Development and validation of a new spectrofluorimetric method for the determination of some beta-blockers through fluorescence quenching of eosin Y. Application to content uniformity test

Abstract: A simple, rapid, sensitive and economic spectrofluorimetric method has been developed and validated for determination of some β-adrenergic blocking agents namely: betaxolol hydrochloride (BTX), carvedilol (CAR), labetalol hydrochloride (LBT), nebivolol hydrochloride (NEB) and propranolol hydrochloride (PRO). The method is based on the quenching effect of the cited drugs on the fluorescence intensity of eosin Y at pH 3.4 (acetate buffer). The fluorescence quenching is due to the formation of an ion-pair complex and was measured without extraction at 545 nm (λex. 301.5 nm). The factors affecting the formation of the ion-pair complex were carefully studied and optimized. Under the optimal conditions, the linear ranges for the relationship between the fluorescence quenching value and the concentration of the investigated drugs were 100-2500, 150-2500 and 50-2250 ng mL⁻¹ for (BTX, CAR), (LBT, NEB) and (PRO) respectively. The method was validated according to ICH guidelines and was applied for determination of the cited drugs in pharmaceutical dosage forms with excellent recoveries. In addition, content uniformity testing of some commercial dosage forms was checked by the proposed method.

Keywords: β–adrenergic blockers; Eosin Y; Ion-pair complexation; Fluorescence quenching; Pharmaceutical analysis

1 Introduction

Beta adrenergic blocking agents (β-blockers) are a well-known and characterized group of pharmaceutical compounds. β-adrenergic blockers are commonly used in the treatment of several cardiovascular disorders such as; congestive heart failure, angina, cardiac arrhythmia and hypertension [1-3]. The most commonly used β-adrenergic blockers are labetalol hydrochloride (LBT), propranolol hydrochloride (PRO), betaxolol hydrochloride (BTX), carvedilol (CAR) and nebivolol hydrochloride (NEB). The chemical structures of the investigated drugs are shown in table 1.

Due to the high importance of these drugs, several methods have been reported for determination of the investigated β-blockers in different matrices. Among these methods are spectrophotometry [4-14], spectrofluorimetry [15-24] liquid chromatography [25-28], gas chromatography [29, 30], electrochemical [31-35] and capillary electrophoresis [36, 37].

Eosin Y, tetrabromofluorescein disodium salt, is an acidic dye exhibiting a yellowish-red colour with green fluorescence. The native fluorescence of eosin Y can be quenched as a result of ion pair complex formation with cationic basic nitrogenous drugs in acidic aqueous solutions [38, 39].

The aim of the present work is to develop a more sensitive, rapid and less expensive new spectrofluorimetric method for determination of the studied drugs in pure and pharmaceutical formulations. The method is based on the binary complex formation between the cited drugs and eosin Y.
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2 Experimental

2.1 Apparatus:

A SCINCO FluoroMate (SCINCO Company, Seoul, Korea), equipped with 150 W Xe-arc lamp, PMT detector for excitation & emission and 1 cm matched quartz cells was used for spectrofluorimetric measurements. The slit width of both excitation and emission monochromators was set at 10 nm.

Sonicator (SONICOR SC-101TH) and pH-meter, model AD11P (Adwa, Romania) were used. All weighing was performed on an electronic single pan balance (Precisa XB 220A, 10 mg-220 mg, Glattburg, Switzerland). Distilled water was prepared by water distiller (TYUMEN-MIDI-A0-25 MO, Russia).

2.2 Materials and reagents

All reagents and solvents were of Analytical Reagent Grade.

Betaxolol hydrochloride of 98.5% purity, was kindly provided by Borg Pharmaceutical Industries, Alexandria, Egypt. Carvedilol of 99.0% purity, was kindly provided by Sigma Pharmaceutical Industries, Cairo, Egypt. Labetalol hydrochloride of 99.8% purity, was kindly provided by Debeiky Pharmaceutical Company, El Obour City, Cairo, Egypt. Nebivolol hydrochloride, of 99.6% purity, was kindly provided by Marcyrl Pharmaceutical Industries, El Obour City, Cairo, Egypt. Propranolol hydrochloride, of 99.4% purity, was kindly provided by El-Kahira Pharmaceutical and Chemical Industry, Cairo, Egypt. Eosin Y disodium salt (Merck, Darmstadt, Germany) was freshly prepared as 3.47 x 10^{-4} M aqueous solution by dissolving 24 mg of eosin Y in 100 mL distilled water. Acetate buffer solution (0.2 M) was prepared by mixing appropriate volumes of 0.2 M sodium acetate and 0.2 M acetic acid and adjusting the pH to 3.4 using pH Meter.

2.3 Pharmaceutical formulations

Betaxolol ® tablets (Borg Pharmaceutical Industries, Alexandria, Egypt) are labeled to contain 10 mg betaxolol hydrochloride per tablet. Karvex® tablets (Sigma Pharmaceutical industries, Cairo, Egypt) are labeled to contain 25 mg carvedilol per tablet. Labipress® tablets (Debeiky Pharmaceutical Company, El Obour City, Cairo, Egypt) are labeled to contain 200 mg labetalol hydrochloride per tablet. Nevilob® tablets (Marcyrl Pharmaceutical Industries, El Obour City, Cairo, Egypt) are labeled to contain 5 mg nebivolol hydrochloride per tablet. Inderal ® tablets (El-Kahira Pharmaceutical and Chemical Industry Company, Cairo, Egypt) are labeled to contain 10 mg propranolol hydrochloride per tablet.

2.4 Standard drug solutions

Stock solutions of betaxolol, labetalol and propranolol were prepared by dissolving 100.0 mg of each drug in distilled water. Carvedilol stock solution was prepared by dissolving 100.0 mg of carvedilol powder in methanol. Nebivolol hydrochloride was prepared by dissolving 100.0 mg of nebivolol hydrochloride powder in 30 mL methanol and further diluted with distilled water. Working standard drug solutions of the required concentrations were prepared by further dilution of the stock solutions with distilled water (methanol was used in the case of carvedilol). The standard solutions were stable for 7 days when refrigerated.

| No. | Name     | Chemical structure | Log p | pka  |
|-----|----------|--------------------|-------|------|
| 1   | Betaxol  | ![Chemical structure](image1) | 2.81  | 9.4  |
| 2   | Nebivol  | ![Chemical structure](image2) | 3.21* | 8.9* |
| 3   | Labetal  | ![Chemical structure](image3) | 3.09  | 9.8* |
| 4   | Carvedil | ![Chemical structure](image4) | 4.19  |      |
| 5   | Propranol| ![Chemical structure](image5) | 3.48  | 9.42 |

* The value is predicted one.

Table 1: Chemical structures, log p, and pk_a of the studied β-adrenergic blockers.

**Notes:**
- The value is predicted one.
2.5 General Analytical Procedure:

One milliliter of the working standard or sample drug solution was transferred into a 10 mL calibrated flask, followed by 1.0 mL of acetate buffer solution of pH 3.4 and 1.0 mL of eosin Y (3.47×10⁻⁴ M) solution. The solution was completed to 10 mL with distilled water and the relative fluorescence intensity of the resulting solution was measured at 545 nm (λₑx.302 nm). A control experiment was carried out simultaneously in the same manner omitting the drug solution.

2.6 Procedure for Tablets

An accurately weighed amount of the powdered 30 tablets, equivalent to 25 mg of each drug, was extracted with methanol in 25 mL calibrated flasks. The volumes were completed to the mark with the same solvent and filtered. The first portion of the filtrate was rejected and a portion of the filtrate was analyzed as described in the general procedure.

2.7 Procedure for Content Uniformity Testing for studied drugs

Ten individual tablets were analyzed using the same procedure designed for the analysis of tablets. The uniformity of the tablet contents was carried out by following the official USP guidelines [40] (Chapter 905: Uniformity of Dosage Units).

3 Results and discussion

3.1 Fluorescence Spectra

The aqueous solution of eosin showed fluorescence activity measured at 545 nm after excitation at 301.5 nm. The addition of the studied drug solution to the reagent solution decreased significantly the fluorescence intensity (Figure 1). The observed fluorescence quenching effect is due to the formation of an ion pair complex between the studied β-adrenergic drugs and eosin Y. There was a linear relationship between the fluorescence quenching of the dye (ΔF) with the concentration of the drug.

3.2 Optimization of the reaction conditions

To find the optimum conditions for the complex formation, various parameters were carefully studied. The investigated parameters included; the solution pH, type and concentration of the buffer, reaction time, temperature, eosin Y concentration and diluting solvent.

3.2.1 Effect of pH and buffer (type & concentration)

The influence of pH on the fluorescence quenching value (ΔF) was investigated in the pH range of 2.8–4.0. At pH values higher than 4.2, a negligible quenching effect was observed. The maximum ΔF was observed in the pH range of 3.3–3.5. Lower or higher pH values gradually decreased the quenching effect (Figure 2), therefore the optimum pH was 3.4. Moreover the general procedure was carried out using various buffer systems such as McIlvain, acetate, Toerell-Stenhagen and Britton-Robinson buffers. The best results were obtained in the presence of an acetate buffer (Supplementary data, SI 1). Finally the effect of acetate buffer concentrations (0.05–0.35 M) was examined. The fluorescence quenching was gradually increased by increasing the concentration of the buffer solution until it reached a maximum value at 0.15 M. The results remained unchanged up to 0.3 M after which a slight decrease was observed (Supplementary data, SI 2). Consequently, 0.2 M acetate buffer solution of pH 3.4 was chosen in the general analytical procedure for all drugs.
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3.2.2 Effect of formation and stability times

The reaction between the studied drugs and eosin Y was completed at room temperature immediately after mixing of the solutions. Furthermore, the fluorescence quenching values of all formed complexes were stable for at least one hour after the final dilution. Therefore the fluorescence intensity was measured directly after mixing the reactants without the need for any standing time.

3.2.3 Effect of volume of eosin

The general analytical procedure was carried out using different volumes (0.20–2.0 mL) of 3.47×10^{-4} M eosin Y. It was found that increasing the concentration of the reagent resulted in a gradual increase in the fluorescence quenching until it reached a maximum value at 0.8 mL of the reagent. Further increase in the reagent concentration did not produce any significant changes in the result up to 2.0 mL. Therefore, 1.0 mL of 3.47×10^{-4} M eosin Y solution was used throughout the work for all the studied drugs (Supplementary data, SI 3).

3.2.4 Effect of diluting solvent:

Different solvents were tried to dilute the formed ion pair complex including; n-butanol, ethanol, methanol, dichloroethane, acetone, dioxan and distilled water. The highest fluorescence quenching values were observed with distilled water which is the best green solvent. Therefore the present procedure is considered both environmentally safe and economically inexpensive. As a result, the suggested procedure is advantageous compared to many of the reported methods.

3.3 The stoichiometry of the reaction

Job’s method of continuous variation [41] was used to study the molar ratios of the formed ion-pairs. The results of Job’s method revealed a 1:1 ratio between drug and eosin for all of the studied drugs (Figure 3). This explains the use of the same optimum eosin Y concentration for all the studied drugs.

3.4 Method Validation

The analytical performance of the proposed procedure was validated according to ICH Q2 guidelines. The investigated parameters included linearity, limit of detection, limit of quantitation, precision, accuracy, and robustness [42].

**Linearity and range**: Under the described experimental conditions, the general analytical procedure was applied for a series of studied drug solutions with different concentrations. The calibration curves for each drug were constructed by plotting the fluorescence quenching values against the corresponding drug concentrations. Linear regression analysis of the obtained result was performed and the different analytical parameters were calculated (Table 2). The linear ranges for the relationship between the fluorescence quenching value and the concentration of the investigated drugs were 100-2500, 150-2500 and 50-2250 ng mL^{-1} for (BTX, CAR, LBT, NEB, PRO).
The correlation coefficients were at least 0.9996 indicating good linearity of the proposed method.

It was found that sensitivity of the method (as indicated by the slope) has a good linear correlation \( r^2=0.9733, n=5 \) with the partition coefficient \( \log p \) of the studied drugs. The following equation could represent this relationship: \( a = 0.58 \times \log p + 1.70 \), where “a” is the slope of the calibration curve of the proposed method. It should be noted that all of the studied drugs have only one basic centre (secondary amino group). Hence, we can conclude that the complex formation between the cited drugs and eosin is highly dependent on the lipophilicity of the target compound.

**Detection and Quantitation Limits:** The following formulas were used to calculate limits of detection (LOD) and quantitation (LOQ); LOD=3.3 \( \sigma /S \) and LOQ=10 \( \sigma /S \), where \( \sigma \) is the standard deviation of intercept and \( S \) is the slope of the calibration curve. The calculated LOD and LOQ were in the ranges of 16-57 and 50-172 ng mL\(^{-1}\) respectively. These low values give an indication of the high sensitivity of the proposed method.

**Precision and accuracy:** The accuracy of the proposed method was checked at three concentration levels of the drug (500, 1000 and 1500 ng mL\(^{-1}\)). The results were expressed in percentage recovery and relative standard deviation. As shown in Table 3, the % recovery values were ranged from 98.27% to 101.70% indicating good accuracy of the developed method and its suitability for quality control measurements.

To assess the intra-day precision of the proposed method, three replicates of the working standard drug solutions at three different concentration levels (500, 1000 and 1500 ng mL\(^{-1}\)) were analyzed using the proposed method within the same day. Inter-day precision was checked by carrying out the analysis on three different days. The results of intra-day and inter-day precisions are summarized in Table 3. The calculated RSD % values for all of the studied drugs were ranged from 0.28 to 1.77% indicating good repeatability and reproducibility of the proposed procedure.

**Robustness:** The robustness of the proposed method was determined by studying the effect of minor changes on the experimental conditions on the \( \Delta F \) of the formed complex. For the determination of the method’s robustness, four factors were examined; pH and concentration of the buffer, volume of the dye and reaction time. From the obtained results in Table 4, it is clear that none of these variables had any significant effect on the determination of investigated drugs. Therefore, the developed spectrofluorimetric method is considered robust and gives an indication of the reliability of the proposed method during routine use.

### 3.5 Application to pharmaceutical formulations

The general analytical procedure was applied to determine the drug contents of different commercial dosage forms. The obtained results were statistically compared with those of the reported methods \([13-16]\) in respect to accuracy and precision (Table 5). No significant difference was found between the results of both methods as the calculated t- and F-tests values did not exceed the tabulated values. Moreover, the standard addition technique was applied to examine the accuracy of the proposed method. A known amount of standard drug solution at three different concentration levels was added to pre-analysed sample solutions and the total content of the drug was re-analysed by the proposed method (Supplementary data, SI 4). The obtained excellent recoveries prove that there is no interference from the frequently encountered excipients. This indicates the suitability of the proposed method for the determination of the studied drugs in their dosage forms in quality control laboratories.

**Content Uniformity Test for studied drugs:** Content uniformity testing is a time consuming process when using

### Table 2: Summary of quantitative parameters and statistical data of the proposed method.

| Studied drugs | Intercept * (a) | Slope* (b) | Linear range * | \( r^2 * \) | LOD * | LOQ * |
|---------------|----------------|------------|----------------|-------------|-------|-------|
| Betaxolol     | 326            | 1.80       | 100-2500       | 0.9997      | 28    | 86    |
| Carvedilol    | 623            | 4.20       | 100-2500       | 0.9997      | 24    | 71    |
| Labetalol     | 618            | 2.11       | 150-2500       | 0.9996      | 57    | 172   |
| Nebivelol     | 212            | 2.91       | 150-2500       | 0.9993      | 46    | 140   |
| Propranolol   | 218            | 1.72       | 50-2250        | 0.9999      | 16    | 50    |

* units are in ng mL\(^{-1}\)  
\( r^2 \) is the correlation coefficient, and number of determinations is eight.
Table 3: Inter-day and Intra-day precision for the determination of the studied drugs by the proposed spectrofluorimetric method.

| The studied drugs | Conc. level (ng mL^-1) | Intra-day precision | Inter-day precision |
|-------------------|------------------------|---------------------|---------------------|
|                   | % Recovery ± SD | %RSD   | % Recovery ± SD | %RSD |
| Betaxolol         | 500        | 99.52 ± 0.32 | 0.32   | 99.52 ± 0.85 | 0.85 |
|                   | 1000       | 99.94 ± 1.21 | 1.21   | 100.78 ± 0.56 | 0.55 |
|                   | 1500       | 101.57 ± 0.28 | 0.28   | 99.65 ± 1.50 | 1.50 |
| Carvedilol        | 500        | 101.60 ± 0.55 | 0.54   | 99.22 ± 0.99 | 1.00 |
|                   | 1000       | 100.96 ± 1.31 | 1.30   | 98.18 ± 1.07 | 1.09 |
|                   | 1500       | 100.53 ± 0.72 | 0.71   | 100.85 ± 0.93 | 0.92 |
| Labetalol         | 500        | 101.30 ± 1.79 | 1.77   | 100.98 ± 0.72 | 0.72 |
|                   | 1000       | 99.30 ± 0.72 | 0.73   | 99.94 ± 1.66 | 1.67 |
|                   | 1500       | 101.27 ± 0.39 | 0.39   | 101.70 ± 0.56 | 0.65 |
| Nebivelol         | 500        | 100.21 ± 1.37 | 1.37   | 100.20 ± 0.69 | 0.69 |
|                   | 1000       | 99.82 ± 0.52 | 0.53   | 99.47 ± 1.30 | 1.31 |
|                   | 1500       | 101.48 ± 1.28 | 1.26   | 101.02 ± 0.29 | 0.29 |
| Propranolol       | 500        | 101.32 ± 1.05 | 1.04   | 100.23 ± 1.16 | 1.16 |
|                   | 1000       | 101.69 ± 0.73 | 0.72   | 98.76 ± 0.89 | 0.90 |
|                   | 1500       | 98.27 ± 1.10 | 1.12   | 100.72 ± 0.81 | 0.80 |

* The value is the mean of three determinations.

Table 4: Robustness of the proposed method for determination of the studied drugs (1.0 µg mL^-1).

| Variations     | Betaxolol % Recovery ± SD | Carvedilol % Recovery ± SD | Labetalol % Recovery ± SD | Nebivelol % Recovery ± SD | Propranolol % Recovery ± SD |
|----------------|---------------------------|----------------------------|---------------------------|---------------------------|----------------------------|
| Optimum pH     |                           |                            |                           |                           |                            |
| 3.3            | 99.89 ± 1.19              | 100.72 ± 1.20              | 98.99 ± 0.99              | 99.36 ± 0.86              | 101.88 ± 1.04              |
| 3.5            | 100.06 ± 0.45             | 98.58 ± 0.77               | 101.91 ± 1.58             | 98.56 ± 0.34              | 100.70 ± 1.54              |
| Buffer conc.   |                           |                            |                           |                           |                            |
| 0.15 M         | 99.98 ± 0.45              | 99.77 ± 1.85               | 101.28 ± 1.09             | 100.96 ± 0.34             | 100.89 ± 0.34              |
| 0.25 M         | 99.96 ± 0.71              | 99.45 ± 0.24               | 98.20 ± 0.47              | 101.19 ± 1.10             | 101.69 ± 0.73              |
| Eosin volume   |                           |                            |                           |                           |                            |
| 0.8 mL         | 101.17 ± 0.15             | 101.00 ± 0.94              | 99.78 ± 1.45              | 101.76 ± 1.30             | 99.73 ± 1.21               |
| 1.2 mL         | 100.02 ± 0.96             | 99.13 ± 0.6                | 101.67 ± 1.52             | 99.13 ± 1.55              | 102.05 ± 1.37              |
| Reaction time  |                           |                            |                           |                           |                            |
| 5 min.         | 100.41 ± 1.00             | 100.96 ± 0.50              | 100.57 ± 0.47             | 98.96 ± 1.38              | 101.59 ± 0.58              |
| 10 min.        | 99.39 ± 1.69              | 99.77 ± 0.96               | 99.70 ± 1.39              | 99.82 ± 1.21              | 98.57 ± 1.34               |

* The value is the average of six replicates.
conventional assay techniques. However, the proposed method has high sensitivity and is able to rapidly measure the fluorescence intensity of a single tablet extract with sufficient accuracy. The proposed procedure was adopted according to the USP [40] procedure. The acceptance value (AV) was calculated and was found to be smaller than the maximum allowed acceptance value (L1). These results demonstrated excellent drug uniformity of the studied dosage forms (Table 6).

Table 5: Analysis of commercial tablets containing the investigated β-blockers by the developed and reported methods [13 – 16].

| Dosage forms            | % Recovery ± SD Proposed method | Reported method | t-value  | F-value  |
|-------------------------|-------------------------------|-----------------|----------|----------|
| Betaxolol® tablets      | 100.17 ± 1.28                 | 99.99 ± 0.90    | 0.25     | 2.03     |
| Karvex® tablets         | 99.93 ± 1.53                  | 100.34 ± 0.98   | 0.51     | 2.47     |
| Labetalol® tablets      | 100.05 ± 1.04                 | 101.49 ± 1.99   | 1.44     | 3.65     |
| Nevilob® tablets        | 99.48 ± 0.51                  | 99.81 ± 0.95    | 0.67     | 3.49     |
| Inderal® tablets        | 101.00 ± 1.43                 | 100.20 ± 1.01   | 1.02     | 1.99     |

* Average of six replicates.
^ Theoretical values at 95% confidence limit; t = 2.228, F = 5.053.

Table 6: Results of content uniformity testing of the studied β-blockers tablets using the proposed method.

| Dosage form         | Percentage of the label claim | % RSD † | % error | AV ‡ |
|---------------------|------------------------------|---------|---------|------|
| Betaxolol® 10 mg tablets | 100.72                       | 1.84    | 0.58    | 4.46 |
| Karvex® 25 mg tablets      | 100.43                       | 1.56    | 0.49    | 3.75 |
| Nevilob® 5 mg tablets       | 100.51                       | 1.38    | 0.44    | 3.32 |
| Inderal® 10 mg tablets     | 99.96                        | 1.33    | 0.42    | 3.20 |

† RSD is the relative standard deviation
‡ AV is the acceptance value

4 Conclusion

A novel and non-extractive fluorescence quenching method was developed and validated for determination of some beta-blockers (betaxolol, carvedilol, labetalol, nebivolol and propranolol). The method is based on ion-pair complex formation with a cheap and commercially available reagent (eosin Y). The method is sufficiently sensitive to permit determinations of the studied drugs as low as 170 ng mL⁻¹. Compared to GC and HPLC, the spectrofluorimetric methods is relatively simple and less time consuming. The proposed method could be applied successfully in content uniformity testing. In addition, the methodology of the proposed method is non-polluting and meets the requirements of green chemistry, since no organic solvents are used in the procedure. Hence, the proposed method should be useful for quality control purposes.

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