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Validated HPLC Methodology for Pregabalin Quantification in Human Urine Using 1-Fluoro-2,4-dinitrobenzene Derivatization

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Pregabalin (PGB) is a synthetic drug used for the treatment of central nervous system disorders and neuropathic pain. PGB is metabolized to N-Methyl pregabalin while the rest is excreted virtually unchanged in the urine. Numerous analytical techniques for measuring pregabalin have been documented. This study aimed to validate a simple, sensitive, and accurate method for PGB quantification in human urine using the HPLC technique with 1-Fluoro-2, 4-dinitrobenzene used as a derivatizing agent. One hundred and twenty urine samples were analyzed by a reversed-phase (C18) column and a mixture of acetonitrile and 50 mM KH2PO4 (pH 2.5) (60:40, v/v) as mobile phase and the flow rate was 1 ml/min and the UV detector wavelength was set to 360nm. The procedure was linear within the 10-1000 µg/ml range of PGB in urine ($r > 0.99$). Intraday and interday RSD precision values fell between 2.8% and 5.9%. 2.5 and 1.5 µg/ml, respectively, were determined to be the method's limits of quantification and detection. The recovery (90.8%) and statistical characteristics show that the suggested method has excellent accuracy and precision. The method is accurate, precise, reproducible, and specific, and it can be applied to regular examinations of pregabalin in urine samples.

Keywords: Pregabalin, 1-Fluoro-2,4-dinitrobenzene, derivatization, validation, HPLC analysis

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Introduction

Pregabalin (PGB) can cause an euphoric state like that of addictive substances such as benzodiazepines [1-3]. In 2004, the FDA (US Food and Drug Administration) permitted PGB for the treatment of central nervous system disorders and neuropathic pain [4-6]. In Egypt, pregabalin has been added to Schedule D controlled substances since 2019.

Pregabalin (figure 1) is lipophilic as it dissolves in both basic and acidic aqueous solutions in addition to water. In the central nervous system, PGB decrease dopamine, glutamate, substance P, noradrenaline, and serotonin excretion. It is a non-opioid analgesic used to treat pathological disorders like neuropathic pain, partial seizures, and generalized anxiety disorder [4, 7].

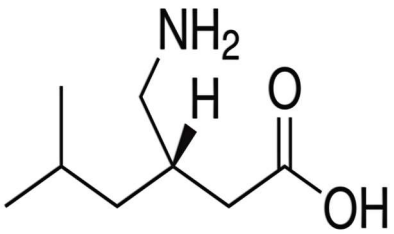

Figure 1. Pregabalin structure

Pregabalin has a 6.3-hour elimination half-life, and just 2% of it is converted to N-methyl pregabalin while the rest is excreted virtually unchanged in the urine [8]. Pregabalin is increasingly being reported as possessing a potential for abuse and misuse [9, 10].

Pregabalin levels have been measured using a variety of analytical techniques, such as tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS) [11-13], gas chromatography-mass spectrometry (GC–MS) [14, 15], GC [16], high-performance liquid chromatography (HPLC) [17, 18], and capillary electrophoresis [19].

Even though the majority of LC-MS-MS techniques are sensitive and trustworthy, most clinical laboratories cannot afford the apparatus. Moreover, the primary analytical issues with LC-MS techniques are carry-over and ion suppression effects, which make routine use of these techniques inappropriate [20, 21].

HPLC and LC-MS-MS systems have been utilized for the detection of PGB in biological fluids [22]. Derivatization with a chromophore has been employed in most reported HPLC methods for PGB determination because PGB has insignificant UV, visible, or fluorescence absorption. In earlier studies, many derivatization reagents have been used, including picryl sulfonic acid, O-phthaldialdehyde, and fluoresce amine with fluorescence detection [23-24]. Also, there have been reports of spectrofluorimetric and spectrophotometric techniques for determining PGB in pharmaceutical dose forms [25]. Straightforward and proven procedures are needed to determine different drugs in biological fluids. Pregabalin is determined in the current investigation using an HPLC technique with UV detection following derivatization with FDNB (1-fluoro-2,4-dinitrobenzene). FDNB has already been applied to

determine a number of other medications with success [17].
The objective of this study was to validate and develop a robust, efficient, and sensitive HPLC analytical technique for the detection and quantification of PGB in human urine.

Experimental section

Ethics Considerations

This study was carried out at the Poison Control Center of Ain Shams University Hospitals, Cairo, Egypt (PCC-ASUH). The study complied with the ARRIVE guidelines and was approved by the Institutional ethical committee of the Faculty of Medicine, Ain Sham University before the start of the study (No: FMASU R347/2023). Patients were also assigned the written informed consent form.

Subjects and specimens

The study included 120 patients (76 males and 44 females) who presented to the PCC-ASUH during the period from December 2023 to March 2024, complaining of disturbed consciousness level, and a history of pregabalin intake. Patients' urine samples (10 ml each) were taken in clean, dry test tubes with labels at admission and before starting any treatment. All specimens were tested by Right Sign rapid test cassette for PGB. Specimens that confirmed positive for pregabalin were assembled into the study. The samples were preserved at -18ºC until the time of analysis.

Chemicals

Pregabalin and amlodipine were bought from LGC GmbH, Biotechnologiepark, Germany. FDNB, the derivatizing reagent, was sourced from Fluka, Switzerland. Merck supplied HPLC-grade acetonitrile (Darmstadt, Germany). Sigma-Aldrich supplied all other analytically grade compounds.

Instrumentation

A Yang Lee 9100 HPLC System equipped with a 9110 Quaternary Pump, 9101 Vacuum Degasser, and 9160 PDA Detector, Clarity Chromatography data system software was used for data processing.

Chromatographic Conditions

A reversed-phase C18 Agilent (5 µm) ODS column, 4.6 mm × 150 mm, at ambient temperature was used for chromatographic separation. The mobile phase was prepared by acetonitrile and 50 mM KH2PO4 (60:40 v/v) at a flow rate of 1.0 mL/min. The final pH of the mobile phase was adjusted to 2.5 with 0.01 M orthophosphoric acid, prepared daily. The UV detector was set to 360 nm. All separations were performed at room temperature with detection at 360 nm.

Preparation of solutions

Standard solutions Preparation: To prepare PGB stock standard solution, 0.5 g of drugs was dissolved in 10 mL of methanol. Working standard solutions for pregabalin were prepared in methanol producing 0.25, 0.5, 1.25, 2.5, 5, 12.5, and 25 mg/ml. Internal standard (IS) amlodipine besylate was prepared at a concentration of 200 µg/mL in water. Up until they were used, all stock and operating standard solutions were kept at 8°C. 1.14 mg of the FNDB reagent was dissolved in 100 mL of acetonitrile to create FNDB 0.06 M (FNDP should be handled cautiously since it is a skin irritant). Borate buffer solution 0.25 M at pH 8.2 was prepared by dissolving 1.97 g of potassium chloride and 1.545 g of boric acid in 90 ml of water. The pH was adjusted to 8.2 by adding 2M NaOH and the volume was completed to 100 ml with H_2O.

Calibration curve

A cleaned-up urine-free drug (0.5 ml) was used to prepare calibrators by spiking 20 µl from the above-mentioned PGB working standards to prepare ultimate concentrations comparable to 10, 20, 50, 100, 200, 500, and 1000 ug/ml. Similar dilutions were done using ultrapure water. Five replicates of the calibrators were made, and the outcomes were used to calculate the calibration equation. In each run, a urine blank sample was also analyzed.

Derivatization procedure

50 µl of IS was added to 500 µl of calibrators, samples, or working standard solutions for pregabalin then mixed well. All test tubes were filled with 500 µL of borate buffer, 100 µL of FDNB reagent, and 2 mL of acetonitrile. The tubes were held at 65ºC for 45 minutes after being vortexed for 30 seconds. Following cooling, each tube received 75 µL of 1.0 M HCl solution, which was then thoroughly vortexed, filtered, and 20 µL of the filtrate was injected to the HPLC apparatus.

Validation of the method

The method that was developed has undergone a comprehensive validation process to ensure its accuracy and reliability. This validation included assessments of linearity, specificity, detection limit, quantification limit, carryover effect, accuracy, and precision. The validation was conducted by following the guidelines made by the Scientific Working Group for Forensic Toxicology [26].

Accuracy and precision

Intraday and inter-day assay precision was determined using six replicates of 5 derivatized working standard levels (20, 50, 100, 500, and 1000 µg/ml), expressed as relative standard deviation (RSD). Six separate analyses were achieved at each concentration level within one day (6 runs per day) and six successive days (6 runs per day within 6 continuous days). Accuracy was determined for

six days and expressed as the percent deviation, calculated according to the following formula:

$$\%\text{Accuracy} = \frac{(\text{Calculated conc. of analyte} - \text{Known conc.})}{\text{Calculated Conc. of analyte}} \times 100$$

Recovery

The extraction recovery (n=5) was calculated by comparing the peak area gained from prepared derivatized spiked samples with those found by the direct injection of drug-derivatized standard solution at the same concentration, and calculated according to the following formula:

$$\text{Recovery \%} = \frac{\text{Measured amount of analyte after derivatization}}{\text{Spiked amount of analyte}} \times 100$$

Linearity

Method linearity was assessed on six calibration curves (seven calibration points from 10 to 1000 ug/ml), three of which were carried out on the same day and the remaining three on 3 different days. The linearity was evaluated by linear regression analysis.

Specificity

Several drugs containing the primary amine group (ephedrine, amitriptyline, imipramine, and clomipramine) were added to blank urine and were analyzed according to the current method for specificity validation.

Sensitivity

Calculating the limit of detection (LOD) and limit of quantification (LOQ), the method's sensitivity was evaluated. Finding the concentration of a diluted reference solution at which the signal-to-noise ratio was about 3 or 10, respectively, allowed for the calculation of the LOD and LOQ.

Stability studies

The stability of pregabalin standards and calibrators was determined by measuring concentration change in the control sample over time and was evaluated with the help of HPLC systems. The stability studies were assessed using two fortified urine samples (low 100 ug/ml and high 1000 ug/ml concentrations of PGB). These strengthened samples were initially analyzed in triplicate to establish time-zero responses (peak area). The average time-zero response for each set of samples was compared to the average signals from each of the following stability studies. The analyte will be considered stable until the average signal compared to the time-zero average signal falls outside of the method's acceptable accuracy. The urine control samples after derivatization were analyzed immediately after derivatization and reinjected and analyzed after 24, 48, and 72 hours.

Robustness

The robustness of the method relative to each operational parameter was checked and investigated. The influences of small changes in the mobile phase composition and buffer pH were studied to determine the robustness of the method, such as the changes in peak area and retention time.

Carryover

To estimate any carryover that could have happened during the test run, a blank was injected immediately following a pregabalin concentration with a high concentration (1000 ug/ml). The injection sequence was as follows: pre-standard blank (B1), standard (1000 ug/ml), and post-standard blank (B2). Carryover is then calculated by:

$$\text{Carryover \%} = \frac{(B_2 \text{ peak area} - B_1 \text{ peak area})}{\text{Standard peak area}} \times 100$$

Statistical analysis

The Microsoft Excel 2010 application was used to compute the mean and standard deviation to fulfill validation calculations in compliance with the standards provided by the Scientific Working Group for Forensic Toxicology (2013) [26].

Results and Discussion

Our first trials to detect PGB in urine samples were carried out on gas chromatography using ethyl chloroformate as a derivatization agent [27]. The results were not satisfactory owing to the lack of sensitivity.

In the current study, PGB was identified in 105 specimens obtained from 120 intoxicated patients using a drug test dip card during the period study. While 97 specimens were positive using the HPLC present method (92.3% prevalence). The median and range (mean) concentration of PGB in samples were 265.4 (2.8–2457) and 315.0 µg/ml, respectively. The PGB and IS retention times were 2.46 and 7.37 min, respectively with a relative retention of 2.99 as shown in Figure 2.

Chromatographic conditions

A number of chromatographic conditions were tested by checking different columns, mobile systems, and different UV wavelengths. Many trials did not provide an appropriate peak shape. The optimized conditions are:- C18 Agilent (5 µm) ODS column, 4.6 mm × 150 mm, a mobile phase composition of acetonitrile and 50 mM KH2PO4 (pH 2.5) (60:40, v/v), flow rate of 1.0 ml/ min and UV detection at 360 nm.

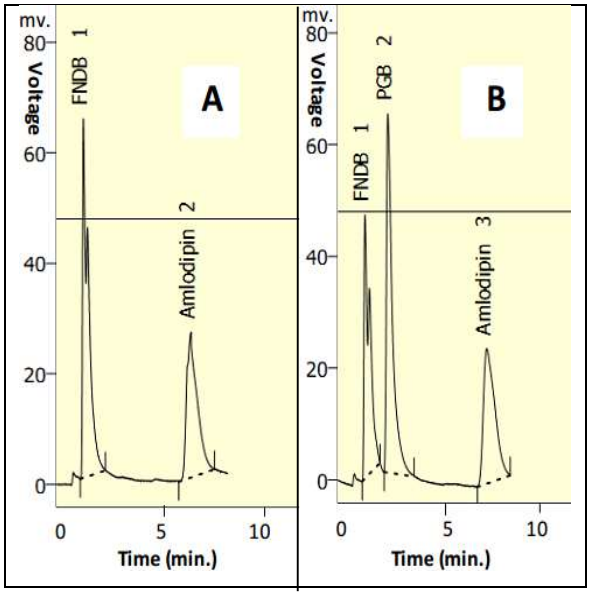

Figure 2. HPLC chromatograms of PGB using FDNB as derivatizing agent and amlodipine as internal standard; A) Blank sample chromatogram showing internal standard and FDNB peaks; B) Spiked sample chromatogram showing PGB and internal standard in addition to the FDNB peaks.

Derivatization reaction

O-phthaldialdehyde, mercaptopropionic acid, and picrylsulfonic acid were utilized in the majority of published procedures [17, 24, 28] to obtain a more accurate result. Regrettably, the majority of these techniques is laborious, intricate, and has poor recovery rates. As far as we are aware, there is just one study [4] on the PGB in human, urine which was measured using HPLC without derivatization. Nevertheless, the sensitivity of this approach was not suitable for routine work.

Using borate buffer (pH 8.2), FDNB can react with PGB (primary amine) in a mildly basic media. A highly absorbing UV derivative that can be detected with a reasonable sensitivity is formed as a result of a nucleophilic substitution process [29, 30]. However, in an acidic media, the reaction's colorful byproduct becomes an uncolored compound when HCL is added, producing a clearer chromatogram.

Numerous experimental factors influencing the derivatization process were examined; they were adjusted at various time intervals and evaluated at varying temperatures to determine the optimal reaction conditions. For 45 minutes at 65° C, the ideal reaction conditions were attained. The reaction rate was sluggish at lower temperatures, and solvent evaporation occurred at greater concentrations. It appeared that the reaction was finished with 100µL of reagent after raising the volume of the reagent from 25µL to 200µL. The peak area was unaffected by higher reagent quantities.

Validation of the method

The current linearity approach was examined by building six calibration curves with the typical process for sample preparation. In the 10-1000 µg/ml range, the data demonstrated strong linearity with an acceptable coefficient of correlation (r>0.99) (Table 1. and Figure 3.) Intraday and interday precision of procedure data are obtained in (Table 2.) with recorded RSD <5.9% and <5.8%, respectively, while the accuracy range was 2.5-5.5%, indicating adequate accuracy and precision of the experiment. The mean recovery (n = 5) was 90.1%. LOQ and LOD were found to be 2.5 µg/ml (RSD <9.7%) and 1.5 µg/ml (RSD <11.3%), respectively (Table 2).

The suggested method's specificity was examined with the presence of many drugs containing the primary amine group (ephedrine, amitriptyline, imipramine, and clomipramine) none of the tested drugs interfered with the PGB assay. On the other hand, no significant carryover (0.12%) of PGB at 1000 ug/ml concentration to the blank urine was detected.

Table 1. Summary of LOQ, LOD and regression characteristics of the proposed method (n= 6 replica, 6 times).

Parameters	Results
Linearity range	10–1000 µg/ml
Regression equation	Y=16.486X–284.23
Correlation coefficient (r)	>0.99
Carryover	0.12 %
LOD	1.5 µg/ml
LOQ	2.5 µg/ml

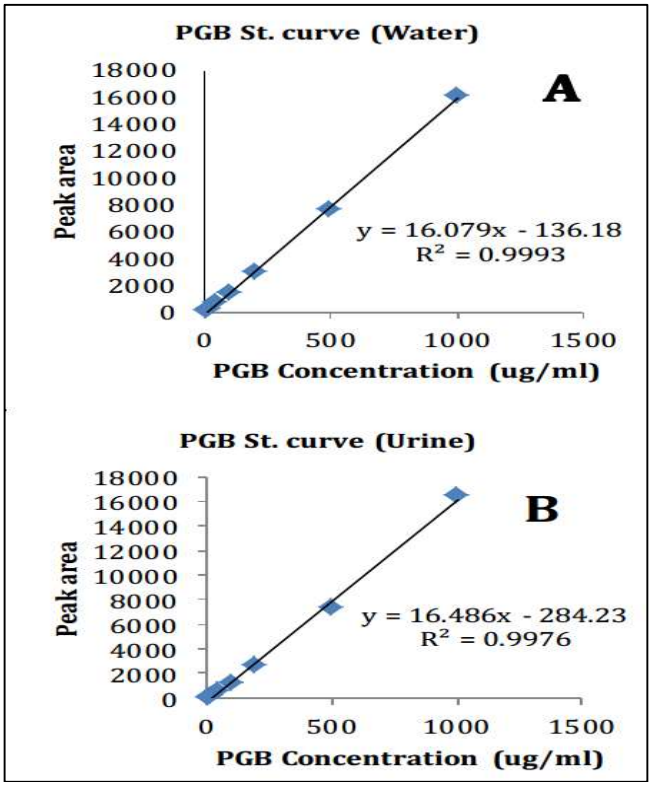

Figure 3. Calibration curves of PGB in water (A) and urine free drug (B).

Table 2. Recovery, Precision and accuracy of method for determination of pregabalin in standard solutions.

Conc.	Mean ± SD	Recovery%(n=5)	Intra-day (n=6)		Inter-day (n=6)	
µg/ml	µg/ml		Precision%	Accuracy%	Precision%	Accuracy%
50	44.65± 1.74	89.3	3.9	2	2.9	-4
100	90.9± 3.99	90.9	4.4	-2.3	2.8	5
200	187.2 ± 9.17	93.6	4.9	1.8	5.8	3.5
500	437.5 ± 25.81	87.5	5.9	-1.25	4.9	0.9
1000	892 ± 36.57	89.2	4.1	2.5	5.5	2.6

Stability

The stock standard solutions and calibrators of PGB when kept at 4ºC demonstrated that for at least two months, the solutions were comparatively stable. The derivatization product remained steady for 24 hours when compared with the method's accuracy of ± 5.0%.

Robustness

It was investigated how minor changes to the optimal technique parameters affected the chromatographic parameters. The peak area values (CV < 1.2%) were not significantly affected by ten percent variations in the pH of the buffer or the mobile phase's composition. Changes in the composition of the mobile phase resulted in notable fluctuations in the retention time but did not affect the method's sensitivity or selectivity.

Conclusions

Compared to the other documented methods, the present method is simpler, cost-effective, fast, and efficient. Also, LOD and LOQ are comparable with other methods. This approach is appropriate for high-throughput analysis since it requires less than 60 minutes for the whole analysis (including sample preparation and instrument run time). Consequently, it can be applied to a great number of samples in a relatively short time without extraction steps. This technique may be applied to the quantitative determination of PGB in urine samples as well as regular screening. The suggested method's high accuracy and precision are demonstrated by the statistical parameter and recovery data.

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Conflict of interest

The authors declare no conflicts of interest

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