

Determination and Validation of Duloxetine Hydrochloride in Capsules by HPLC with Pre-Column Derivatization and Fluorescence Detection

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A high-performance liquid chromatographic (HPLC) method is described for the determination of duloxetine hydrochloride in capsules. The method was based on pre-column derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole using the fluorimetric detection technique. Duloxetine hydrochloride was analyzed by HPLC using an Inertsil C18 column (5 μ m, 150 \times 4.6 mm) and mobile phase consisted of methanol and water (65:35, *v/v*). The fluorescence detector was adjusted at excitation and emission wavelengths of 461 and 521 nm, respectively. The linearity of the method was in the range of 10–600 ng/mL. Limits of detection and quantification were 0.51 and 1.53 ng/mL, respectively. The proposed method was successfully applied for determination of duloxetine hydrochloride in its pharmaceutical preparation. The results were in good agreement with those obtained using a reference method.

Introduction

Duloxetine hydrochloride (DH) is chemically known as (+)-(*S*)-*N*-Methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine hydrochloride (1). DH is a newer selective serotonin and norepinephrine reuptake inhibitor (SSNRI) used for major depressive disorders (2, 3).

A few methods are available for the determination of DH in biological fluids, including liquid chromatography with single-quadrupole mass spectrometry (LC–MS) (4), LC–tandem mass spectrometry (MS–MS) (5, 6), LC with atmospheric pressure ionization–tandem mass spectrometry (7), gas chromatography–mass spectrometry (GC–MS) (8), high-performance liquid chromatography HPLC (9) and capillary electrophoresis (10).

Several methods have been reported for the determination of DH in pharmaceutical preparations. Among the methods are HPLC with ultraviolet (UV) detection (11), spectrofluorimetry (12, 13) and spectrophotometry (14).

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) has been used as a derivatizing reagent in the spectrofluorimetric determination of many pharmaceutical amines (15–20). The reaction of NBD-Cl with DH has not yet been investigated. Therefore, the present study was devoted to investigating the reaction between DH and NBD-Cl, and to employ the reaction in the development of a sensitive and simple HPLC method for determination of DH in capsules.

Materials and Methods

DH was supplied from Eli Lilly (Istanbul, Turkey) and its pharmaceutical preparation, Cymbalta capsule (60 mg), was

purchased from a local pharmacy. Ranitidine (internal standard; IS) was supplied from Sigma (St. Louis, MO). NBD-Cl was purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical or HPLC grade.

Apparatus

Fluorescence spectra were recorded with a Shimadzu (Kyoto, Japan) RF-1501 spectrofluorimeter. HPLC analyses were performed on Shimadzu equipment consisting of a LC 20 AT system controller with LC-10AT pump, with an SIL-20AHT autosampler with a 5 μ L loop, RF-10AXL fluorescence detector and CTO-10AS column oven. Compounds were separated on an Inertsil analytical column (150 \times 4.6 mm, 5 μ m) and guard column (4 \times 3 mm, 5 μ m, Inertsil). The mobile phase was methanol–water (65:35, *v/v*). The mobile phase flow rate was maintained at 1.2 mL/min, and the column temperature was 40°C. The excitation and emission wavelengths were 461 and 521 nm, respectively.

Preparation of solutions

A stock solution of DH and IS (1,000 μ g/mL) was prepared in methanol. The standard solutions of 10 μ g/mL DH and 10 μ g/mL IS were prepared in methanol. NBD-Cl was prepared as 0.2% (*w/v*) methanol solution. Borate buffer solution (0.025M) was prepared by dissolving sodium tetraborate in water and pH of the sodium hydroxide solution was adjusted to pH 8.5.

Analysis of capsules

Contents of 20 capsules were weighed and net content of a capsule was calculated. Capsule powder equivalent to 100 mg of DH was weighed and transferred to a 100-mL calibrated flask, 50 mL of methanol was added and the solution was sonicated for 15 min. It was then made up to volume with methanol, mixed well and filtered. The prepared solution was quantitatively diluted with methanol to obtain a suitable concentration (10 μ g/mL) for the analysis.

Derivatization

Aliquots of DH working standard solution covering the concentration range of 10–600 ng/mL were transferred into a series of 10-mL stoppered tubes. This was followed by addition of 100 μ L pH 8.5 borate buffer, 100 μ L NBD-Cl and 50 μ L IS solutions. The solution was mixed well, heated at 70°C for 30 min

in a thermostatically controlled water bath and then cooled to room temperature. The NBD derivatives were then acidified with 100 μL of 0.1N HCl. The NBD derivatives were extracted three times with 2.0 mL of chloroform. The combined organic phases were adjusted to 10 mL with the chloroform. The organic phases were dried on anhydrous sodium sulfate. A 5-mL aliquot of the extract was evaporated under nitrogen at 45°C. The residue was then dissolved in 1 mL of the mobile phase. Typically, 5 μL aliquots of this solution are used for determination by HPLC.

Method Validation

The method was validated in accordance with International Conference on Harmonization guidelines (21) for validation of analytical procedures.

Linearity

Calibration curve of duloxetine-NBD derivative was constructed by linear regression using internal standard technique. The plots of peak area ratios versus concentrations of the associated compound were employed.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) of drugs by the proposed methods were determined using calibration standards. LOD and LOQ were calculated as 3.3 and 10 σ/S , respectively, where S is the slope of the calibration curve and σ is the standard deviation of intercept of regression equation.

Precision and accuracy

The precision and accuracy of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). The intra-day precision was calculated as the relative standard deviation (RSD) of results from three standard samples, during the same day, and the inter-day precision was studied by comparing the assays on three different days. The accuracy of the method was expressed by relative mean error (RME).

Recovery

The percent recovery of the added pure drug was calculated as: % recovery = $[(C_t - C_s)/C_a] \times 100$, where C_t is the total drug concentration measured after standard addition; C_s , drug concentration in the formulation sample; C_a , drug concentration added to the formulation.

Robustness

The robustness of the method was evaluated by introducing small variations from the optimum condition. These variations included mobile phase, flow rate and column oven temperature.

Specificity

The specificity of the method was investigated by observing any interference encountered from the capsule excipients, which did not interfere with the proposed methods.

System suitability

To ascertain the resolution and reproducibility of the HPLC method, system suitability tests were performed using the working standard solution of DH. Resolution (R_s), theoretical plate number (N), capacity factor (k') and tailing factor (T) were measured as the criteria for system suitability testing.

Results and Discussion

Derivatization

NBD-Cl reacts with primary amines and secondary aliphatic amines, producing intensely fluorescent derivatives (22). In the present study, NBD-Cl was used for the fluorescence labeling of the secondary amino group of DH (Figure 1). The fluorimetric reaction of the drug with NBD-Cl exhibited its highest fluorescence intensity at emission at 521 nm after excitation at 461 nm (Figure 2).

The different experimental parameters affecting the development of the reaction product were carefully studied and optimized. Such factors were changed individually while others were kept constant. These factors include pH, type of buffer, temperature, time and concentration of reagents.

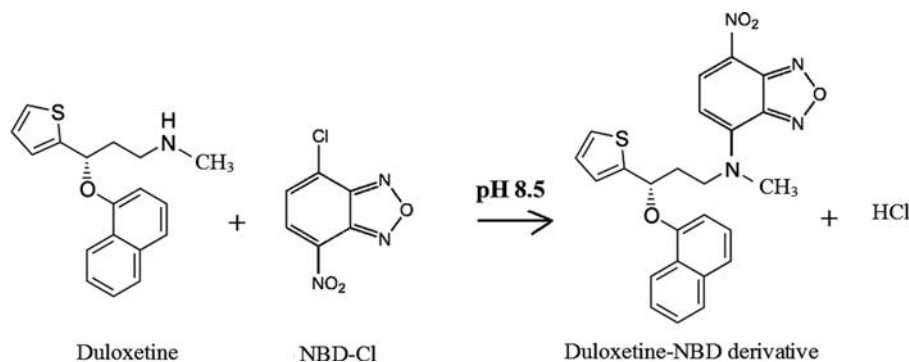


Figure 1. The reaction between duloxetine and NBD-Cl.

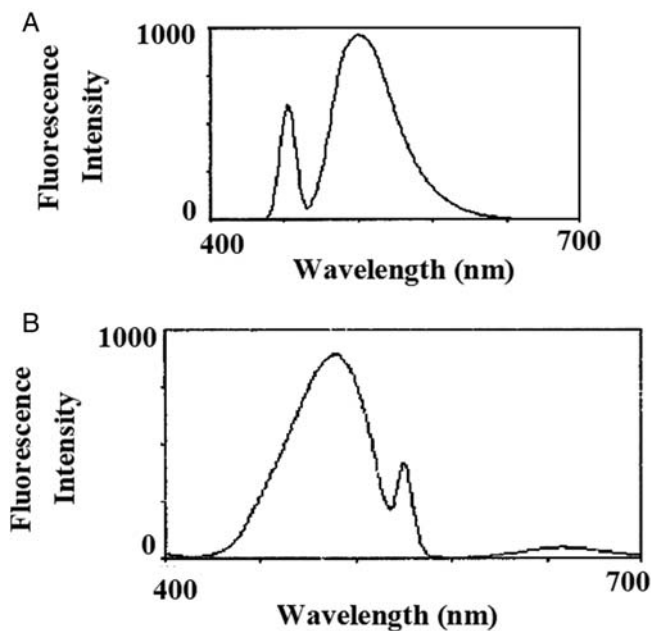


Figure 2. Emission spectra of the duloxetine-NBD derivative (A); excitation spectra of the duloxetine-NBD derivative.

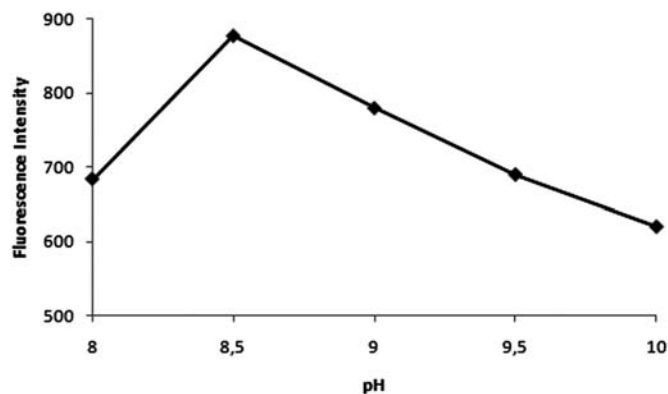


Figure 3. Effect of pH on the intensity of the reaction of DH with NBD-Cl.

The influence of pH on the fluorescence intensity of the reaction product was investigated using 0.1M borate buffer over the pH range of 8.0–10.0 (Figure 3). Maximum fluorescence intensity was obtained at pH 8.5.

The effect of temperature on the color intensity was studied in the range from 50 to 80°C for different periods of time. The color intensity increased on increasing the applied temperature up to 70°C (Figure 4). The effect of the reaction time on the reaction course was studied by measuring the corresponding fluorescence intensity at constant temperature for different periods of time. The optimum reaction time was found to be 30 min.

Studying the effect of different NBD-Cl reagent concentrations on the produced fluorescence intensity revealed that

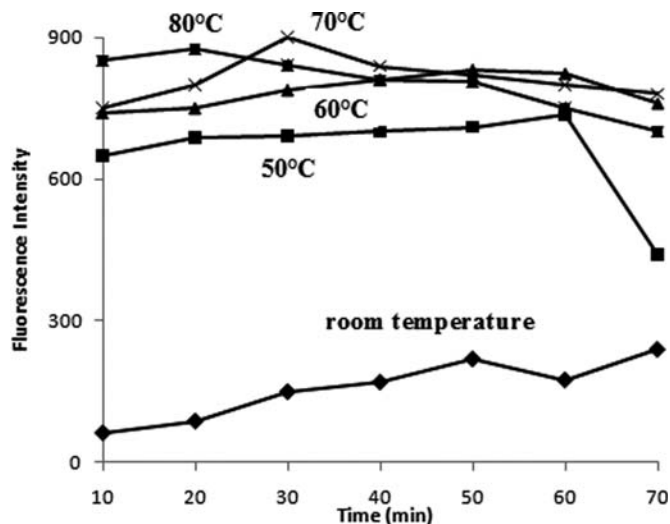


Figure 4. Effect of time and temperature on the reaction of DH with NBD-Cl.

highest fluorescence intensity was over the concentration of NBD-Cl reagent in the final solution between 0.1 and 0.4% (*w/v*). Therefore, the concentration of 0.2% (*w/v*) was selected for further experiments.

Different solvents (methanol, acetonitrile, dichloromethane, chloroform and ethyl acetate) were attempted to dilute the reaction mixture throughout the study. Chloroform was observed to give the highest fluorescence intensity.

The fluorescence of the hydrolysis product of NBD-Cl, namely, 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH), is quenched by decreasing the pH of the reaction medium to less than one. It is known that the maximum association between NBD-Cl and the target compound is realized at the basic medium. However, NBD-Cl is also hydrolyzed in alkaline solution. Thus, the system was stabilized by acidifying the reaction mixture to pH 2 (by adding 100 μ L 0.1N HCl) before measurement.

HPLC method development

Several parameters were examined for the optimization of HPLC analysis of DH. The first attempt was to find out the consistency of the mobile phase.

Different mixtures of methanol and double distilled water were tried as mobile phase, from 90:10 to 85:15, 80:20, 70:30 and 65:35, *v/v*. The most suitable peaks were appeared when 65:35, *v/v* solvent system was utilized with a flow-rate of 1.2 mL/min.

The elution order was DH ($t_r = 6.23$ min) and IS ($t_r = 2.79$ min). Typical chromatograms of DH beside that of the reagent blank are shown in Figure 5.

Method validation

Linearity

The calibration curve of DH was linear over the concentration range of 10–600 ng/mL (10, 25, 50, 100, 150, 300 and 600 ng/mL) with a correlation coefficient of 0.9999.

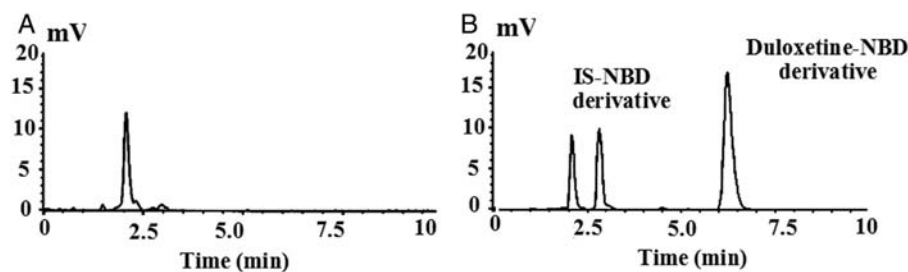


Figure 5. HPLC chromatograms obtained from: blank sample (A); DH (500ng/mL) and IS (500ng/mL) (B).

Table I

Results of Linearity Data of DH ($n = 3$)

Parameters	Values
Slope \pm SD	$0.0077 \pm 1.0 \times 10^{-3}$
Intercept \pm SD	$0.0053 \pm 1.2 \times 10^{-3}$
LOD (ng/mL)	0.51
LOQ (ng/mL)	1.53
Correlation coefficient	0.9999

Table II

Intra-Day and Inter-Day Precision and Accuracy of the DH Assay ($n = 3$)

Added concentration (ng/mL)	Found concentration (ng/mL) mean \pm SD	Precision (RSD%)	Accuracy (RME%)
Intra-day			
10	$9.98 \pm 2.0 \times 10^{-2}$	0.20	-0.20
100	$100.2 \pm 1.3 \times 10^{-2}$	0.01	+0.20
600	$600.3 \pm 2.0 \times 10^{-2}$	0.01	+0.05
Inter-day			
10	$9.97 \pm 3.1 \times 10^{-2}$	0.31	-0.30
100	$100.4 \pm 1.5 \times 10^{-2}$	0.01	+0.40
600	$600.5 \pm 2.3 \times 10^{-2}$	0.01	+0.08

LOD and LOQ

The LOD was found to be 0.51 ng/mL. Under the developed HPLC conditions, LOQ was determined to be 1.53 ng/mL (Table I). These values are much lower than those obtained by many other methods (9–10, 12–14).

Precision and accuracy

The intra-day and inter-day reproducibilities expressed as RSD were found to be 0.01–0.31%. RME values were found to be 0.08–0.20%; therefore, it is obvious that the method is remarkably accurate, which ensures that reliable results are obtained (Table II).

Recovery

The recovery of DH from the capsule matrix were investigated by standard addition technique. The results (Table III) showed that the recoveries were in the range of 99.54–100.23%.

Robustness

The robustness is a measurement of the method capacity to remain unaffected by small, but deliberate, variations in method parameters and was studied by testing the influences of small changes in mobile phase composition and column oven. All

Table III

Recovery Results from the Standard Addition Method ($n = 3$)

Concentration of drug in formulations (ng/mL)	Concentration of pure drug added (ng/mL)	Recovery (mean \pm SD)	RSD (%)
80	20	100.23 ± 0.72	0.72
80	70	99.67 ± 0.38	0.38
80	520	99.54 ± 0.97	0.97

Table IV

Robustness Data for the Proposed Method (10 ng/mL) ($n = 3$)

Parameter	Modification	Mean \pm SD (ng/mL)	RSD (%)
Mobile phase ratio (v/v)	64:36	$9.98 \pm 2.0 \times 10^{-2}$	0.20
	66:34	$9.96 \pm 1.9 \times 10^{-2}$	0.19
Flow rate (mL/ min)	1.1	$10.02 \pm 2.1 \times 10^{-2}$	0.21
	1.3	$9.95 \pm 1.8 \times 10^{-2}$	0.18
	41°C	$10.03 \pm 2.3 \times 10^{-2}$	0.22
Column oven	41°C	$10.03 \pm 2.3 \times 10^{-2}$	0.22
	42°C	$9.98 \pm 2.0 \times 10^{-2}$	0.20

Table V

System Suitability Parameters ($n = 3$)

Capacity factor (k')	Tailing Factor (T)	Theoretical plates (N)	Resolution (Rs)
18.35	1.0	21,559	2.41

critical separations were achieved with the indicated minimum baseline resolution (Table IV).

System suitability

System suitability was tested on the basis of results obtained from several representative chromatograms. According to ICH guidelines the system is suitable when: $RS > 2$, $N > 2,000$, $k' > 2.0$ and $T \leq 2$. The values obtained for this method ($RS = 2.41$, $N = 21559$, $k' = 18.35$ and $T = 1.0$) were within the acceptable ranges (Table V) (21).

Specificity

No interference was observed when a capsule matrix of gelatin, hydroxypropyl methylcellulose, sodium lauryl sulfate, sucrose, talc and titanium dioxide was used.

Table VI

Determination of DH in Pharmaceutical Preparations by the Proposed and Reference Method (Cymbalta 60 mg)*

	Proposed method	Reference method (13)
Recovery (%) \pm SD	99.18 \pm 0.78	99.13 \pm 0.81
t		0.17
F		1.06

*n = 6; the tabulated values of t and F at 95% confidence limit are 2.23 and 5.05, respectively.

Application

The proposed method was applied to the determination of DH in capsules. A comparative determination of the same samples was also investigated by a reference method (13). Statistical comparison of the results by the Student's *t*-test and the variance-ratio *F*-test at the 95% confidence level revealed no significant difference between the accuracy and precision of the two methods (Table VI).

Conclusions

This method proposes, for the first time, the development of a sensitive and simple HPLC method with fluorescence detection for the determination of DH after pre-column derivatization with an NBD-Cl reagent. The chromatographic separation is based on a reversed-phase mechanism performed with isocratic elution for a run time of only 10 min. The analytical results demonstrated that the proposed method is suitable for the accurate determination of DH in pharmaceutical preparations at concentrations as low as 0.51 ng/mL with a wide linear range. Compared with previously reported methods, this method provided the highest sensitivity in measuring very low concentrations of DH. The proposed method can be applied for routine analysis of DH in dosage forms.

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