# Highly Sensitive Determination and Validation of Gabapentin in Pharmaceutical Preparations by HPLC with 4-Fluoro-7-Nitrobenzofurazan Derivatization and Fluorescence Detection

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## Abstract

**A sensitive HPLC method with pre-column fluorescence derivatization using 4-Fluoro-7-Nitrobenzofurazan (NBD-F) has been developed for the determination of gabapentin in pharmaceutical preparations. The method is based on the derivatization of gabapentin with (NBD-F) in borate buffer of pH 9.5 to yield a yellow, fluorescent product. The HPLC separation was achieved on a Inertsil $C_{18}$ column (250 mm × 4.6 mm) using a mobile phase of methanol–water (80:20, v/v) solvent system at 1.2 mL/min flow rate. Mexiletine was used as the internal standard. The fluorometric detector was operated at 458 nm (excitation) and 521 nm (emission). The assay was linear over the concentration range of 5–50 ng/mL. The method was validated for specificity, linearity, limit of detection, limit of quantification, precision, accuracy, robustness. Moreover, the method was found to be sensitive with a low limit of detection (0.85 ng/mL) and limit of quantitation (2.55 ng/mL). The results of the developed procedure for gabapentin content in capsules were compared with those by the official method (USP 32). Statistical analysis by *t*- and *F*-tests, showed no significant difference at 95% confidence level between the two proposed methods.**

## Introduction

Gabapentin (GB) [1-(aminomethyl)cyclohexaneacetic acid] is a γ-aminobutyric acid (GABA) analog used for treatment of partial seizures in adults and children (1).

It has also been shown to be effective for neuropathic pain (2). It has a milder side effect profile when compared with older generation anti-epileptics (3) GB is structurally related to the neurotransmitter γ-aminobutyric acid (GABA). It was originally designed as a GABA-mimetic agent that freely crosses the blood-brain barrier (4). GB has been shown to increase GABA levels in the brain clinically (5).

Several methods for GB determination in biological fluids have been reported in the literature; these methods employ gas chromatography (GC) (6,7), gas chromatography-mass spectrometry (GC–MS) (8–10), high-performance liquid chromatography (HPLC) (11–23), liquid chromatography-mass spectrometry (LC–MS) (24–27), and capillary electrophoresis (CE) (28). A few published methods have been developed for the determination of GB in bulk or pharmaceutical dosage forms which include spectrofluorometry (29), spectrofluorimery and spectrophotometry (30), flow analysis (31), CE (32), and HPLC (33).

In this study, a sensitive HPLC method for the assay of GB in capsules by means of the derivative formed with NBD-F, which is a specific reagent in the analysis of primary and secondary aliphatic amines. This reagent has been proposed as a replacement for chloro analog, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), as the former is 50–100 times more reactive as a fluorogenic reagent for amines. NBD-F is self fluorescent. It has good detection limit for the secondary and primary amines (34). NBD-F was chosen as the fluorogenic reagent for GB an attempt to develop an alternative method that is sensitive and simple enough for the determination of the drug in dosage form and in biological fluids.

A new fluorimetric method, which has high reproducibility and highly sensitivity has been developed for the determination of GB in capsules. In literature research, GB, for the first time has been derivatized by a reagent and has been determined by using a fluorescent system with a 0.85 ng/mL limit of detection (LOD) and a 2.58 min run time.

## Experimental

### Materials and reagents

GB was kindly supplied by Pfizer (Istanbul, Turkey). Pharmaceutical preparations Neurontin capsules (100 mg) were purchased from a local pharmacy. Mexiletine (IS) was supplied from Sigma (St. Louis, MO). NBD-F was purchased from Fluka (Buchs, Switzerland). All solvents were of analytical grade. Ultra pure water was prepared by using aquaMAX ultra, Young Instrument (Anyang, South Korea) ultra water purification.

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**Instrumentation**

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, LC-10AT pump, with an SIL-20AHT autosampler with a 5 µL loop, RF-10AXL fluorescence detector, and CTO-10AS column oven. The analytical column was a Inertsil $C_{18}$ column (Tokyo, Japan) (150 mm × 4.6 mm i.d., 5 µm) with a guard column (Tokyo, Japan) (4 mm × 3 mm i.d., Inertsil) packed with the same material. The mobile phase was methanol–water 80:20 (v/v); the flow rate of 1.2 mL/min. The fluorescent detector was set at 458 and 521 nm for the excitation and emission wavelength, respectively.

**Solutions**

Stock solution of GB was prepared by dissolving accurately weighed 100 mg of the drug in 100 mL of water (final concentration, 1 mg/mL). This solution was diluted with water to give standard solution of 1.0 µg/mL. Mexiletine (internal standard) stock solution was made at an initial concentration of 1 mg/mL. The internal standard (IS) stock solution was diluted with methanol to a final concentration of 1.0 µg/mL. NBD-F was prepared as a 0.02% (w/v) solution of methanol. A borate buffer (0.10 M) was prepared by dissolving 0.62 g of boric acid and 0.75 g of potassium chloride in 100 mL water. The pH was adjusted to 9.5 with 0.10 M sodium hydroxide solution and the volume was made up to 200 mL with water. All the solutions were stored at 4°C and protected from light.

*Sample solution*

Twenty GB capsules were finely powdered and weighed. An accurately weighed quantity of the mixed powder containing an equivalent of 100 mg of GB was dissolved in water. The solution was then sonicated in an ultrasonic bath for 30 min, filtered through a 0.45 µm syringe filter and diluted to 100 mL with water. The working sample solution (1.0 µg/mL) obtained by dilution of supernatant was used to set up the concentrations in the range of calibration studies.

**Assay procedure and derivatization**

To a set of 12 mL volumetric flasks, increasing volumes from the standard solution of the GB were quantitatively transferred so as to contain the drug within the concentration range 5–50 ng/mL. Next, 50 µL IS and 100 µL borate buffer pH 9.5 and 25 µL NBD-F solutions were added, and the reaction mixture was kept at 70°C for 30 min in a water bath. The mixture was cooled and acidified with 100 µL of 0.1 N HCl. The derivative was extracted with 2 × 2.5 mL ethyl acetate, and the organic layer was transferred to a tube. The organic phases were dried on anhydrous sodium sulfate. A 4.5 mL aliquot of the extract was evaporated under nitrogen at 45°C. The residue was then dissolved in 1.0 mL of the mobile phase. Typically, 5 µL aliquots of this solution are used for determination by HPLC.

**Validation**

The validity of the method was tested regarding; linearity, limit of detection, limit of quantification, precision, accuracy, recovery, and robustness according to ICH Q2B recommendations (35).

*Linearity*

The regression plots showed that there was a linear dependence of the peak area ratio value on the concentration of the drug over the range of 5–50 ng/mL. The peak area ratio of the drug to the IS was considered for plotting the linearity graph. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

*LOD and LOQ*

Limits of detection (LOD) and quantification (LOQ) were estimated from the signal-to-noise ratio. The limit of detection was calculated by LOD = 3.3r/S, where r is the standard deviation of the response of the blank and S is the slope of the calibration curve. The limit of quantification was calculated by LOQ = 10r/S under the ICH guidelines (35).

*Precision and accuracy*

As a part of determining accuracy of the proposed methods, different levels of GB concentrations were prepared from independent stock solution and analyzed ($n$ = 6). Accuracy was assessed as the relative mean error (RME). Precision was expressed as the relative standard deviation (RSD%).

*Recovery*

The % recovery of the added pure drug was calculated as, % recovery = [($C$t – $C$s)/$C$a] × 100, where $C$t is the total drug concentration measured after standard addition, $C$s the drug concentration in the formulation sample and $C$a is the drug concentration added to formulation.

*Specificity*

In the formulation samples of GB, potential excipients (microcrystalline cellulose, magnesium stearate, hydroxypropylmethylcellulose, and titanium dioxide) were checked to make sure they did not interfere with the peak of GB and IS.

*Robustness*

The robustness of the method was evaluated during the development by making small but deliberate changes to the method parameters. The factors chosen for this study were the flow (mL/min), mobile phase composition, and wavelengths.

*System suitability*

Resolution ($R_S$), number of theoretical plates (N), capacity factor (k), and tailing factor (T), were measured as criteria for system-suitability testing. According to ICH guidelines, recommended values of these properties for system suitability are $R_S$ > 2, N > 2000, k > 2.0 and T ≤ 2 (35).

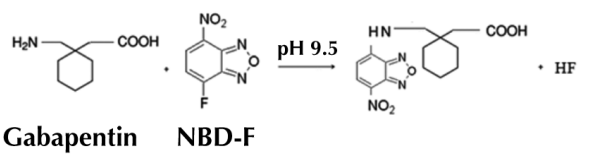


**Figure 1.** The reaction between gabapentin and NBD-F.

## Results and Discussion

### Pre-column derivatization

The reaction between GB and IS with NBD-F in borate buffer of pH 9.5 produces a yellow colored product with maximum fluorescence at 521 nm (Figure 1).

GB-NBD produced a highly fluorescent derivative with excitation and emission maxima of 458 and 521 nm, respectively. The different experimental parameters affecting the intensity of the color derivatized were studied and optimized to obtain maximum color intensity. The pH was varied over the pH range of 7–10 using borate buffer where the maximum fluorescence was obtained at pH 9.5 NBD-F is hydrolyzed in alkaline medium. Therefore, it was necessary to acidify the reaction mixture to pH 2 (by adding 100 µL 0.1 N HCl) before the measurement was carried out. The influence of different heating temperatures and times was studied using a water bath. Effect of heating time at different temperatures 50–80°C for NBD derivatives. The best results were obtained at 70°C within 30 min.

### Conditions of chromatography

This isocratic-mode method with fluorescence detection was developed for the determination of GB. To develop a rugged and suitable HPLC method for the quantitative determination of GB, different mobile phases were employed. The mobile phase consisted of methanol–water at various ratios (75:25, 80:20, 85:15, 90:10, v/v) was tested as starting solvent. The variation at the mobile phase leads to considerable changes in the chromatographic parameters. However, the proportion methanol–water at a ratio of 80:20 (v/v) at a flow rate of 1.2 mL/min yielded the best results. On the other hand, the mobile phase in the proposed method methanol–water instead of buffered systems used in previously reported HPLC methods (11,13). Therefore flushing of the column after analysis is not required. GB and the IS were well resolved with good symmetry with respective retention times of 2.58 and 3.60 min (Figure 2).

The method uses a simple mobile phase composition, easy to prepare with little or no variation. Besides, according to the other methods the retention time is quite short (13). It is a highly specific and precise analytical procedure and its chromatographic run time of 2.58 min allows the analysis of a large number of samples in a short period of time.

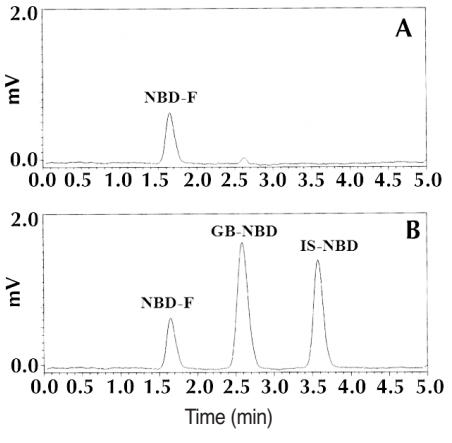


**Figure 2.** Chromatogram of blank solution (A) and 10 ng/mL GB-NBD derivative (B).

### Method validation

*Specificity*

Because analytical problems can be caused by interfering materials, studies were performed to investigate the effect of the other ingredients in the pharmaceutical formulations. It was found that the proposed method can be used for determination of GB in a variety of pharmaceutical preparations without analytical problems. None of the excipients present in the capsules interfered with analysis of the drug.

*Linearity*

Calibration plots obtained by plotting GB-to-IS peak-area ratio against drug concentration were linear over the range 5–50 ng/mL. As can be seen from the data, the method is much more sensitive than most of the reported methods (30,32). The regression equation of the calibration plot for GB was $A = 7.76 \times 10^{-2}c - 3.5 \times 10^{-3}$, where $A$ is the peak-area ratio and $c$ the concentration.

*LOD and LOQ*

Under these HPLC conditions the LOD was 0.85 ng/mL and the LOQ was 2.55 ng/mL, indicating the sensitivity of the method is good. The results compare favorably with others reported in the literature (30,32). As spectrofluorometry is among the most sensitive methods of analysis, it has been chosen for developing a method of analysis of GB. This drug contains an amino group, which makes it a suitable candidate for derivatization by fluorogenic reagent such as NBD-F. This reagent has been proposed as a replacement for chloro analog, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), as the former is 50–100 times more reactive as a fluorogenic reagent for amines (34). NBD-F was chosen as the fluorogenic reagent for GB an attempt to develop an alternative method that is sensitive and simple enough for the determination of the drug in dosage form and in biological fluids. Optical characteristics and statistical data are given in Table I.

*Precision and accuracy*

The method indicated very good precision and accuracy. Intra- and inter-day precision and accuracy for GB data are shown in Table II. The intra- and inter day precision and accuracy were measured to be between 0.09–0.98 and –0.1–2.0%.

**Table I. Results of Regression Analysis of the Linearity Data of GB-NBD Derivative ($n$ = 6)**

| | Mean ± SD* | SE* |
|---|---|---|
| Slope | $0.0776 \pm 7.1 \times 10^{-2}$ | $2.9 \times 10^{-2}$ |
| Intercept | $0.0035 \pm 2.0 \times 10^{-2}$ | $8.1 \times 10^{-3}$ |
| Correlation coefficient (r) | $0.9997 \pm 1.5 \times 10^{-4}$ | |
| LOD | 0.85 | |
| LOQ | 2.55 | |

* Standard deviation: SD; standard error: SE.

**Table II. Intra- and inter-day precision and accuracy of GB-NBD derivative (*n* = 6)***

| | Intra-day | | | Inter-day | | |
|---|---|---|---|---|---|---|
| Conc. added (ng/mL) | Conc. found (mean ± SD ng/mL) | Precision (RSD%) | Accuracy (RME%) | Conc. found (mean ± SD ng/mL) | Precision (RSD%) | Accuracy (RME) |
| 5.0 | 5.10 ± 0.05 | 0.98 | +2.00 | 4.98 ± 0.02 | 0.40 | –0.4 |
| 30.0 | 29.97 ± 0.03 | 0.10 | -0.10 | 29.95 ± 0.05 | 0.16 | –0.16 |
| 50.0 | 50.05 ± 0.05 | 0.09 | +0.1 | 50.15 ± 0.15 | 0.29 | +0.3 |

* RSD: relative standard, RME: relative mean error, SD: standard deviation.

**Table III. Recovery of GB-NBD Samples (*n* = 5)**

| Conc. of drug in formulations (ng/mL) | Conc. of pure drug added (ng/mL) | % Analytical drug found (ng/mL) | Recovery (± SD) |
|---|---|---|---|
| 5.0 | 5.0 | 9.98 | 99. 98 ± 0.52 |
| 5.0 | 25.0 | 30.26 | 101.90 ± 0.34 |
| 5.0 | 45.0 | 50.27 | 99.97 ± 0.48 |

**Table IV. Experimental Data for Robustness Testing and Obtained Responses (*n* = 6)**

| Parameter | Modification | % Recovery |
|---|---|---|
| Mobile phase ratio (v/v) | 85:15 (Optimum) | 99.95 |
| | 90:10 | 99.54 |
| | 80:20 | 99.76 |
| Flow rate (mL/min) | 1.2 (Optimum) | 99.95 |
| | 1.3 | 99.09 |
| | 1.1 | 99.85 |
| Wavelength | 458 nm, 521 nm (Optimum) | 99.95 |
| (λex, λem nm) | 457 nm, 520 nm | 99.48 |
| | 459 nm, 522 nm | 99.23 |

**Table V. System Suitability Data of HPLC Method**

| Parameters | GB-NBD | IS-NBD |
|---|---|---|
| Retention time ($t_R$) | 2.58 | 3.60 |
| Capacity factor (k′) | 2.423 | 2.852 |
| Tailing factor (T) | 1.049 | 1.017 |
| Resolution ($R_s$) | 2.664 | 2.773 |
| Theoretical plates N | 3545.340 | 4258.300 |

**Table VI. Assay of Gabapentin in Capsule Formulations***

| Drug | | Proposed method | Official method (36) |
|---|---|---|---|
| Neurontin (100 mg) | % Recovery ± SD | 100.28 ± 0.51 | 101.02 ± 0.91 |
| | t | 1.60 | |
| | F | 3.18 | |

* *n* = 6, standard F-values at 95% confidence level = 5.05; standard t-values at 95% confidence level = 2.23.

*Recovery*

The results showed that (Table III) the recoveries were in the range of 99.97–101.90 %.

*Robustness*

The robustness of a method is its resilience to minor changes in the analytical conditions, for example mobile phase composition, flow rate and wavelengths. From the results listed in Table IV it can be concluded that this HPLC method is robust, because slight variation of these experimental conditions have little or no effect on the results.

*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: $R_S > 2, N > 2000, k' > 2.0$, and $T \leq 2$. The values obtained for this method were within the acceptable ranges (35) (Table V).

*Method application*

GB capsules were subjected to analysis by the proposed method and the official method (36). Mean percentage recovery, relative to the label claims, obtained by use of the proposed method ranged from 101.02% to 100.28% (Table VI). *t* and *F*-tests showed there were no significant differences, at the 95% confidence level, between calculated and theoretical values for both the proposed and the official methods.

## Conclusion

A new highly sensitive, quick, analytical method has been developed to be applied in the analysis of commercially available dosage forms. Compared with the existing methods, the described method exhibits some remarkable advantages of the derivatization procedure, short analysis time, low derivatization concentration limit of detection and low consumption of sample and reagent. In this study, derivatization and extraction processes do not take much time; additionally, short retention time is an advantage (13). On the other hand, in the mobile phase in the proposed method consists of methanol–water instead of buffered systems was used in previously reported HPLC methods (13). Therefore flushing of the column after analysis is not required. It is not probable for the column to get blocked and dirty. Hence, this HPLC method can be used for the routine drug analysis.

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