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Research Article

VALIDATED SPECTROFLUORIMETRIC METHOD FOR THE DETERMINATION OF DORZOLAMIDE HYDROCHLORIDE IN ITS PURE FORM AND PHARMACEUTICAL FORMULATIONS VIA DERIVATIZATION WITH 4-CHLORO-7-NITROBENZO-2-OXA-1, 3-DIAZOLE (NBD-CL)

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ABSTRACT

Objectives: Simple spectrofluorimetric method was applied and validated for the determination of dorzolamide hydrochloride in its pure form and pharmaceutical formulations. **Method:** Based on nucleophilic substitution reaction of dorzolamide with 4-chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) to form a highly fluorescent fluorophore measured at 541 nm after excitation at 404 nm. **Results:** Under optimum condition, the proposed method obeys Beer's law in range (1- 8 µg/mL). **Conclusion:** The method was validated according to ICH guideline for accuracy, precision and was successfully applied for the determination of dorzolamide its pure form and pharmaceutical formulations. Statistical comparison of the obtained results with the other results of the reported method indicated the good agreement of the proposed method.

Keywords: Dorzolamide hydrochloride; 4-Chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl); Spectrofluorimetric method.

INTRODUCTION

Dorzolamide hydrochloride (DOR), [(4S,6S)-4-ethylamino-6-methyl-5, 6-dihydro-4H thieno [2,3b] thiopyran-2-sulphonamide 7,7-dioxide hydrochloride] Figure (1) is an inhibitor of the carbonic anhydrase enzyme which mainly used in the management of open-angle glaucoma and ocular hypertension either alone or as an adjunct to a topical beta blocker [1].

Several methods have been reported for the determination of DOR including spectrophotometric [2-4], capillary electrophoretic [5], electrochemical[6, 7] and chromatographic [7-14] methods in pharmaceutical dosage form.

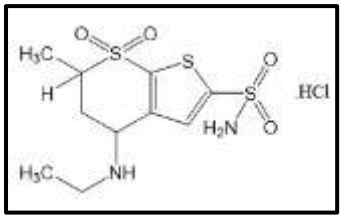

Fig 1 Chemical structure of dorzolamide hydrochloride

4-Chloro-7-nitrobenzo-2-oxa-1, 3-diazole Figure (2) also known as 7-chloro-4-nitrobenzo-furazan (NBD-Cl) is a stable non-fluorescent pale-yellow solid[15]. In spectrophotometric and spectrofluorimetric methods, NBD-Cl applied as derivatizing reagent for estimation of many amines[15-18]. The aim of the present work is to develop a spectrofluorimetric method for the determination of DOR via derivatization with 4-chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl).

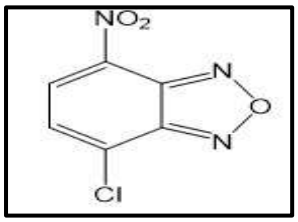

Fig 2: Chemical structure of 4-chloro-7-nitrobenzo-2oxa-1, 3-diazole (NBD-Cl).

M. wt. 199.56 g/mol.

MATERIALS AND METHODS

Instruments

Jasco FP6200 single beam spectrofluorometer (Japan).

Chemicals and reagents

- DOR (certified to contain 99.92%) was kindly supplied by Orchidia for pharmaceuticals (Obour city, Egypt).
- Trusopt ® eye drops: (batch number 2199730) each 1 mL is claimed to contain 22.26 mg dorzolamide hydrochloride equivalent to 20 mg dorzolamide, manufactured by Orchidia for pharmaceuticals (Obour city, Egypt), were purchased from local market.
- 0.1% NBD-Cl (99%) (Sigma Chemical Co., St. Louis, USA) was freshly prepared by dissolving 100mg in 100 mL methanol and protected from light.
- 1M hydrochloric acid, 0.2M sodium bicarbonate (El-Nasr Co., Egypt). methanol, ethanol, acetonitrile, acetone, propanol (sigma-Aldrich, USA). Water used throughout the procedures was freshly double distilled.

Standard solutions

- Dorzolamide stock solution (100 µg/mL) was prepared by dissolving 10 mg of the drug powder in 80 mL water, and the volume was then completed to 100 mL with water.
- Working dorzolamide solution (50 µg/mL) was obtained by dilution of the stock solution with water.

Linearity and construction of calibration curves

Different concentrations covering the linearity range (1-8 µg/mL) of dorzolamide hydrochloride were accurately prepared into a test tube set. 1mL (0.1% NBD-Cl) was added and followed by 1.5 mL of (0.2M) NaHCO3. Processing of The reaction mixtures was done in thermostatically controlled water bath at 60 °C for 30 minutes, and then cooled to room temperature. After cooling, the reaction mixture was acidified by adding 1mL of 1M HCl, and completed to volume with water. The relative fluorescence intensity was recorded at λem.= 541 nm after excitation at λex.= 404 nm.

Application to pharmaceutical preparation

0.45 ml of Trusopt ® eye drops was transferred into **100**-mL volumetric flask and completed to volume with distilled water to obtain a concentration of 100 µg/mL of DOR. The procedures were repeated for different prepared working concentration range of the pharmaceutical preparation. The concentrations of DOR were calculated from the corresponding regression equation.

REPORTED METHOD [2]

The reported method depends on direct determination for dorzolamide hydrochloride at 253 nm in phosphate buffer pH 7.4.

RESULTS AND DISCUSSION

Dorzolamide hydrochloride doesn't have a native fluorescence. Derivatization of the studied drug with fluorigenic reagent enables its spectrofluorimetric determination. 4-chloro-7-nitrobenzo-2-oxa-1,3diazole (NBD-Cl), a common derivatizing reagent, which seems to be target for good nucleophiles. Upon the reaction of dorzolamide hydrochloride with (NBD-Cl), a yellow-colored fluorescent derivative was formed, which exhibited maximum fluorescence intensity (λem.= 541 nm) after its excitation at wavelength (λex.= 404 nm). The excitation and emission spectra for the reaction product of dorzolamide hydrochloride with (NBD-Cl) was shown in Figure (3).

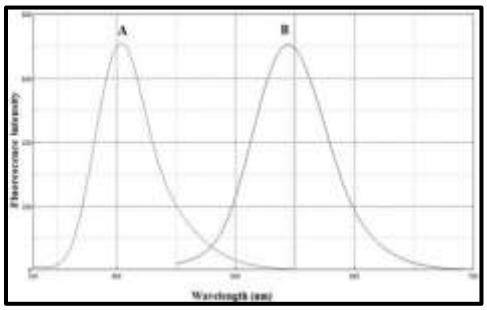

Fig 3. Excitation (A) and emission (B) spectra of the reaction product of dorzolamide hydrochloride (6 µg/mL) with 0.1% NBD-Cl.

Optimization of experimental conditions

Different experimental conditions affecting the fluorescence intensity were checked to recognize the most optimum recommended parameters.

Effect of reagent volume

The effect of NBD-Cl concentration was evaluated using different volumes of 0.1% (w/v) NBD-Cl solution ranging from (0.25-2 mL). 1mL of 0.1% (w/v) NBD-Cl gave the highest FI and beyond which the FI decreased, Figure (4).

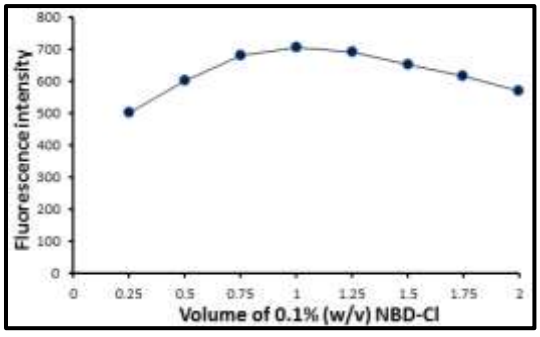

Fig 4: Effect of volume of 0.1% NBD-Cl on the fluorescence intensity of dorzolamide-NBD fluorophore at λem 541 nm.

Effect of $NaHCO_3$ concentration

In order to generate the nucleophile from dorzolamide hydrochloride, the reaction of dorzolamide hydrochloride with NBD-Cl should be done in alkaline medium (pH ~8.3). The influence of $NaHCO_3$was checked using different volumes of 0.2 M $NaHCO_3$ solution ranging from (0.25-2.5 mL). 1.5 mL (0.2 M) $NaHCO_3$ gave the highest FI and above and beyond which the FI decreased, Figure (5).

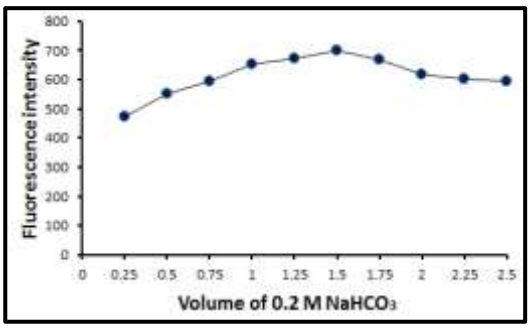

Fig 5: Effect of volume of 0.2 M $NaHCO_3$ on the fluorescence intensity of dorzolamide-NBD fluorophore at λem 541 nm.

Effect of temperature

The influence of temperature was checked at different temperatures (30-70 ˚C). The reaction seem to be dependent on the temperature and the FI increased as the temperature increased and the maximum FI was obtained at 60 ˚C, Figure (6).

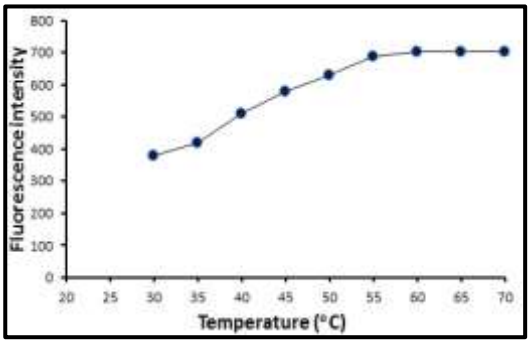

Fig 6. Effect of heating temperature (°C) on the fluorescence intensity of dorzolamide-NBD fluorophore at λem 541 nm.

Effect of reaction time

In order to recognize the time required for completion of the reaction, the reaction was done at different reaction time interval (5-40 min.). The results confirmed that the optimum time was 30 min Figure (7).

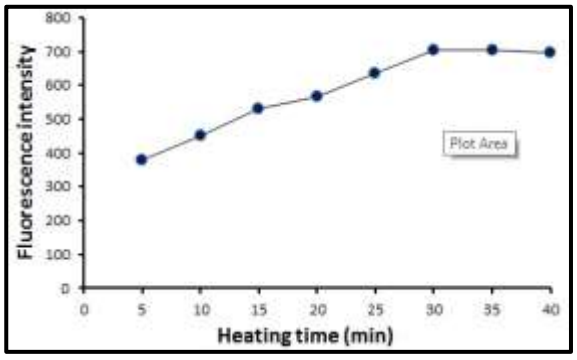

Fig 7: Effect of heating time at 60 °C on the fluorescence intensity of dorzolamide-NBD fluorophore at λem 541 nm.

Effect of HCl concentration

Before measurement of the FI, addition of HCl[15] to the reaction mixture was necessary for remarkably decreasing the background fluorescence (duo to the hydrolysis product of NBD-Cl to the corresponding hydroxyl derivative namely, 7-hydroxy-4

nitrobenzoxadiazole (NBD-OH) [19]. In strong acidic medium (pH ≤ 1), the fluorescence of NBD-OH was found to be quenched. The reaction was done using different volumes of 1M HCl ranging from (0.25-2mL). The optimum concentration of HCl required for acidification was found to be 1 mL of 1M HCl Figure (8).

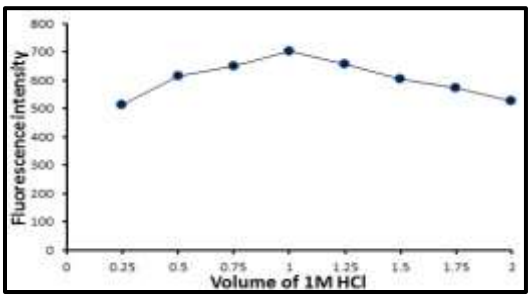

Fig 8: Effect of volume of 1M HCl on the fluorescence intensity of dorzolamide-NBD fluorophore at λem 541 nm.

Effect of diluting solvent

To recognize the most appropriate solvent for diluting the reaction solution, different solvents contain: water, methanol, ethanol, propanol, acetone, acetonitrile were studied. The highest FI was obtained upon using water or methanol but water was used as a diluting solvent because it is economic Figure (9).

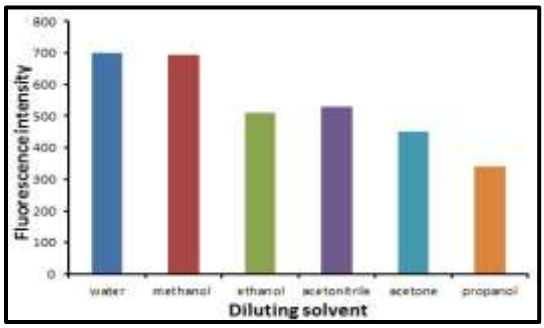

Fig 9: Effect of diluting solvent on the fluorescence intensity of dorzolamide-NBD fluorophore at λem 541 nm.

Mechanism of the reaction:

Dorzolamide hydrochloride has free terminal amino group which react with NBD-Cl through nucleophilic substitution mechanism forming highly fluorescent golden yellow fluorophore, the suggested reaction pathway was shown in Figure (10).

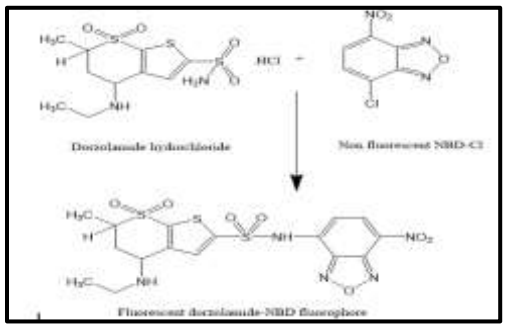

Fig 10: The Proposed reaction pathway between dorzolamide hydrochloride and NBD-Cl.

Stability of fluorescent fluorophore:

The stability of the fluorescent dorzolamide-NBD fluorophore is time dependant, so it was studied by measuring the FI at different time intervals. It was found that the FI values remain constant for at least 24 hours at room temperature.

The optimum variables affecting the reaction of dorzolamide hydrochloride with NBD-Cl were summarized in Table (1).

Table 1: Optimization of variables affecting the reaction of dorzolamide hydrochloride with NBD-Cl.

Variable	Studied range	Optimum
Excitation wavelength (nm)	350 – 520	404
Emission wavelength (nm)	490 – 600	541
(0.1 %, w/v) NBD-Cl	0.25-2 mL	1 mL
0.2 M NaHCO3	0.25-2.5 mL	1.5 mL
Temperature (°C)	30 – 70	60
Heating time (min)	5-40	30
1M HCl	0.25-2 mL	1mL
Diluting solvent	Water, methanol, ethanol, propanol, acetone, acetonitrile.	Water
Stability of dorzolamide-NBD fluorophore	1- 24 hr.	24 hr. at room temp.

Method validation [20]

The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines in terms of linearity, range, LOD, LOQ, accuracy and precision.

Linearity and range

The method obeys the Beer's law in the range of 1-8 µg/mL. The regression data of the method were presented in Table (2).

Limits of detection and quantitation

LOD was found to be 0.147 µg/mL, while LOQ was found to be 0.444 µg/mL, as shown in Table 2.

Accuracy and precision

Accuracy of the described method expressed as %recovery was found to be 99.92. Intra-day precision expressed as %RSD was found to be 1.071, while inter-day precision was found to be 1.186, Table 2. Good %R and %RSD confirm the accuracy and precision of the described method.

The validity of the proposed procedure is further evaluated by applying the standard addition technique showing mean recovery of added ± SD of 99.73 ± 1.644 %. Results are presented in Table 3.

Table 2: Assay validation sheet of the proposed method.

Parameters		Spectrofluorimetric method
λex. & λem.		404 & 541 (nm)
Linearity range (µg/mL)		1 – 8
LOD (µg/mL)		0.147
LOQ (µg/mL)		0.444
Regression Equation		F* = 119.20 C** + 5.006
Coefficient of determination (r)		0.9996
Accuracy (mean ± SD)		99.92± 1.070
Precision	Intra-day	1.071
	Inter-day	1.186

F* is the fluorescence intensity. C** is concentration in (µg/ml).

Table 3: Recovery study of dorzolamide hydrochloride in Trusopt® eye drops by the proposed spectrofluorimetric method by adopting standard addition technique

Pharmaceutical Taken(µg/mL)	Pure added (µg/mL)	Pure found (µg/mL)	Recovery %
2	2	1.96	98.40
	3	3.06	101.95
	4	3.99	99.96
	5	4.93	98.59
Mean ± SD%			99.73± 1.644

Statistical analysis

Statistical comparison between results obtained by applying the proposed procedure and those obtained by applying the reported method [2] showed less calculated t and F values than the tabulated ones revealing no significant difference in accuracy and precision, as shown in Table 4.

Table 4: Statistical comparison for the results obtained by the proposed method and the reported method for the analysis of dorzolamide hydrochloride in trusopt ® eye drops

Parameters	Proposed method	Reported method [2]
n*	5	5
$\bar{X}$	100.03	99.74
SD	1.025	1.186
t**	0.406 (**2.3**06)	——
F**	1.339 (6.**3**88)	——

*** No. of experiments.**
**** The values in the parenthesis are tabulated values of t and F at p= 0.05 level of significance.**

CONCLUSION

This work provided an accurate spectrofluorimetric method for the determination of dorzolamide hydrochloride in its pure form and eye drops based on nucleophilic substitution reaction with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to form a highly fluorescent yellow fluorophore. The proposed method is suitable for the routine analysis of dorzolamide hydrochloride in quality control laboratories.

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