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SPECTROFLUORIMETRIC DETERMINATION OF CARVEDILOL IN DOSAGE FORM AND SPIKED HUMAN PLASMA THROUGH DERIVATIZATION WITH 1-DIMETHYLAMINONAPHTHALENE-5-SULPHONYL CHLORIDE

A sensitive, simple and selective spectrofluorimetric method was developed for the determination of carvedilol (CA) in pharmaceutical formulation and a biological fluid. The method is based on the reaction between the drug and 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) in the presence of mixture (acetone:0.5 M sodium carbonate, 3:2) at pH 10 to yield a highly fluorescent derivative that is measured at 445 nm after excitation at 350 nm. Different experimental parameters affecting the development and stability of the reaction product were carefully studied and optimized. The fluorescence concentration plot was rectilinear over the range of 5.0-80.0 ng ml⁻¹ with a lower detection limit (LOD) of 1.90 ng ml⁻¹ and the limit of quantitation (LOQ) of 5.15 ng ml⁻¹. Quantum yield, formation constant (K) and free energy change (ΔG) values were calculated. The proposed method was successfully applied to the analysis of commercial tablets. The results obtained were in good agreement with those obtained using the official titrimetric method [1,2]. Furthermore, the method was applied for the determination of CA in spiked human plasma, the mean % recovery (n = 5) is 97.82±0.373. A proposal of the reaction pathway was presented.

Key words: carvedilol; 1-dimethylaminonaphthalene-5-sulphonyl chloride; dosage form; spiked human plasma.

Carvedilol (CA), (2RS)-1-(9H-carbazol-4-yloxy)-3-{[2-(2-methoxyphenoxy) ethyl]amino}-propan-2-ol (Figure 1) is a beta-adrenoceptor antagonist, an arteriolar vasodilator that is indicated for the treatment of hypertension, angina pectoris and heart failure. CA is rapidly and well absorbed after oral administration, but is subjected to considerable first - pass metabolism in the liver; the absolute bioavailability is about 25%. Peak plasma concentrations occur 1 to 2 h after administration [3].

CA is the subject of monographs in British pharmacopoeia [1] and European pharmacopoeia [2] whereby a non-aqueous titrimetric method is recommended for its determination, as bulk powder. Several methods have been published for its determination either in bulk powder or in pharmaceutical preparation and biological fluids. These methods include: non-aqueous titration [4], spectrophotometry [4-9], fluorimetry [10,11], Chemiluminescence method [12], electrochemical method [13], gas chromatography [14], HPLC [5,15-18] and Capillary electrophoresis [19-21]. The suggested method has the advantage of being simple, sensitive and suitable for routine analysis in a quality control laboratory.

Dansyl chloride is a useful derivatizing agent for primary amines, secondary amines, imidazole and phenols, etc. Several drugs products have been determined through this approach [22-24].

Figure 1. Structure of carvedilol.
The present work studies the reaction of CA with dansyl chloride to develop a simple and sensitive spectrofluorimetric method for its determination in a drug product and human spiked plasma.

**EXPERIMENTAL**

**Apparatus**
- SHIMADZU RF - 1501 Spectofluorimeter, equipped with Xenon arc lamp, using quartz cell ((1x1x4.5) cm).
- Shaker, Heidolph Duomax 1030, Germany.

**Materials and reagents**
All chemicals used were of analytical grade and solvents were of spectroscopic grade.

Carvedilol was kindly supplied by Eva. Co., Egypt, and assayed for purity according to the official titrimetric methods [1,2] to contain 99.80±0.19%.

Dilator tablets, B.N. 19503, each tablet was labeled to contain 25 mg of carvedilol, are manufactured by Chemipharm Pharmaceutical Industries S.A.E.

Dansyl chloride reagent (BDH chemicals), 0.1 w/v mgml⁻¹, was freshly prepared in acetone (Lab-chemicals, Poland).

Sodium carbonate, 0.5 M aqueous solution (pH 10), was from El Nasr. Co. Egypt.

Human serum plasma, 20 %, (Cealb Co., Amsterdam, B.N. 07126H122A, Biotest Pharma GmbH 63303 Dreieich, Germany, Lot. N. A137054) were kindly supplied from Vacsera, Egypt, and four samples were obtained from National Blood Bank, Egypt.

**Stock solution**
An accurately weighted 5.0 mg of CA was transferred into 100-ml volumetric flask which contained 50-ml of mixture of acetone - 0.5 M sodium carbonate (3:2) and the mixture was sonicated till dissolved. The volume was completed to the mark with mixture (acetone - 0.5 M sodium carbonate, 3:2).

**Working standard solution**
Aliquots equivalent to 50 µg was transferred from its stock solution into 100-ml volumetric flask. The volume was completed to the mark with mixture (acetone - 0.5 M sodium carbonate, 3:2).

**General procedure**
Aliquots of CA working standard solution equivalent to (50-800 ng ml⁻¹) were transferred into a series of 10-ml volumetric flasks; 0.5-ml of 0.1% w/v dansyl chloride reagent was added. The solution was stand for 25 min. The volume was completed to the mark with acetone. The fluorescence intensity of the reaction product was measured at 445 nm after excitation at 350 nm. A blank experiment was carried out simultaneously. The corrected fluorescence intensity was plotted vs. the final drug concentration (ng ml⁻¹) to get the calibration graph. Alternatively, the corresponding regression equation was derived.

**RESULTS AND DISCUSSION**
Dansyl chloride was first introduced for the determination of some primary, secondary amines, imidazoles and phenols [25-27]. In recent reports, dansyl chloride was further used as a fluorogenic reagent for the determination of some pharmaceutical compounds [28-30]. In the present study, CA was found to react with dansyl chloride at pH 10.0 forming a highly yellow fluorescent derivative with $\lambda$ maximum emission at 445 nm after excitation at 350 nm (Figure 2).
duct and its stability were carefully studied and optimized. Such factors were changed individually while the others were kept constant. The factors include pH, the concentration of the reagent, the temperature, the reaction time and dilution.

**Effect of pH**

The influence of pH on the fluorescence intensity of the reaction product was studied. The maximum fluorescence intensity was obtained upon using the mixture of acetone:0.5 M sodium carbonate (3:2) solution. The pH of the reaction mixture was found to be 10.0. This value was found to be the optimum pH for dansylation because the labeling of most amino acids, amines, imidazoles and phenols has been found to be optimal at pH 9.5-10.0 [31]. The rate of dansylation process is increased with increasing the pH value; this is due to an increase in the rate of hydrolysis of dansyl chloride into dansyl hydroxide [31]. The latter shows strong fluorescence and hence interferes seriously in the determination. However, under the proposed chosen conditions and wavelengths used there was no interference arising from any dansyl hydroxide formed, as indicated by the low fluorescence intensity of the reagent.

**Effect of concentration of dansyl chloride**

The influence of the concentration of dansyl chloride was studied using different volumes of 0.1 % w/v of the reagent solution. It was found that the reaction of dansyl chloride with CA started upon using 0.1 ml of the reagent at pH 10.0. Increasing the volume of the reagent produces a proportional increase in the fluorescence intensity of the reaction product till 0.4 ml and remains constant up to 0.7 ml. Therefore, 0.5±0.1 ml of 0.1 % w/v of dansyl chloride solution was chosen as the optimal volume of the reagent (Figure 3).

**Effect of temperature**

Increasing the reaction temperature higher than the room temperature would result in a subsequent decrease in the fluorescence intensity of the reaction.

**Effect of the reaction time**

Different time intervals were tested to ascertain the time after which the solution attains its highest fluorescence intensity and remains stable at room temperature for 2 h (Figure 4).

**Effect of diluting solvent**

Different solvents were tried to dilute the reaction mixture throughout the study. It was observed that acetone gave the highest fluorescence intensity. Dilution with water or 0.5 M sodium carbonate, or the mixture of acetone:0.5 M sodium carbonate (3:2) produces no fluorescence. Using of acetonitrile and isobutylmethylketone produce relatively low fluorescence intensity. The increase of fluorescence intensity due to the use of acetone may be attributed to lowering the fluorescence of the blank reagent (Figure 5). The stoichiometry of the reaction was studied by the Job’s method of continuous variation [32] and it was found to be 1:1 for drug and dansyl chloride (Fi-
Based on the observed molar reactivity of the reaction, and depending on the presence of secondary amino group and by analogy to similar reports dealing with the reaction of dansyl chloride with compounds containing secondary amino group.

The fluorescence signal was linearly ranged to the concentration in the range (5-80 ng ml$^{-1}$), with mean percentage recovery 99.99±0.008%. The regression equation was computed as shown in Table 1.

Scheme 1. The suggested structure of the formed reaction product between carvedilol and dansyl chloride.
Table 1. Validation report obtained by applying the suggested method for determination of carvedilol in a drug substance

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range, ng ml⁻¹</td>
<td>5.0-80.0</td>
</tr>
<tr>
<td>Slope</td>
<td>9.352</td>
</tr>
<tr>
<td>SE of slope</td>
<td>0.0548</td>
</tr>
<tr>
<td>Confidence limit of slopeᵇ</td>
<td>9.211-9.493</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.0448</td>
</tr>
<tr>
<td>SE of intercept</td>
<td>2.3488</td>
</tr>
<tr>
<td>Confidence limit of interceptᵇ</td>
<td>-4.992-7.086</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9998</td>
</tr>
<tr>
<td>SE of r</td>
<td>3.7267</td>
</tr>
<tr>
<td>Accuracy (mean±RSD, %)</td>
<td>99.99±0.008</td>
</tr>
<tr>
<td>Intraday precision, %</td>
<td>0.025</td>
</tr>
<tr>
<td>Interday precision, %</td>
<td>0.010</td>
</tr>
<tr>
<td>LOD / ng ml⁻¹</td>
<td>1.90</td>
</tr>
<tr>
<td>LOQ / ng ml⁻¹</td>
<td>5.15</td>
</tr>
</tbody>
</table>

ᵇAt 95% confidence limit

**Determination of fluorescence quantum yield of CA and dansyl chloride**

Diluted solution of quinine sulfate dissolved in 0.05 mol/L sulfuric acid with fluorescence quantum yield of 0.55 was used as reference reagent, and an equation shown was used to calculate the fluorescence quantum yield of CA [33]:

\[ Y_q = Y_s \left( \frac{F_u}{F_s} \right) \left( \frac{A_q}{A_s} \right) \] (1)

\( Y_q \) and \( Y_s \) referred to the fluorescence quantum yield of CA and quinine sulfate, respectively; \( F_u \) and \( F_s \) represented the integral fluorescence intensity of CA and quinine sulfate, respectively; \( A_q \) and \( A_s \) referred to the absorbance of CA and quinine sulfate at the excited wavelength, respectively. The concentration was selected so that the absorbance was less than 0.05 to minimize an error arising from the inner effect [34]. The fluorescence quantum yield was found to be 0.42 of the dansylation of CA.

The formation constant (K) of CA and the dansyl chloride reaction product was calculated by Benesi-Hildebrand’s method [35], and found to be 5780 L mol⁻¹.

The high free energy change (ΔG) obtained for CA-dansyl chloride reaction product (-2.1465×10⁴ J K mol⁻¹) indicates the stability of complex [36].

**Validation of the proposed method**

The validity of the method was tested regarding: linearity, specificity, accuracy, precision, robustness, ruggedness, limit of detection (LOD), and limit of quantification (LOQ) according to ICH Q2B recommendations [37].

**Linearity**

By using the above procedure, a linear regression equation was obtained. The regression plots showed that there was a linear dependence of the fluorescence signal on the concentration of the drug over the ranges cited in Table 1. The linear regression analysis of the data gave the following equation:

\[ F = 9.3529c + 1.0448 \ (r = 0.9998) \] (2)

where \( F \) is the fluorescence signal, \( c \) is the concentration of the drug, in ng ml⁻¹, and \( r \) is the correlation coefficient.

The limit of quantification (LOQ) was determined by establishing the lowest concentration that can be measured according to ICH Q2B [37]. The results are shown in Table 1. The limit of detection (LOD) was determined by establishing the minimum level at which the analyte can be reliably detected, and the results are also abridged in Table 1.

\[ \text{LOQ} = 10 \sigma S \]
\[ \text{LOD} = 3.3 \sigma S \]

where \( \sigma \) is the standard deviation of intercept of regression line and \( S \) is the slope of the calibration curve (Table 1). The proposed methods were evaluated for the accuracy and precision as percent relative standard deviation (%RSD) (Table 1).

**Accuracy**

The accuracy of the proposed method was determined by investigating the recovery of CA six levels each three times ranging from (10-70 ng ml⁻¹) for CA-dansyl chloride reaction product (Table 1). The results of the proposed method were statistically compared with those obtained by the official method. Statistical analysis of the results, using Student’s t test and variance ratio F test revealed no significant difference between the performance of the proposed and reference method (Table 2). The validity of the methods was proved by statistical evaluation of the regression lines, using the standard error of intercept and standard error of slope. The results are abridged in Table 1. The small values of the figures point out to the low scattering of the points around the calibration line and high precision.

**Precision**

The intraday precision for CA was evaluated by assaying a freshly prepared solution in triplicate in the concentration range (15-60 ng ml⁻¹) of the dansylation of CA and a relative standard deviation (%RSD) was found to be ±0.025%. The interday precision was cal-
culated by assaying a freshly prepared solution in triplicate for three days. The relative standard deviation (%RSD) was found to be ±0.01% (Table 1).

Table 2. Application of the proposed and official method to the determination of carvedilol in a drug substance

<table>
<thead>
<tr>
<th>Item</th>
<th>Results</th>
<th>Official titrimetric method [1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean, %</td>
<td>99.99</td>
<td>99.98</td>
</tr>
<tr>
<td>SD</td>
<td>0.008</td>
<td>0.012</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.008</td>
<td>0.012</td>
</tr>
<tr>
<td>Variance (V)</td>
<td>6.4×10⁻⁶</td>
<td>1.44×10⁴</td>
</tr>
<tr>
<td>SE</td>
<td>3.2×10⁻³</td>
<td>4.89×10⁻³</td>
</tr>
<tr>
<td>t-Test (2.228)⁶</td>
<td>1.754</td>
<td>-</td>
</tr>
<tr>
<td>F-Test (5.1)⁶</td>
<td>2.250</td>
<td>-</td>
</tr>
</tbody>
</table>

⁵Mean of six experiments; ⁶theoretical values at p = 0.05

Robustness of the method

The robustness of the method adopted is demonstrated by the constancy of the fluorescence intensity with the deliberated minor change in the experimental parameters such as the change in the volume of dansyl chloride (0.1% w/v), 0.5±0.1 ml, and the change in reaction time 25±5 min.

These minor changes that may take place during the experimental operation did not affect the fluorescence intensity of the reaction product.

Ruggedness of the method

Five sets of experiments for this drug were carried out using two different laboratories and different analysts; no significant difference was obtained between the results in this study.

Specificity

The specificity of the method was investigated by observing any interference encountered from the common tablet excipients such as talc, lactose, starch, and magnesium stearate. These excipients did not interfere with the proposed method.

Method validation for drug product

The validity of the suggested method was assessed by applying the standard addition technique by adding CA to the previously analyzed pharmaceutical preparations. Statistical comparison of the results obtained by the proposed method with those obtained by HPLC reported method [38] showed that the recommended procedures are simpler and sensitive without any loss of accuracy or precision (Table 3).

Application to spiked human plasma

The high sensitivity of the proposed method allowed the determination of CA in spiked human plasma. CA is rapidly absorbed after oral administration, and peak plasma concentration occurs 1 to 2 h after administration. It is extensively metabolized in liver, the metabolites being excreted mainly in the bile. The elimination half-life is about 6-10 h.

Table 3. Application of the proposed method for the determination of carvedilol in its drug product

<table>
<thead>
<tr>
<th>Item</th>
<th>Results</th>
<th>Reported HPLC method [38]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean, %</td>
<td>100.50</td>
<td>100.15</td>
</tr>
<tr>
<td>SD</td>
<td>0.835</td>
<td>0.415</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.836</td>
<td>0.416</td>
</tr>
<tr>
<td>Variance (V)</td>
<td>0.697</td>
<td>0.172</td>
</tr>
<tr>
<td>SE</td>
<td>0.341</td>
<td>0.169</td>
</tr>
<tr>
<td>t-Test (2.228)⁶</td>
<td>0.921</td>
<td>-</td>
</tr>
<tr>
<td>F-Test (5.1)⁶</td>
<td>4.05</td>
<td>-</td>
</tr>
</tbody>
</table>

⁶Mean of six experiments; ⁷theoretical values at p = 0.05

CA is given orally in an initial dose of 12.5 mg once a day, and increased after two days to 25 mg [3]. This leads to a final blood level concentration (Cmax) of about 4.198 µg ml⁻¹, i.e., higher than the upper limit of the working concentration range of the proposed method.

The validity of the method was proved for analysis of the drug in spiked human plasma according to ICH Q2B (linearity, accuracy, intraday precision, interday precision, specificity, LOD and LOQ). The results are abridged in Table 4. The high sensitivity of the proposed method allowed the determination of CA in spiked human plasma (Table 4).

Table 4. Validation and application of the suggested method for determination of carvedilol in spiked human plasma

<table>
<thead>
<tr>
<th>Items</th>
<th>The results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range, ng ml⁻¹</td>
<td>20-70</td>
</tr>
<tr>
<td>Slope</td>
<td>13.577</td>
</tr>
<tr>
<td>SE of slope</td>
<td>0.1623</td>
</tr>
<tr>
<td>Confidence limit of slope⁵</td>
<td>13.126-14.027</td>
</tr>
<tr>
<td>Intercept</td>
<td>-237.638</td>
</tr>
<tr>
<td>SE of intercept</td>
<td>7.812</td>
</tr>
<tr>
<td>Confidence limit of intercept⁵</td>
<td>From -259.328 to -215.947</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9994</td>
</tr>
<tr>
<td>SE of r</td>
<td>6.790</td>
</tr>
<tr>
<td>Accuracy (mean±RSD, %)</td>
<td>97.82±0.373</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday precision, %</td>
</tr>
<tr>
<td>Interday precision, %</td>
</tr>
<tr>
<td>LOD / ng ml⁻¹</td>
</tr>
<tr>
<td>LOQ / ng ml⁻¹</td>
</tr>
</tbody>
</table>

⁵At 95% confidence limit

The specificity was assessed using six blank human plasma samples, randomly selected from different sources, that were subjected to the extraction
procedure and assayed to determine the extent to which endogenous plasma components could interfere in the analysis of CA. The results were compared to the solution containing 5.20 ng ml⁻¹ of CA.

Precision
The intra-day precision was evaluated through a replicate analysis of plasma samples spiked with different concentrations of the drug. The percentage recoveries based on the average of four separate determinations were 97.37±0.390%, thus indicating the high precision of the method Table 4. The inter-day precision was also evaluated through a replicate analysis of plasma samples spiked with 30 ng ml⁻¹ of the drug on four successive days. The percentage recoveries based on the average of four separate determinations were 97.29±0.144%. The results are shown in Table 4.

Under the optimum experimental conditions obtained, the fluorescence intensity (FI) showed a rectilinear relation with CA and dansyl chloride in spiked human plasma concentration over range (20-70 ng ml⁻¹).

CONCLUSION
The proposed method has the advantage of being simple, sensitive and suitable for the routine analysis in the quality control laboratory. Also, it is suitable for the determination of CA in the drug substance and spiked human plasma with a minimum detection limit lower than the reported value. In addition, it could be applied for the determination of CA in its drug product.

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SPEKTROFOTOMETRIJSKO ODREĐIVANJE KAREDI-LOLA U LEKOVIMA I OBOGAČENOJ HUMANOJ PLAZMI DERIVATIZACIJOM SA 1-DIMETILAMINONAFTALEN-5-SULFONIL HLORIDOM

U radu je opisana jednostavna, senzitivna i selektivna spektrofotometrijska metoda za određivanje karvedilola (CA) u farmaceutskim preparatima i u biološkom fluidu. Metoda se zasniva na reakciji leka i 1-dimetilaminonaftalan-5-sulfonil hlorida (dansil-hlorida) u prisustvu smeše aceton:0,5 M natrijum-karbonat 3:2 na pH 10, pri čemu se dobija visoko fluorescentni derivat koji se meri na 445 nm posle eksitacije na 350 nm. Ispitan je i optimizovan uticaj različitih eksperimentalnih parametara koji uticaju na razvoj i stabilnost reakcionih produkata. Fluorescentni koncentracijski grafik je pravolinjski za opseg 5,0-80,0 ng ml⁻¹ sa limitom detekcije od 1,90 ng ml⁻¹ i limitom kvantifikacije od 5,15 ng ml⁻¹. Izračunat je kvantitativni prinos, konstanta formiranja i promena slobodne energije. Predložena metoda je uspešno primenjena za analizu komercijalnih tableta. Dobijeni rezultati su u dobroj saglasnosti sa oficijalnom titrimetrijskom metodom [1,2]. Određivanjem CA u obogaćenoj humanoj plazmi u 5 uzoraka postignuto je iskorišćenje od 97,8±0,37%. U radu je predstavljena reakcija schema.

Ključne reči: karvedilol; 1-dimetilaminonaftalen-5-sulfonil-hlorid; formulaciona doza; obogaćena humana plazma.