.

Table 8

Reported results for CAR determination.

Instrumental methodology	Experimental details	DL (mgL^{-1})	Samples	Reference
Spectrophotometry	Method A: reaction of CAR with PDAB[a], pH=4, λmax = 601 nm, molar absorptivity = 0.92×10^3 L $mol^{-1}cm^{-1}$	1.65		
	Method B: is based on the charge transfer complex formation of CAR with *p*-chloranil; λmax=662 nm, molar absorptivity = 0.257×10^4 L $mol^{-1}cm^{-1}$	0.6626	Pharmaceuticals	7
Spectrophotometry	1)Reaction of CAR with NIN[b] in basic medium, λmax = 402 nm, molar absorptivity = 2.57×10^4 L $mol^{-1}cm^{-1}$	0.139	Pharmaceuticals	8
	2) Reaction of CAR with AA[c] in presence of sodium nitroprusside in basic medium, λmax=558 nm, molar absorptivity =1.617×10^4 L $mol^{-1}cm^{-1}$.	0.157		
HPLC	Brownlee C8 column, isocratic elution and on-line deproteination	8×10^{-4} (LOQ)	Human plasma	10
Flow injection spectrofluorimetry	λex = 286 nm, λem=341 nm, Sampling rate: 30 samples h^{-1}, K_B[d] = 3.2×10^2 L mol^{-1}.	1.47×10^{-3}	Pharmaceuticals	14

Gas chromatography-mass spectrometry	Target compounds were extracted with liquid–liquid extraction. The extracts were completely derivatized with MSTFA[e] and MBTFA[f] and analyzed by GC–MS using an Ultra-2 ((5%-phenyl)- methylsiloxane) column.	*o*-DMC[i] 3×10^{-4} 4-HPC[j] 7.5×10^{-4}	Human urine	16
Liquid chromatography	Protein precipitation with methanol, mobile phase consisted of acetonitrile-30 mM potassium dihydrogenphosphate buffer, pH= 2 (30:70 v/v). Column: Develosil 3*μm ODS 100*×4.6 mmI.D.	1.3×10^{-3} (LOQ)	Human plasma	17
Spectrofluorimetric	Reaction of CAR with 1-dimethylaminonaphthalene-5-sulphonyl chloride in the presence of mixture (acetone: 0.5 M, sodium carbonate 3:2), pH=10, λem= 445 nm, λex =350 nm.	1.9×10^{-3}	Human plasma	19
Chemiluminometric	Reaction: oxidation of luminol by hypochlorite. Multi-pumping flow system multiple solenoid actuated *μ*-pumps.	3.53×10^{-3}	Pharmaceuticals	18
Spectrophotometry	Reaction of CAR with nitrite.			
	*λ*max= 250 nm, molar absorptivity = 3.41×10^{6} $Lmol^{-1}$ cm^{-1}	1.76×10^{-4}	Human urine	This work
	*λ*max= 278 nm, molar absorptivity = 3.19×10^{5} $Lmol^{-1}$ cm^{-1}	8.12×10^{-4}		

[a]. *p*-dimethylaminobenzaldehyde.

[b]. Ninhydrin.

[c]. Acetaldehyde.

[d]. Binding constant

[e]. *N*-methyl- *N*-trimethylsilyltrifluoroacetamide

[f]. *N*-methyl*bis*- trifluoracetamide.

[g]. *o*-desmethyl carvedilol.

[h]. 4-hydroxyphenyl carvedilol.

[i] . *o*-desmethyl carvedilol

[j] . 4-hydroxyphenyl carvedilol

Conclusions

A simple, sensitive, rapid and cost-effective spectrophotometric method was developed and validated for the determination of CAR. The reagent utilized in the proposed method is cheap and readily available and the procedure does not involve any critical reaction conditions or tedious sample preparation. The method is more selective and sensitive than many of the reported spectrophotometric methods. Moreover, its analytical characteristics are superior over expensive methods like GC-MS and HPLC in determination of CAR. This method can be used as general method for the determination of CAR in bulk powder, dosage forms and biological samples. The method has many advantages over the separation techniques such as HPLC and includes reduced cost and speed with high accuracy. Hence, the method can be used in routine analysis of drug in quality control laboratories.

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