DRUG FORMULATIONS AND CLINICAL METHODS

A Highly Sensitive Spectrofluorometric Method for the Determination of a New Antidepressant Drug, Reboxetine, in Tablets

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A highly sensitive, selective, and rapid spectrofluorometric method has been developed for the determination of reboxetine (REB) in tablets. The method is based on derivatization with 7-chloro-4-nitrobenzofurazan. The product showed an absorption maximum at 476 nm and a fluorescence emission peak at 533 nm in ethyl acetate. The optimum conditions of the reaction were investigated, and it was found that the reaction proceeded quantitatively at pH 8.5, 70°C in 5 min. The calibration graph is rectilinear over the range of 0.02–0.40 μg/mL. The relative standard deviation values for intraday and interday precision were 0.40–0.93 and 0.54–1.37%, respectively. The proposed method was applied to the assay of REB in tablets. Mean recovery of REB from the tablets ranged between 99.91–100.20%. The results were compared statistically with those obtained by a method reported in the literature. The method is sensitive, simple, and selective, and can be used for routine quality control analysis.

Depression is a common, recurring disorder that ranges in severity from mild to very severe. Reboxetine (REB), (RS)-2-[(RS)-α-(2-ethoxyphenoxy)benzyl] morpholine (Figure 1), is a noradrenaline selective reuptake inhibitor (1) that has been shown to be effective in the treatment of depression with a relatively safe side effect profile (2, 3). REB's receptor affinity is low, hence its use is not accompanied by the typical adverse effects of tricyclic antidepressants (3, 4). With respect to fluoxetine and other selective serotonin reuptake inhibitors, REB seems to have greater efficacy against a lack of confidence and motivation and to reduce the risk of suicide (5, 6).

There is no official analytical method for REB in pharmacopeias. A literature search revealed that there are only 2 papers reporting the analysis of REB in tablets. The methods used were derivative spectrophotometry and capillary zone electrophoresis (7) or spectrophotometry (8).

Among the various methods available for the determination of drugs, spectrophotometry and spectrofluorometry continue to be very popular because of their simplicity, specificity, and low-cost. Detection of the drugs at ultraviolet (UV) wavelength tends to be interfered with by sample matrix components having shortwave UV-absorbing chromophores. Spectrofluorometric analysis constitutes a widespread, effective technique to improve analysis selectivity and sensitivity. Obviously, for routine quality control or determination of traces of drugs in serum and plasma, the development of a simple, rapid, selective, and sensitive spectrofluorometric method is highly desirable. In this study, a new, highly sensitive, and reliable spectrofluorometric method has been developed for the assay of REB in tablets. Derivatization is better than the other methods to prevent the interference of inactive ingredients in the pharmaceutical preparation. The new method is based on a derivatization with 7-chloro-4-nitrobenzofurazan (NBD-Cl) reagent, which is widely used to produce fluorescent derivatives of compounds with primary and secondary amine groups (9, 10). The proposed method is comparable to previously reported methods in tablets (7, 8) in terms of sensitivity. The applicability of the developed method was evaluated by the determination of REB in tablets.

Received September 13, 2005. Accepted by JM November 16, 2005.
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Experimental

Apparatus

(a) *Spectrofluorometer.*—Fluorescence spectra and measurements were taken on a spectrofluorometer Model RF-1501 (Shimadzu, Tokyo, Japan) equipped with a xenon lamp and 1 cm quartz cells. Excitation and emission wavelengths were set at 476 and 533 nm, respectively. The measuring system of the instrument was calibrated daily by using sodium fluorescein (reference standard) solution in 0.1 M NaOH at an appropriate concentration.

(b) *pH meter.*—Model 526 digital pH meter, calibrated with buffer solution (pH 7.0; WTW, Weilheim, Germany).

Reagents and Solutions

REB methansulfonate and its pharmaceutical preparation (Edronax®) containing 4 mg REB/tablet were kindly supplied by Pfizer (Istanbul, Turkey). NBD-Cl and other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals and reagents were of analytical reagent grade.

A stock solution of REB methansulfonate containing 1 mg/mL (calculated as free base) was prepared in water and

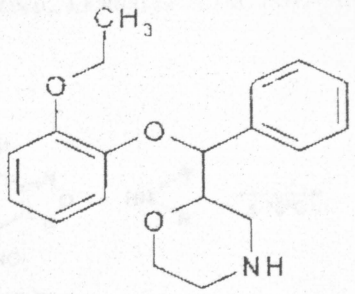

Figure 1. Chemical structure of REB.

diluted further with the water to obtain standard solutions of 10 μg/mL.

For the preparation of sample solution, 20 tablets were weighed and powdered, and then a portion of tablet powder equivalent to 100 mg REB was weighed. The drug was extracted with ca 50 mL water by shaking mechanically for 15 min in a 100 mL volumetric flask. The solution was filtered after the volume was adjusted with water, and 1 mL of the filtrate was diluted to 100 mL with water in a volumetric flask. Then proceeded as described under *General Procedure*. The nominal contents of the tablets were calculated using either the calibration graph or the corresponding regression equation.

NBD-Cl solution was freshly prepared in methanol at 3.5 mg/mL concentration. Buffer solution was prepared as follows: 0.620 g boric acid and 0.750 g potassium chloride were dissolved in 100 mL water. The pH was adjusted to 8.5 with 0.1 M sodium hydroxide solution, and the volume was made up to 200 mL with water.

General Procedure

Aliquots of 0.010–0.20 mL standard solution at 10 μg/mL concentration were mixed with 0.2 mL buffer solution in a glass, stoppered tube. After addition of 0.2 mL NBD-Cl solution, the mixture was heated at 70°C for 5 min in a thermostatted water bath. The mixture was cooled in the ice batch and acidified with 0.2 mL of 0.1 M HCl solution. The derivative was extracted with 5 mL ethyl acetate on a Vortex mixer after the phases had been separated by centrifugation.

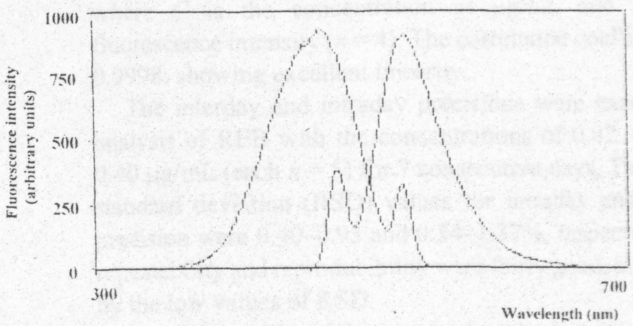

Figure 2. Excitation and emission spectra (excitation maximum, 476 nm, and emission maximum, 533 nm) of the reaction product of REB (0.35 μg/mL) with NBD-Cl in ethyl acetate.

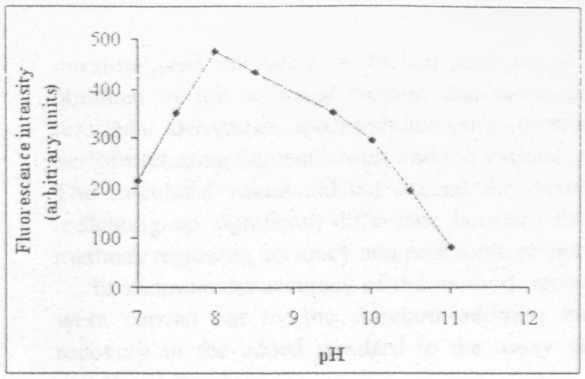

Figure 3. Effect of pH on the development of the reaction product of REB (0.12 μg/mL) with NBD-Cl.

The fluorescence intensity of the derivative was measured at 533 nm while exciting at 476 nm against a blank prepared similarly. The fluorescence intensity of reference standard solution, sodium fluorescein, was also measured at the same wavelength combination. The relative fluorescence intensity (I_F) was then calculated by the following equation:

$$I_F = x/y \times 100$$

where x and y represent the fluorescence intensities of the sample and reference standard solutions, respectively ($n = 4$).

Assay Procedure

A 0.2 mL aliquot of sample solution was reacted with NBD-Cl as described above ($n = 5$). The amount of REB was calculated using the regression equation of the calibration graph.

Results and Discussion

REB contains a secondary amino group that is known to react with NBD-Cl in alkaline medium to yield a fluorescent derivative (11). Under the described experimental conditions, the fluorophore exhibits its highest fluorescence intensities at excitation and emission wavelengths of 476 and 533 nm, respectively, in ethyl acetate. Figure 2 shows the excitation and emission spectra of the fluorescent product.

The different experimental parameters affecting the intensity of the color produced were studied and optimized to obtain maximum color intensity. First, the influence of pH on the absorption was studied. Phosphate and borate buffer systems were tested, and higher fluorescence intensities were obtained when the reaction was carried out using borate buffer system. The pH was varied over the range of 7–11 using borate buffer, and the maximum fluorescence intensity was obtained at pH 8.5 as shown in Figure 3. NBD-Cl is hydrolyzed in alkaline medium to give NBD-OH, which has maximum absorbance at 462 nm. Therefore, it was necessary to acidify the reaction mixture to pH 2 (by adding 0.2 mL of 0.1 M HCl) before the measurement was carried out. At this acidic pH, the reagent blank did not show any significant absorption peak between 420 and 500 nm (12).

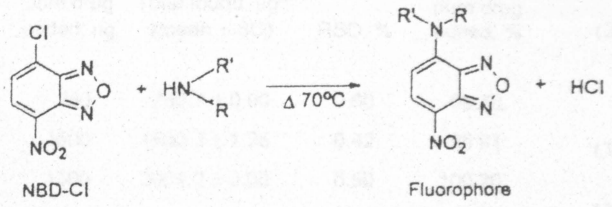

Figure 4. Proposed pathway of the reaction between NBD-Cl and REB.

The influence of temperature on color development was also studied in the range from 60 to 80°C. It was found that heating at 70°C gave the most reproducible results. The effect of time on the course of the reaction was studied by measuring the corresponding absorbance at 70°C for different periods of time. It was found that the optimum reaction time was 5 min. The effect of concentration of NBD-Cl on the color intensity was studied over the range 0.1–0.4%. It was found that 0.2 mL of 0.35% NBD-Cl solution was the most suitable volume of the reagent. The derivative has maximum intensity in ethyl acetate, and it was stable in this solvent for at least 48 h at 4°C in the dark. Furthermore, the molar ratio of NBD-Cl to REB in the reaction mixture was studied according to Job's method of variation (13). Utilizing equimolar solutions of REB and NBD-Cl (4×10^{-1} mmol), the reaction stoichiometry continuous was found to be a good approximation of a 1:1 ratio (drug:reagent), confirming that 1 molecule of REB reacted with 1 molecule of NBD-Cl. Accordingly, the reaction was postulated to proceed as proposed in Figure 4.

Under the optimum reaction conditions, relative fluorescence intensity was found to be linearly correlated to REB concentration over the range of 0.02–0.40 μg/mL. Linear regression analysis of the concentration-fluorescence intensity data gave the following equation:

$$I_f = 0.2072\ C + 4.816$$

where C is the concentration in μg/mL and I_f is the fluorescence intensity ($n = 4$). The correlation coefficient was 0.9998, showing excellent linearity.

The interday and intraday precisions were examined by analysis of REB with the concentrations of 0.02, 0.20, and 0.40 μg/mL (each $n = 5$) for 7 consecutive days. The relative standard deviation (RSD) values for intraday and interday precision were 0.40–0.93 and 0.54–1.37%, respectively. The repeatability and reproducibility were fairly good, as indicated by the low values of RSD.

The applicability of the proposed method was tested by the determination of REB in tablets. The results obtained were satisfactorily accurate and precise, as indicated by the excellent recovery values and standard deviation (SD) <2 (Table 1). Common tablet excipients, such as talc, lactose, starch, avisil, gelatin, or magnesium stearate, did not interfere with the assay. Statistical analysis of the results obtained by the proposed method and those given by the reference derivative spectrophotometric method (7) was performed using Student's t-test and the variance ratio F-test. The calculated values did not exceed the theoretical ones, indicating no significant difference between the compared methods regarding accuracy and precision, respectively.

To examine the accuracy of the method, recovery studies were carried out by the standard addition method. The recovery of the added standard to the assay samples was calculated from:

$$\text{Recovery, \%} = [(C_t - C_u)/C_a] \times 100$$

where C_t is the total concentration of the analyte found, C_u is the concentration of the analyte present in the formulation, and C_a is the concentration of the pure analyte added to the formulation. The results are given in Table 2. The average recoveries obtained were quantitative (99.78–100.20%), indicating good accuracy of the method.

Conclusions

The proposed spectrofluorometric method provides very sensitive, accurate, and reproducible quantitative determination of REB in tablets. This method exhibits high sensitivity that makes it possible to measure concentrations down to 0.02 μg/mL. This value is comparably sensitive to other methods reported in the literature (7, 8). The suggested method can readily be applied for routine analysis in quality control testing. Being more sensitive, the fluorometric method is more advantageous than UV-spectrophotometric methods. It allows the analysis of the drug at low concentrations and might be applicable for the assay of REB in biological fluids. Determination of REB in biological fluids, such as urine or plasma, requires sensitive analytical methods. The proposed spectrofluorometric method developed for the determination of REB in tablets can also be applied for this purpose after chromatographic separation by high-performance liquid

Table 1. Statistical evaluations of the results obtained by the proposed and comparison methods for the assay of REB in tablets containing 4 mg of the drug ($n = 5$)

Statistical value	Proposed method[a]	Reference method (ref. 7)
Mean	4.01	3.96
Recovery, %	100.21	99.1
RSD, %	0.85	0.92
Confidence limits	3.98–4.04	3.92–4.00
t-test of significance[b]	2.18	
F-test of significance[c]	0.87	

[a] Edronax tablet (4 mg).
[b] Five independent analyses.
[c] $P = 0.05$, $t = 2.23$, $F = 5.05$.

Table 2. Results of recovery studies by the standard addition method

Amount of drug in tablet, μg[a]	Amount of pure drug added, μg	Total found, μg[b] (mean ± SD)	RSD, %	Recovery of pure drug added, %
501.25	250	750.7 ± 0.90	0.60	99.78
501.25	1000	1500.3 ± 1.25	0.42	99.91
501.25	1500	2004.2 ± 2.00	0.50	100.20

[a] Edronax tablet, 4 mg.
[b] Five independent analyses.

chromatography and use of a fluorescence detector. The applicability of the method to the biological samples is now under investigation.

Acknowledgments

The author would like to thank the Research Fund of Istanbul University for support of this work (Project No. 416/13092005).

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