Iranian Journal of Pharmaceutical Sciences
2023: 19 (4): 350- 359
https://journals.sbmu.ac.ir/IJPS

Original Article

Spectrofluorimetric Determination Method for Pregabalin in Dosage Forms Using 9-Fluorenylmethyl Chloroformate

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Abstract

The present study produces a new, accurate, and simple spectrofluorimetric technique for the determination of pregabalin after derivatization with 9-fluorenylmethyl chloroformate in mild basic medium. Pregabalin, containing a primary amine group, reacts with 9-fluorenylmethyl chloroformate, a fluorophore reagent, in borate buffer and produces a fluorescent derivative. The maximum fluorescence intensity of the derivative and reagent was obtained at 315 nm after excitation at 280 nm. The fluorescence intensity of the reagent increases after derivatization with pregabalin, and the difference in the fluorescence intensity is proportional to the concentration of pregabalin. Experimental parameters which affect the derivatization reaction were studied. The best derivatization reaction between pregabalin and 9-fluorenylmethyl chloroformate was obtained after 30 sec. when 40 µg mL^{-1} of the reagent was used in a borate buffer of pH 9.0. Under optimized conditions, the linearity range (6-150 µg mL^{-1}) of the method with a correlation coefficient of r^2 0.9998, accuracy (Error<1.7%), and precision (CV<2.0%) was acceptable. The validated method was utilized for quantifying pregabalin in dosage form without interferences and showed good agreement with a reference method.

Keywords: Pregabalin, 9-fluorenylmethyl chloroformate, Derivatization, Spectrofluorimetry, Determination, Dosage form.

1. Introduction

Pregabalin has the chemical name of (3S)-3-(aminomethyl)-5-methylhexanoic acid ($C_8H_{17}NO_2$). The compound's molar mass equals 159.23 g/mol, and its melting point is 194-196 °C. Pregabalin can create hydrogen bonds due to carboxylic acid and amine groups in its structure. This drug is available in the pharmaceutical market as capsules, tablets, and oral solutions. Its bioavailability is more than 90%, its half-life elimination is about 6.3 hours, and the steady state is achieved after 1-2 days of administration. This drug has no protein

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Cite this article as: Souri E, Haramipour H, Barazandeh Tehrani M, Shabani Ravari N, Hariri R, *Spectrofluorimetric Determination Method for Pregabalin in Dosage Forms Using 9-Fluorenylmethyl Chloroformate*, Iran. J. Pharm. Sci., 2023, 19 (4): 350- 359.

DOI: https://doi.org/10.22037/ijps.v19i4.43503

binding and is excreted by urine (90% as an unchanged drug) [1-3]. Pregabalin is a potent agonist for $\alpha_2\delta$ protein of Q-type calcium channels, which are voltage dependent. Pregabalin is prescribed for partial seizure, neuropathic pain, and anxiety disorders [4-9]. After binding of pregabalin to its receptor, the release of glutamate, dopamine, noradrenaline, substance P, and serotonin is reduced in the central nervous system [10, 11]. Therefore, due to its many uses, the measurement of this drug becomes important in pharmacy [12, 13].

Different LC/MS/MS [14-17] or HPLC [18-22] methods are available for the quantification of pregabalin either in biological fluids or dosage forms. Furthermore, the USP proposed a method for determining pregabalin using HPLC [23]. As there is no chromophore group in the pregabalin structure, derivatization with different derivatizing reagents is needed to increase the UV, visible, or fluorescence absorption. Picryl sulfonic acid [19], fluorescamine [24, 25], O-phthaldialdlyde [20, 26, 27], 1-fluoro-2-4-dinitrobenzene [21, 22, 28, 29], salicylaldehyde [30], xanthone analog [31], ninhydrin and phenylacetaldehyde [32], acetyl butyrolactone [33], carbon quantum dots [34], and 7-chloro-4-nitrobenzoxadiazole [35] have been used before as derivatizing reagent for the analysis of pregabalin in biological fluids or dosage forms. There are also few spectrofluorimetric or spectrophotometric methods for quantifying pregabalin after derivatization with different reagents [25, 36-39].

The main purpose of this study was to validate a selective and sensitive spectrofluorimetric method for quantifying pregabalin. 9-Fluorenylmethyl chloroformate (FMOC-Cl) ($C_{15}H_{11}ClO_2$) is available as a white to pale yellow crystalline powder with a molar mass of 258.7 g/mol and a melting point of 62-64 °C. This composition is sensitive to moisture and must be stored in appropriate conditions. This compound is a fluorogenic derivatizing reagent that can react with primary and secondary amines in mild basic conditions. In the present study, this reagent was successfully used as a derivatizing reagent for quantifying pregabalin in dosage forms for the first time.

2. Materials and Methods

2.1. Chemicals

Bakhtar Bioshimi Co., Kermanshah, Iran, kindly donated pregabalin (Sun Pharma, India). Pregabalin 75 mg capsules were from Razak Laboratories, Tehran, Iran. Other chemicals, solvents, and 9-fluorenylmethyl chloroformate were from Merck (Darmstadt, Germany). Double distilled water was used for the preparation of solutions.

2.2. Instruments

A spectrofluorimeter (RF-5000) from Shimadzu (Japan) equipped with a 150 W Xenon lamp with a bandwidth of 5 nm and 1 cm diameter quartz cells was used for spectrofluorimetric measurements.

2.3. Standard solutions

A standard solution of pregabalin (200 µg mL^{-1}) was prepared in water. The 9-fluorenylmethyl chloroformate solution (200 µg mL^{-1}) in acetonitrile was also prepared daily.

Using appropriate amounts of KCl and H_3BO_3, a 0.25 M borate buffer was prepared, and the pH was adjusted to 9.0. The solutions were stable in the refrigerator for at least seven days.

2.4. General procedure

Into a 10 mL volumetric flask, 2 mL of pregabalin solution was transferred, and borate buffer (pH 9.0) (2.5 mL), reagent solution (2 mL), and acetonitrile (3 mL) were added. After mixing, the solution was allowed to stand at room temperature (24±2°C) for 30 sec. The volumetric flask comprised 10 mL of distilled water, and the fluorescence intensity was read at 315 nm after excitation at 280 nm (F_1). A blank solution containing 2 mL of distilled water instead of pregabalin solution was also prepared. The fluorescence intensity (F_2) of a blank solution was read. The fluorescence intensity (F_2-F_1) increase was calculated which depends on the pregabalin concentration.

2.5. The effect of different factors on derivatization reaction

According to other reports, the reaction between pregabalin and reagent was conducted at room temperature [40-42]. Higher temperatures did not show any effect on the derivatization yield. The effect of other parameters on the derivatization reaction, such as reagent amount, pH of the buffer, and reaction time, were carefully studied. The effect of each variable was studied, maintaining the other parameters constant.

2.6. Effect of pH

Different pH values of borate buffer in the range of 7.5-10.0 were checked using the general procedure for analysis of a pregabalin solution (200 µg mL^{-1}) to study whether the pH affect the derivatization reaction.

2.7. Effect of reagent volume

To find out the effect of reagent amount, a volume of 0.5-4.0 mL of the reagent (200 µg mL^{-1}) to produce the final concentration of 20-80 µg mL^{-1} in the reaction solution was used for determination of a pregabalin solution (200 µg mL^{-1}) and maximum fluorescence intensity of the formed derivative was measured and compared.

2.8. Effect of time

The reaction between pregabalin and 9-fluorenylmethyl chloroformate was followed for 60 min to determine the derivative's stability over time.

2.9. Robustness

The robustness of the method was checked by performing minor changes in the parameters such as the pH value (9.0±0.2), the reagent volume (2.0±0.2), and time (5±2min) on the derivatization reaction.

2.10. Linearity

Six series of standard pregabalin solutions in the concentration range of 6-150 µg mL^{-1} (6, 15, 30, 60, 90, 120, and 150 µg mL^{-1}) were prepared by consequent dilution of a pregabalin stock solution. The calibration solutions were analyzed based on the general procedure, and statistical analysis was performed after constructing the calibration graphs.

2.11. Accuracy and precision

Triplicate analysis of quality control solutions of pregabalin (6, 60, and 150 µg mL^{-1}) was performed to check the within-day accuracy and precision of the determination method. The same analysis was repeated for three consecutive days to check the between-day accuracy and precision.

2.12. Application of the proposed method

The content of 10 pregabalin 75 mg capsules was mixed and crushed to reach a fine powder. Precisely one capsule's worth of powder was added to a 100 mL volumetric flask containing 70 mL of water, sonicated for 15 minutes, and made of volume. After filtration using a 0.45 µm Syringe filter, the solution was diluted ten times and analyzed based on the general procedure. The same process was applied to a standard solution to determine the amount of pregabalin in the sample solution.

2.13. Relative recovery

The conventional addition method was utilized to assess the pregabalin's relative recovery. An amount of capsule powder equal to one capsule (about 75 mg of pregabalin) was transferred to a 100 mL volumetric flask. After adding 70 mL of distilled water and a standard pregabalin solution containing 75 mg, the mixture was sonicated for 15 minutes. Following volumetric adjustment and tenfold dilution, the material underwent standard protocol treatment. After deducting the fluorescence intensity of an unspiked sample made using the same method, the fluorescence intensity of this solution was compared to that of a standard solution.

3. Results and Discussion

3.1. Fluorescence measurements

Few spectrophotometric or spectrofluorimetric methods have been reported for quantifying pregabalin based on derivatization with different reagents. A summary of the reagents used and the linearity range and the reaction conditions are shown in **Table 1**. As shown in Table 1, most of the previously reported methods need heating at 55-95°C for at least 10 minutes for derivatization. Only two of the derivatization methods were performed at room temperature. Two other methods also need extraction after the derivatization reaction.

The purpose of this study was to develop a simpler method for the quantification of pregabalin. 9-Fluorenylmethyl chloroformate is a derivatization reagent reacting with primary and secondary amines and hydroxyl groups. Derivatization of the amino group of pregabalin with 9-fluorenylmethyl chloroformate occurs in a relatively mild alkaline condition (**Figure 1**). The formed derivative showed the same fluorescence profile with higher intensity than the blank solution. The magnitude of the fluorescence increase was proportional to the pregabalin concentration (**Figure 2A**).

3.2. The effect of different factors on derivatization reaction

3.2.1. Effect of pH

The derivatization procedure was conducted at various pH values within the range of 7.5-10.0. It was noticed that the largest rise in fluorescence intensity occurred at pH 9 (**Figure 2B**).

Table 1: Comparison of some previously reported spectrophotometric and spectrofluorimetric methods for the analysis of pregabalin.

Method and reagent	Linearity (µg mL^{-1})	Remarks	Ref.
Spectrophotometry using 7-chloro-4-nitrobenzofurazon	0.5-7.0	10 min. at 80°C and extraction with chloroform	(36)
Spectrofluorimetry using 7-chloro-4-nitrobenzofurazon	0.04-0.40	10 min. at 80°C and extraction with chloroform	(25)
Spectrofluorimetry using fluorescamine	0.01-0.30	At room temperature	(25)
Spectrophotometry using 1,2-naphthoquinone-4-sulphonate	2.0-25.0	20 min. at 55±5°C	(37)
Spectrophotometry using 2,4-dinitrofluorobenzene	0.5-8.0	20 min. at 70±10°C	(37)
Spectrophotometry using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone	2.0-30.0	15 min. at 60°C	(38)
Spectrophotometry using 7,7,8,8-tetracyanoquinodimethaneane	1.5-10.0	5 min. at room temperature	(38)
Spectrophotometry using ninhydrin	40.0-180.0.0	1 min. at 95°C	(38)
Spectrophotometry using ninhydrin	50.0-1000.0	20 min. at 70-75°C	(39)

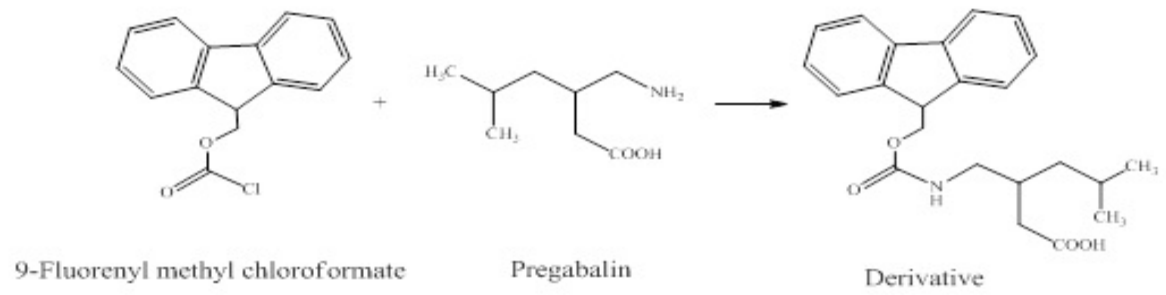

Figure 1. Plausible reaction pathway for pregabalin and 9-fluorenylmethyl chloroformate.

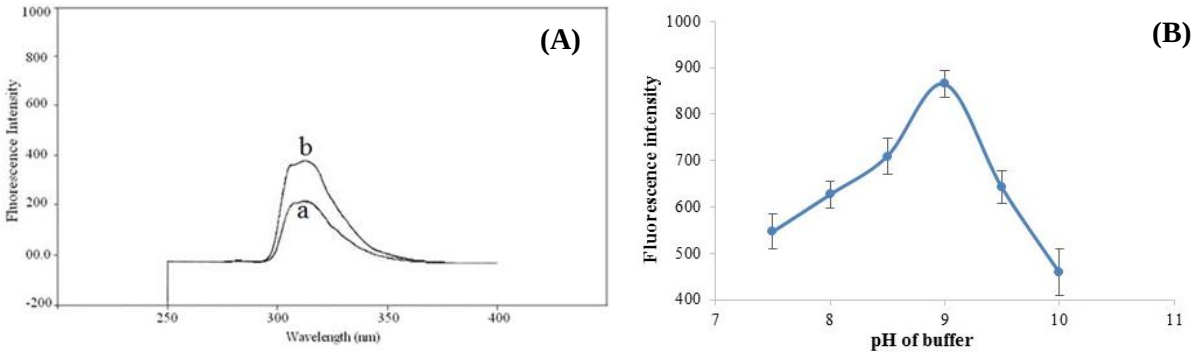

Figure 2. (**A**) Emission spectra of (a) 9-fluorenylmethyl chloroformate solution (2 mL, 200 µg mL^{-1}) at pH 9.0; (b) formed derivative of pregabalin (50 µg mL^{-1}) and 9-fluorenylmethyl chloroformate (2 mL, 200 µg mL^{-1}) at pH 9.0. (**B**) The effect of pH of the borate buffer on derivatization of pregabalin (2 mL, 200 µg mL^{-1}) and 9-fluorenylmethyl chloroformate (2 mL, 200 µg mL^{-1}).

As the amine group could react with the reagent, mild alkaline pH is needed, and maximum reaction is observed at pH 9.0. By increasing the pH value higher than 9.0, the formation of the corresponding alcohol compound (FMOC-OH) will be increased, and the fluorescence intensity decreased as the concentration of the reagent is lower.

3.2.2. Effect of reagent volume

Different reagent volumes (200 µg mL^{-1}) ranging from 0.5-4 mL were used. The fluorescence intensity was increased by increasing the reagent volume up to 2 mL (equal to 40 µg mL^{-1} in the reaction mixture). Higher values of the reagent did not affect the derivatization, and 2 mL of the reagent was used for other experiments (**Figure 3**).

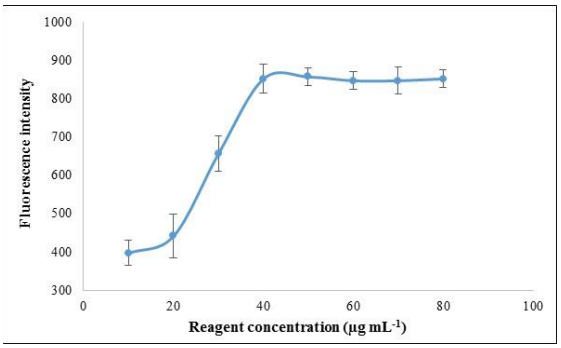

Figure 3. The effect of derivatization reagent final concentration on derivatization of pregabalin (2 mL, 200 µg mL^{-1}) and 9-fluorenylmethyl chloroformate at pH 9.0.

3.2.3. Effect of reaction time

The reaction was rapid and showed maximum fluorescence immediately after mixing. Increasing the reaction time (0–30 min) did not increase fluorescence intensity. Therefore, the measurements were performed after 30 seconds. The fluorescence intensity was decreased by about 10% after 60 min.

3.3. Robustness

The impact of minor adjustments to the parameters, such as the reagent volume (2.0±0.2) and the pH value of the borate buffer (9.0±0.1), on the reaction was checked in order to assess the method's robustness. In each experiment, one parameter was changed based on the one-factor-at-a-time methodology. Based on the results (**Table 2**), no significant effect was observed in the fluorescence intensity, which shows the robustness of the proposed method.

Table 2: Effect of small variations in the derivatization reaction of pregabalin and fluorenyl methyl chloroformate (robustness) (n=3).

pH of the borate buffer	Reagent volume (mL)	Fluorescence intensity
8.9	2.0	652.3±14.2
9.0	2.0	659.0±13.9
9.1	2.0	658.0±19.7
9.0	1.8	652.7±20.9
9.0	2.0	658.3±20.5
9.0	2.2	657.0±12.2

3.4. Linearity

The suggested approach demonstrated linearity throughout the 6-150 µg mL^{-1} range, exhibiting a satisfactory correlation coefficient. **Table 3** displays the statistical information of the repeated calibration curves.

Table 3: Statistical data of calibration curves for pregabalin in standard solutions (n = 6).

Parameters	***Spectrofluorimetry***
Linearity range	6-150 µg mL^{-1}
Regression	y = 4.23 x + 13.06
Slope SD	0.007
Slope relative SD %	0.17
Intercept SD	1.56
Correlation coefficient (r^2)	0.9998
LOQ (µg mL^{-1})*	3.96
LOD (µg mL^{-1})*	1.22

* LOQ= 10 σ/s and LOD= 3.3 σ/s where σ is the SD of intercept and s is the slope of the calibration curve

3.5. Accuracy and precision

The suggested method was tested using different concentration levels of pregabalin to determine its accuracy and precision within and between days. At each concentration level, three replicate measurements were made over one day and three days in a row. The method for quantitative spectrofluorimetric determination of pregabalin was found to have good precision and accuracy, as demonstrated by the tiny values of the coefficient of variation and error (**Table 4**).

Table 4: Precision and accuracy of the method for determination of pregabalin in standard solutions (n=9; 3 sets for three days).

Concentration added (µg mL^{-1})	Found (µg mL^{-1})	CV (%)	Error (%)
Within-day			
6.00	6.10±0.12	1.97	1.67
60.00	60.61±0.59	0.97	1.02
150.00	149.40±0.85	0.59	-0.40
Between-day			
6.00	6.05±0.10	1.65	0.83
60.00	60.14±0.58	0.96	0.23
150.00	149.35±0.78	0.52	-0.43

3.6. Stability

The pregabalin solutions kept at 4°C showed relative stability for at least seven days (recovery > 99%). Also, the solution showed good stability at room temperature for at least two days (recovery > 99%).

3.7. Application of the proposed method

Pregabalin was measured in capsules by using the proposed method. Using a previously reported HPLC method [43] did not show significant differences (**Table 5**).

Table 5: Comparison of the developed method with the reference method for determining pregabalin in capsules.

Method	Label claimed (mg)	Found (mean±sd*)	Statistical tests**
Spectrofluorimetry	75.00	74.87±0.21	t= 0.056 F= 0.547
HPLC method	75.00	74.49±0.13	

* Standard Deviation, **Theoretical values of t and F at p=0.05 are 4.3 and 19.0, respectively.

3.8. Relative recovery

The assay solutions of pregabalin were spiked with an accurate standard concentration of the drug and determined using the general procedure to evaluate the relative recovery. The high obtained relative recovery (99.85±0.65%) shows that there are not any significant interferences with the excipients.

4. Conclusion

The sensitivity of spectrofluorimetric methods is comparable with other complicated methods. In this report, 9-fluorenylmethyl chloroformate was used for the first time as a labeling reagent for the spectrofluorimetric quantification of pregabalin. The reaction between pregabalin and reagent was performed at room temperature, and was relatively fast, and was completed in 30 seconds. Also, the reaction conditions are not critical. A minimal amount of organic solvent is needed for the reaction, and there is no need for tedious sample preparations. The validated quantification method shows linearity over the range of 6-150 µg mL^{-1} with an acceptable correlation coefficient (r^2 0.9998), accuracy (Error<1.7%), and precision (CV<2.0%). The low-cost

proposed method, which showed acceptable accuracy and precision, is suitable for quality control studies of bulk pregabalin or dosage forms.

Acknowledgments

The authors acknowledged Bakhtar Bioshimi Co. for kindly preparing the bulk powder of pregabalin.

Conflict of interest

The authors declare to have no conflict of interest.

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