International Journal of Analytical Chemistry

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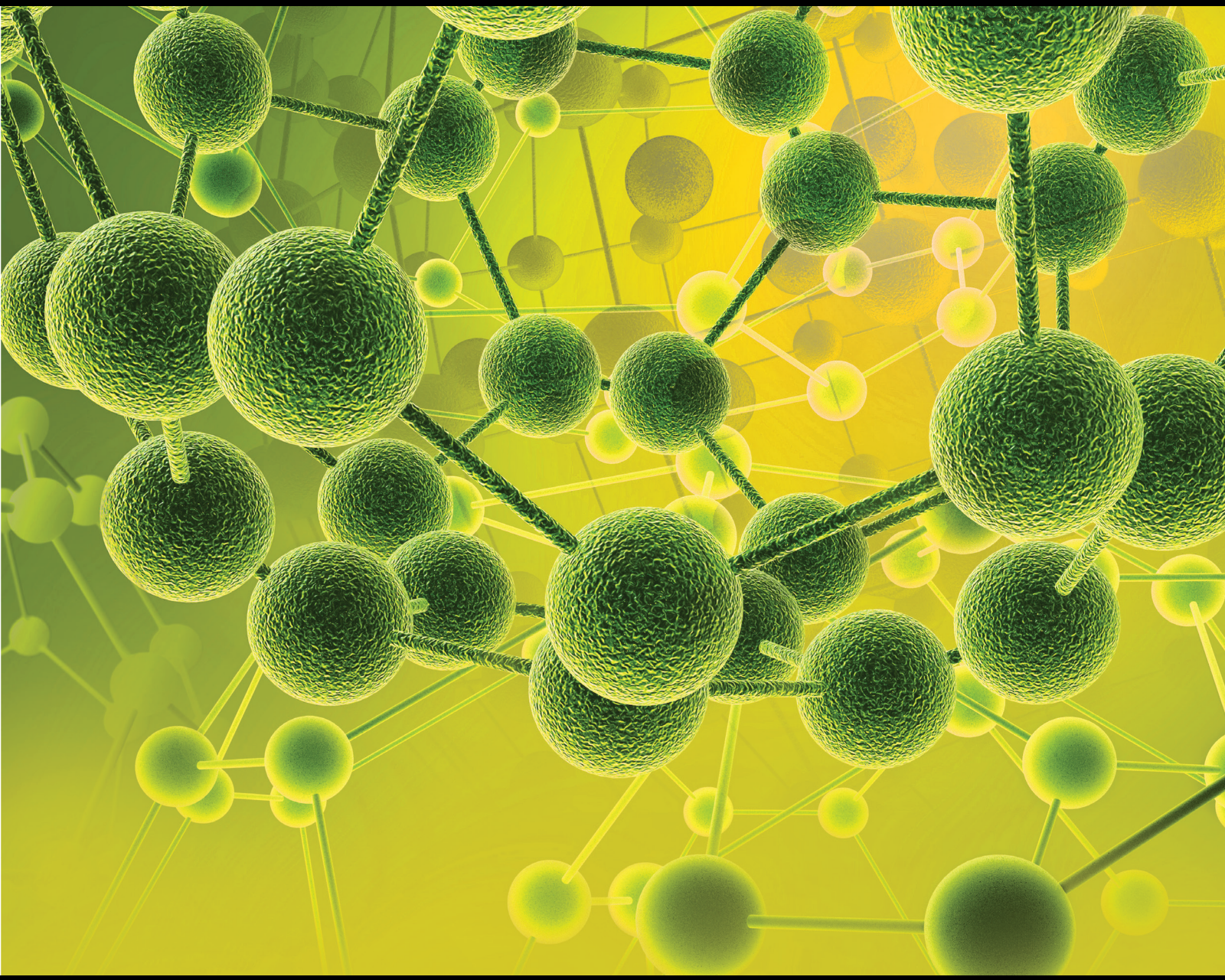

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# Chief Editor



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*Research Article*

# A Fast and Validated HPLC Method for the Simultaneous Analysis of Five 5-$HT_3$ Receptor Antagonists via the Quantitative Analysis of Multicomponents by a Single Marker

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In this study, a new strategy for the simultaneous quantization of five serotonin 5-hydroxytryptamine receptor antagonists—ondansetron, azasetron, ramosetron, granisetron, and tropisetron—either in infusion samples or in injection dosage form was first established based on high-performance liquid chromatography combined with a quantitative analysis of multiple components by a single marker. The quantitative analysis of multicomponents by a single marker method was conducted with ondansetron as an internal reference substance and performed using relative retention time and ultraviolet spectral similarity as the double indicator. The quantitative analysis of the 5-$HT_3$ receptor antagonists was calculated and investigated based on the relative correction factors. Chromatographic separation was achieved using a $C_{18}$ column (150 mm × 4.6 mm, 5.0 $\mu$m), and the mobile phase was composed of acetonitrile-0.05 mol·$L^{-1}$ potassium dihydrogen phosphate (pH 4.0) (25 : 75) at a flow rate of 1.0 mL·$min^{-1}$ and detection wavelengths of 307 nm (ondansetron, azasetron, ramosetron), 302 nm (granisetron), and 285 nm (tropisetron). In addition, the accuracy of the quantitative analysis of multicomponents by a single marker method was compared with an external standard method, and no significant difference was observed between the two methods. The established method is rapid, is easy, and does not require many reference substances, and it can been successfully applied as part of the quality control of the five 5-$HT_3$ receptor antagonists in their injection dosage form and infusion sample drugs in hospitals.

## 1. Introduction

The effects of chemotherapy, radiation, irritable bowel syndrome, opioid analgesic drugs, anesthesia, and postoperative-induced nausea and vomiting are among the most distressful side effects in patients. These side effects of nausea and vomiting can significantly cause poor appetite, body-weight loss, decreased social skills, and more severe clinical consequences such as dehydration, electrolyte imbalances, and prolonged hospital stays [1]. The 5-hydroxytryptamine (5-$HT_3$) receptor antagonists, informally known as "setrons," are a class of antiemetic medications that inhibit the release of 5-HT and vagal 5-HT receptors in the central nervous system. Serotonin 5-$HT_3$ receptor antagonists, including ondansetron (ODT), granisetron (GNT), tropisetron (TPT), azasetron (AZT), and ramosetron (RMT), are particularly effective in the treatment and prevention of nausea and vomiting [2–6]. The chemical structures of the five 5-$HT_3$ receptor antagonists are shown in Figure 1.

At present, the rapid detection of drugs mainly involves infrared spectroscopy. However, near-infrared spectroscopy technology is only applicable to the initial screening of drugs, and a quantitative model is more complex and rare, resulting in the quantitative requirements of rapid drug detection not being met. HPLC is commonly used to verify rapid detection systems due to its attractive features, such as high peak efficiencies, great resolution, high sensitivity, good repeatability, and wide application range. HPLC analytic methods for the detection of 5-$HT_3$ receptor antagonists such as ODT, GNT, TPT, AZT, and RMT are already mature, and most of them use retention data in the qualitative analysis, while the external standard method (ESM) is

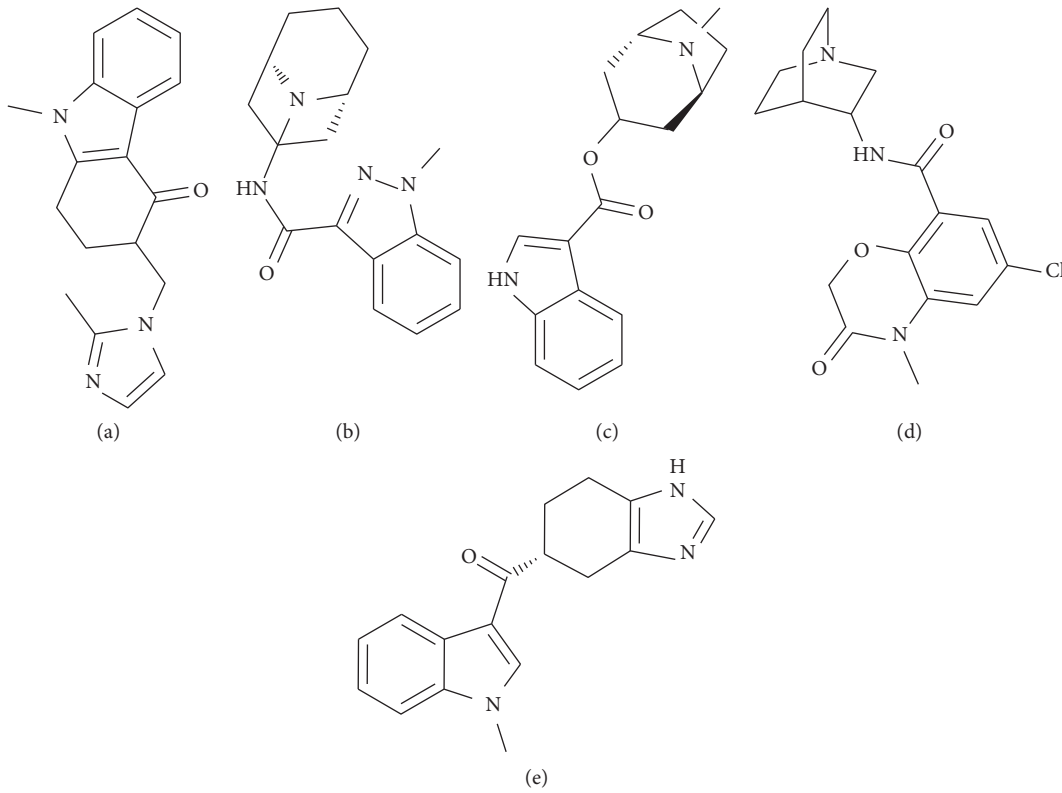


Figure 1: Chemical structure of ODT (a), GNT (b), TPT (c), AZT (d), and RMT (e).

applicable in quantitative analysis [7–24]. However, some of these methods may take a long time, and the specific drugs to be tested must correspond to reference substances, which cannot meet the requirements of rapid drug detection.

To solve the above problems, a unique quantitative analysis of multicomponents by a single marker (QAMS) analytical method was adopted in this paper. The QAMS method is able to simultaneously identify and quantify itself and the other analytes by a single reference standard, which greatly reduces the cost and analysis time of the experiment [25–32]. To date, there are no reports concentrating the QAMS or ESM method on the simultaneous quantification of ODT, GNT, TPT, AZT, and RMT in infusion samples and in injection dosage form. Thus, the purpose of this study was to establish a rapid HPLC-QAMS method for the detection of five 5-$HT_3$ receptor antagonists by using two indexes of relative retention time and UV spectrum similarity for qualitative analysis and a relative correction factor for quantitative analysis. This method was successfully employed for the routine quality control of 5-$HT_3$ receptor antagonist injection, infusion products, and the preliminary screening of unknown drugs in hospitals.

## 2. Experimental Procedure

*2.1. Chemicals and Reagents.* Six reference substances (ODT, GNT, TPT, AZT, RMT, and urine pyrimidine) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Each of six reference substances had a purity of more than 99.5%. HPLC-grade acetonitrile and AR-grade potassium dihydrogen phosphate, phosphoric acid, and triethylamine were supplied by the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Ultrapure water was obtained from a Millipore Milli-Q system (Millipore, USA).

*2.2. Pharmaceutical Formulations.* The following dosage forms were analyzed: commercial injection of ODT hydrochloride from Qilu Pharmaceutical Ltd. (Shandong, China) claimed to contain 2 mg of ONT per mL. Commercial injection of GNT hydrochloride from Cinkate Pharmaceutical (Suzhou, China) claimed to contain 1 mg of GNT per mL. Commercial injection of TPT hydrochloride from Qilu Pharmaceutical (Shandong, China) claimed to contain 5 mg of TPT per mL. Commercial injection of AZT hydrochloride from Wanma Pharmaceutical (Zhejiang, China) was labeled to contain 5 mg of AZT per mL. Commercial injection of RMT hydrochloride from Cisen Pharmaceutical (Shandong, China) was labeled to contain 0.3 mg of RMT per mL.

*2.3. Instrumentation and Chromatographic Conditions.* Chromatographic analysis was performed on an HPLC system (Dionex, Germany) consisting of an UltiMate® 3000 quaternary pump, an autosampler, a column-heating compartment, and an ultraviolet detector (DAD). Chromatographic data were collected and analyzed using Chromeleon® 7.2 software. The simultaneous separation of five 5-$HT_3$ receptor antagonists was performed using an InertSustain $C_{18}$ column (4.6 mm × 150 mm, 5 $\mu$m) supplied

by SHIMADZU (China) Co., Ltd. The mobile phase consisted of acetonitrile-0.05 mol·L$^{-1}$ potassium dihydrogen phosphate (0.1% phosphoric acid, pH 4.0) (25 : 75, v/v) with a flow rate of 1.0 mL·min$^{-1}$ for 15 min. All determinations were carried out at ambient temperature 30°C. The detector wavelengths for ODT, GNT, TPT, AZT, and RMT were 307, 302, 285, 307, and 307 nm, respectively.

*2.4. Preparation of Standard Solutions.* Stock solutions were prepared by weighing accurately and dissolving the five 5-HT$_3$ receptor antagonists' standard references at 5.0 mg each and immersing them in ultrapure water in a 10 mL volumetric flask with a concentration of approximately 0.5 mg/mL as the reference standard. A standard stock solution of urine pyrimidine (20 $\mu$g/mL) was prepared by dissolving 2.0 mg of urine pyrimidine with ultrapure water in a 100 mL volumetric flask. Mixed 5-HT$_3$ receptor antagonist working standard solutions were prepared by appropriate dilution of the stock solutions with ultrapure water to the required concentrations for plotting the calibration curves. All standard solutions were stored at −20°C until use and sonicated for 10 min for injection.

*2.5. Method Validation.* The method was validated according to the International Conference of Harmonization (ICH) guidelines [33]. The following parameters were investigated: linearity, precision, stability, accuracy, limit of detection, and robustness. The limit of detection for each 5-HT$_3$ receptor antagonist was determined at a signal-to-noise ratio (S/N) of 3. For the limit of quantification, the ratio considered was 10 : 1 with an RSD% value less than 10% [34].

*2.5.1. Construction of Calibration Graphs.* The calibration graphs of the method were evaluated with a series of different concentrations of working standards (mixture of all five 5-HT$_3$ receptor antagonists). The concentration range was selected at six different concentrations, viz 5, 10, 20, 50, 75, and 100 $\mu$g/mL for ODT, GNT, TPT, and AZT and 0.5, 3, 6, 20, 30, and 50 $\mu$g/mL for RMT. A 20 $\mu$L aliquot of each working solution was injected in triplicate into a chromatographic system ($n = 3$). The peak areas of the five 5-HT$_3$ receptor antagonists were plotted against the corresponding concentrations of each drug to obtain the calibration curve.

*2.5.2. Precision.* For method precision, three concentration levels of mixed standard solutions (10.0, 20.0, and 50.0 $\mu$g/mL for ODT, GNT, TPT, AZT; 1.0, 3.0, and 6.0 $\mu$g/mL for RMT) were assessed in triplicate during a single day and three consecutive days. The percent relative standard deviation (% RSD) of five analytes was calculated according to the peak area of each component in a single day and on different days to obtain the intraday precision and interday precision.

*2.5.3. Stability.* The sample solutions of the five 5-HT$_3$ receptor antagonists were prepared with ultrapure water stored at room temperature and injected into HPLC at 0, 1, 2, 4, 6, and 8 h after being prepared. The stability of the sample solutions was investigated by the RSD of variation in the peak area of the five analytes.

*2.5.4. Accuracy.* The accuracy of the method was determined as the recoveries of known added amounts of five 5-HT$_3$ receptor antagonist reference substance into the previously analyzed commercial injections in triplicate using three concentration levels.

*2.5.5. Robustness Test.* The robustness test was conducted by four deliberate variations to some chromatographic parameters such as the column temperature, acetonitrile percent, flow rate, and pH value of the mobile phase. The acetonitrile percent, flow rate, and pH value of the mobile phase were changed by ±2, ±0.02, and ±0.2, respectively. The column temperature was altered to ±1°C (from 29 to 31°C). The separation degree and RSD% of the five 5-HT$_3$ receptor antagonists were investigated.

*2.6. Qualitative Investigation*

*2.6.1. UV Spectral Similarity.* The mixed working standard solutions of the five 5-HT$_3$ receptor antagonists were injected into the HPLC system according to the chromatographic conditions given in Section 2.3. Then, the spectral and 1st UV spectra were recorded. The cosine of the vector angle was calculated for similarity evaluation of UV spectra among the five 5-HT$_3$ receptor antagonists. The angle cosine formula is expressed in equation (1) in which $X_{ki}$ and $X_{kj}$ are the peak point absorbances of the reference and test samples, respectively. The similarity values can quantitatively reflect the similarity degree of the different UV spectra. When the similarity value is close to 1, the similarity degree of the different UV spectra is high. In addition, when the similarity value is close to 0, the difference between the UV spectra is large.

$$C_{ij} = \frac{\sum_{k=1}^{n} X_{ki} X_{kj}}{\sqrt{\sum_{k=1}^{n} X_{ki}^2 \sum_{k}^{n} X_{kj}^2}}. \quad (1)$$

*2.6.2. Relative Retention Time.* The same mixed standard solutions of the five analytes and urine pyrimidine were tested under the chromatographic conditions as described in Section 2.3 to identify the relative retention time (RRT) and the RSD%. The RRTs are calculated by the following equation:

$$\mathrm{RRT} = \frac{t_x - t_0}{t_i - t_0}, \quad (2)$$

where $t_0$, $t_i$, and $t_x$ represent the retention time of urine pyrimidine, ONT, and analyte, respectively.

*2.7. Calculation of Relative Correction Factors Using the QAMS Method.* In this study, due to the low price, availability, chemical stability, good separation, and chromatographic peaks having no interference with other 5-

$HT_3$ receptor antagonists, ODT was chosen as the internal standard substance for the QAMS method. The relative correction factors (RCF, f) between ODT and the other 5-$HT_3$ receptor antagonists were calculated using equation (3). The concentration of each 5-$HT_3$ receptor antagonist in the sample solution can be calculated using equation (4) [25–32].

$$\mathrm{RCF}_x = \frac{f_x}{f_i} = \frac{A_x/C_x}{A_i/C_i}, \tag{3}$$

$$C_x = \frac{C_i}{\mathrm{RCF}_x} \times \frac{A_x}{A_i}, \tag{4}$$

where $A_i$ is the peak area of an internal reference substance (ODT) under test, and $C_i$ is the concentration of internal reference substance under test. $A_x$ is the peak area of other investigated components, and $C_x$ is the concentration of other investigated components in the sample solution.

### 2.8. Sample Analysis

*2.8.1. Analysis of the Five 5-$HT_3$ Receptor Antagonists in Infusion Samples.* All infusion samples of the five 5-$HT_3$ receptor antagonists were prepared under aseptic conditions in laminar flow hoods by licensed central intravenous additive services in hospitals. The most commonly submitted infusion preparations for ODT, GNT, TPT, AZT, and RMT were 80.0, 30.0, 50.0, 100, and 3.0 $\mu$g/mL diluted in 0.9% sodium chloride injection. Accurate volumes of each 5-$HT_3$ receptor antagonist's infusion preparations were transferred into a set of 10 mL volumetric flasks and then diluted to volume with ultrapure water to keep the concentrations of the drugs within the linear ranges.

*2.8.2. Analysis of the Five 5-$HT_3$ Receptor Antagonists in Commercial Injections.* Commercially available injections of the five 5-$HT_3$ receptor antagonists were prepared with ultrapure water and injected into HPLC under the chromatographed conditions described above. Subsequently, the chromatographic peak area of each of the five 5-$HT_3$ receptor antagonists was recorded. Then, the content of the five 5-$HT_3$ receptor antagonists was calculated by the ESM and QAMS methods.

## 3. Results and Discussion

*3.1. Optimization of Chromatographic Conditions.* According to the literature [7–24], acetonitrile and acidic water media were selected to optimize the mobile phase composition. A series of concentrations, pH values, the ratio of aqueous potassium dihydrogen phosphate solution, and different chromatographic column types were studied to ensure good resolution and appropriate retention time of the five 5-$HT_3$ receptor antagonists. The HPLC chromatograms are shown in Figures 2 and 3. The best result was achieved by comparing the peak shapes and resolutions of the investigated drugs at a pH of 4.0 and acetonitrile-50 mM $KH_2PO_4$ buffer (25 : 75; v/v) at a flow rate of 1.0 mL/min.

*3.2. Calibration Curves, Precision, Stability, and Accuracy.* The linearity range, precision, stability, and recovery are shown in Tables 1 and 2. High coefficient of determination values ($r^2 > 0.999$) showed good linearity for all five 5-$HT_3$ receptor antagonists. The RSDs of intraday and interday variability ranged from 0.4 to 1.9%, which showed good instrument precision. The RSDs of the peak areas of ODT, GNT, TPT, AZT, and RMT were 0.8%, 0.6%, 0.9%, 1.8%, and 1.2% (RSD% ≤ 2.0%), which indicates that the five 5-$HT_3$ receptor antagonists tested sample solution were stable within 8 hours (average time of analysis) and can be evaluated under normal laboratory environment without any significant loss. The recoveries ranged from 98.3% to 101.8%, with RSD < 2.0%, illustrating that the method was accurate.

*3.3. UV Spectral Similarity and Relative Retention Time.* The differences in UV spectra are based on the differences in the structure of different 5-$HT_3$ receptor antagonists (Figure 1). The results of the original and 1st UV spectral similarity are shown in Tables 3 and 4. According to the results, there are certain differences between the original UV spectra of the five 5-$HT_3$ receptor antagonists. In particular, the 1st UV spectral similarity effectively magnified the difference in the original UV spectrum (Figures 4 and 5). Therefore, qualitative identification using 1st UV spectral similarity can effectively distinguish different 5-$HT_3$ receptor antagonists.

The qualitative HPLC method is usually compared with the retention time of the reference substance. The retention time is influenced by several factors such as column packing, mobile phase, and instrument. It is difficult for the five 5-$HT_3$ receptor antagonists with similar physical properties or structures to identify accurately using only the retention time. In this study, the relative retention time (RRT) was used to qualitatively determine the different 5-$HT_3$ receptor antagonists. The reproducibility of the data could be improved by correcting the dead volume. In this experiment, the effects of different LC instruments (UltiMate 3000, Agilent 1260, and SHIMADZU LC-20A), brand columns (Agilent Zorbax Extend $C_{18}$, InertSustain $C_{18}$ column, and Kromasil $C_{18}$), temperatures (±1°C), pH values (±0.2), and flow rates (±0.02) of the mobile phase on RRTs were investigated. The results are shown in Table 5. The results showed that the RRTs of the five 5-$HT_3$ receptor antagonists were significantly different, but the changes in pH of the mobile phase and the chromatographic column had some effects on the RRTs. The differences between RRTs can mainly be attributed to different manufacturers being associated with the properties and preparation of packing materials. To this end, we herein restricted conditional parameters and performed spectral similarity as a double indicator to qualify the qualitative analysis.

*3.4. Robustness Test of QAMS.* To evaluate the robustness of the RCFs, the influence of different LC instruments (UltiMate 3000, Agilent 1260, and SHIMADZU LC-20A), brand

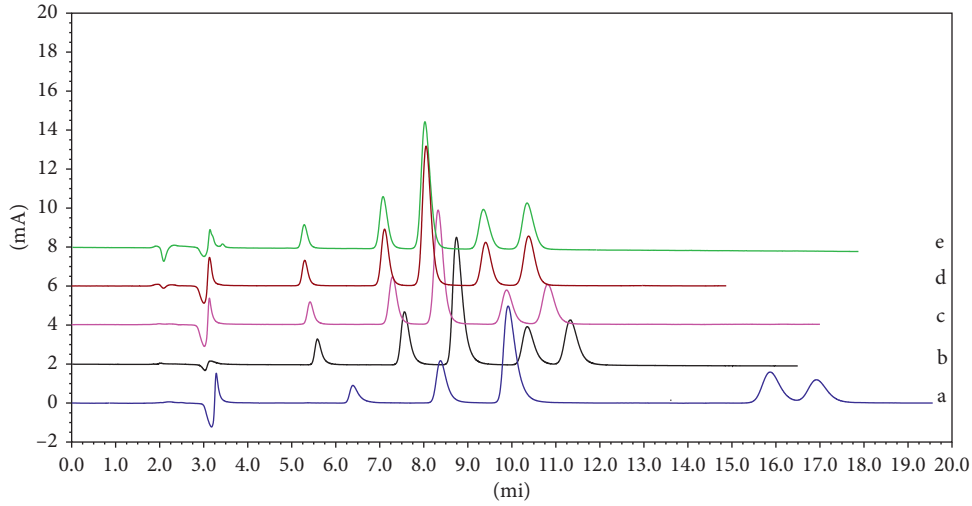


Figure 2: Typical HPLC chromatogram for simultaneous separation of ODT, GNT, TPT, AZT, and RMT. Chromatographic conditions were acetonitrile-0.05 mol·L$^{-1}$ potassium dihydrogen phosphate (25 : 75, v/v) except the buffer pH was varied: (a) pH 3.0; (b) pH 3.5; (c) pH 4.0; (d) pH 4.5; and (e) pH 5.0.

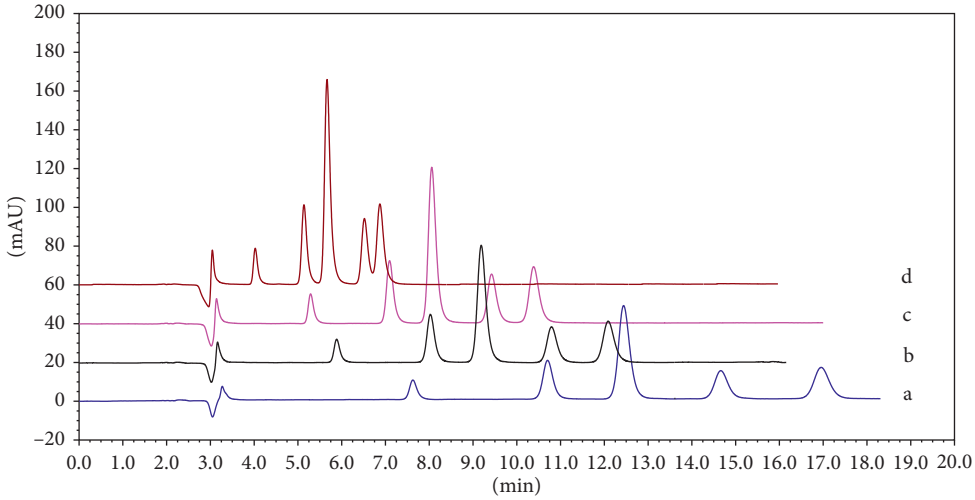


Figure 3: Typical HPLC chromatogram for simultaneous separation of ODT, GNT, TPT, AZT, and RMT. Chromatographic conditions were acetonitrile-0.05 mol·L$^{-1}$ potassium dihydrogen phosphate (phosphoric acid to adjust pH to 4.0), except the acetonitrile content was varied: (a) acetonitrile content 20; (b) acetonitrile content 25; (c) acetonitrile content 30; and (d) acetonitrile content 35.

Table 1: Regression equation, correlation coefficient ($r$), linear range, and detection limit of the five 5-HT3 receptor antagonists.

| Compound | Regression equation | $r$ | Linear range ($\mu$g·mL$^{-1}$) | Detection limit ($\mu$g·mL$^{-1}$) | Quantification limit ($\mu$g·mL$^{-1}$) |
|---|---|---|---|---|---|
| Azasetron | $Y = 0.6321X + 1.3833$ | 0.9996 | 5.0–100 | 0.2 | 0.6 |
| Granisetron | $Y = 0.6957X + 0.5444$ | 0.9999 | 5.0–100 | 0.1 | 0.4 |
| Tropisetron | $Y = 1.0634X + 4.8592$ | 0.9997 | 5.0–100 | 0.05 | 0.2 |
| Ondansetron | $Y = 1.8585X + 5.2914$ | 0.9999 | 5.0–100 | 0.1 | 0.3 |
| Ramosetron | $Y = 2.3167X + 8.6551$ | 0.9999 | 0.5–50 | 0.1 | 0.2 |

Table 2: Accuracy and precision results for the HPLC method.

| Drug | Measured concentrations ($\mu$g/mL) | Accuracy (%) | Precision RSD (%) | |
|---|---|---|---|---|
| | | | Intraday | Interday |
| Azasetron | 10.0 | 99.7 | 1.5 | 1.7 |
| | 20.0 | 99.1 | 1.4 | 1.5 |
| | 50.0 | 99.2 | 1.2 | 1.8 |

Table 2: Continued.

| Drug | Measured concentrations ($\mu$g/mL) | Accuracy (%) | Precision RSD (%) | |
|---|---|---|---|---|
| | | | Intraday | Interday |
| Granisetron | 10.0 | 101.7 | 0.7 | 1.1 |
| | 20.0 | 98.9 | 1.0 | 1.4 |
| | 50.0 | 99.0 | 0.8 | 1.4 |
| Tropisetron | 10.0 | 101.8 | 0.4 | 1.6 |
| | 20.0 | 99.3 | 1.6 | 1.8 |
| | 50.0 | 100.9 | 1.4 | 1.9 |
| Ondansetron | 10.0 | 101.8 | 1.1 | 1.5 |
| | 20.0 | 99.2 | 0.7 | 1.9 |
| | 50.0 | 98.3 | 0.9 | 1.7 |
| Ramosetron | 1.0 | 101.5 | 1.4 | 1.6 |
| | 3.0 | 101.6 | 1.3 | 1.6 |
| | 6.0 | 99.5 | 1.2 | 1.9 |

Table 3: UV spectra similarity of the five 5-HT3 receptor antagonists.

| | Azasetron | Granisetron | Tropisetron | Ondansetron | Ramosetron |
|---|---|---|---|---|---|
| Azasetron | 1.0000 | | | | |
| Granisetron | 0.8964 | 1.0000 | | | |
| Tropisetron | 0.9049 | 0.9364 | 1.0000 | | |
| Ondansetron | 0.8719 | 0.9166 | 0.9238 | 1.0000 | |
| Ramosetron | 0.8969 | 0.9164 | 0.9078 | 0.9823 | 1.0000 |

Table 4: 1st UV spectra similarity of the five 5-HT3 receptor antagonists.

| | Azasetron | Granisetron | Tropisetron | Ondansetron | Ramosetron |
|---|---|---|---|---|---|
| Azasetron | 1.0000 | | | | |
| Granisetron | 0.1764 | 1.0000 | | | |
| Tropisetron | 0.1069 | 0.5472 | 1.0000 | | |
| Ondansetron | 0.3419 | 0.3880 | 0.4861 | 1.0000 | |
| Ramosetron | 0.4371 | 0.5021 | 0.5515 | 0.8837 | 1.0000 |

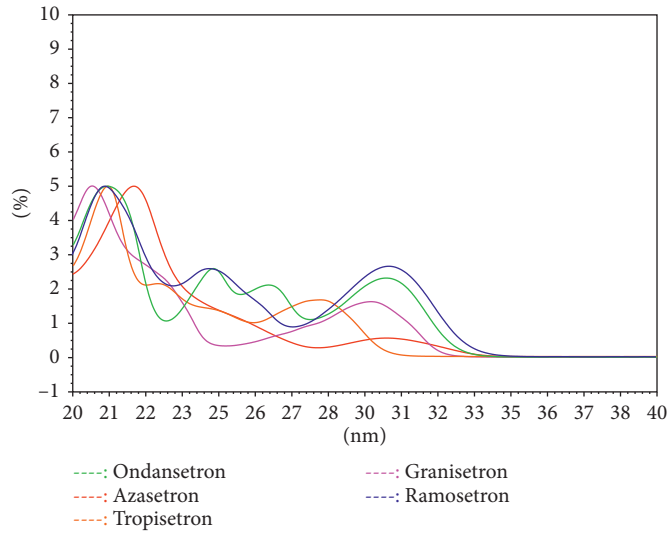


Figure 4: The UV spectra of ODT, GNT, TPT, AZT, and RMT.

columns (Agilent Zorbax Extend $C_{18}$, InertSustain $C_{18}$ column, and Kromasil $C_{18}$), temperatures (±1°C), pH values (±0.2), and flow rates (±0.02) of the mobile phase on the RCFs was investigated. The RCFs of the five 5-$HT_3$ receptor antagonists are shown in Table 6. The experimental results show that the values of RCF have good repeatability (RSDs

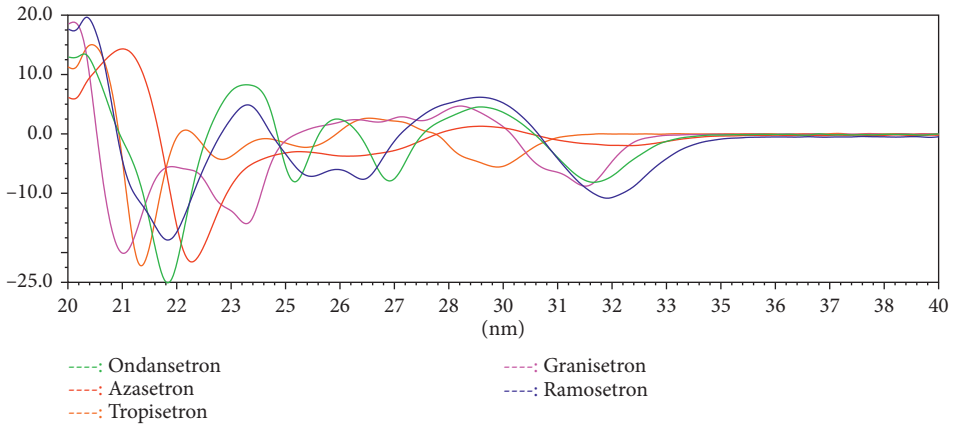


Figure 5: The 1st UV spectra of ODT, GNT, TPT, AZT, and RMT.

Table 5: Effects of instrument, column, flow rate, pH value of mobile phase, and column temperature upon RRT of the five 5-$HT_3$ receptor antagonists.

| Compound | RRT | RSD (%) | | | | |
|---|---|---|---|---|---|---|
| | | Instrument | Column | Flow rate | Temperature | pH value |
| Azasetron | 0.431 | 0.3 | 4.2 | 0.9 | 0.5 | 2.3 |
| Granisetron | 0.679 | 0.7 | 2.8 | 1.3 | 0.9 | 1.9 |
| Tropisetron | 0.812 | 0.3 | 2.1 | 0.4 | 1.2 | 2.5 |
| Ondansetron | 1.000 | 0 | 0 | 0 | 0 | 0 |
| Ramosetron | 1.132 | 0.5 | 2.6 | 0.6 | 0.6 | 1.6 |

Table 6: Effects of HPLC column, flow rate, pH value of mobile phase, and column temperature upon RCF of the five 5-$HT_3$ receptor antagonists.

| Compound | RCF | RSD (%) | | | | |
|---|---|---|---|---|---|---|
| | | Instrument | Column | Flow rate | Temperature | pH value |
| Azasetron | 0.340 | 0.9 | 2.8 | 1.6 | 0.3 | 2.1 |
| Granisetron | 0.374 | 1.2 | 3.0 | 1.0 | 0.6 | 1.7 |
| Tropisetron | 0.572 | 0.8 | 2.3 | 1.5 | 1.2 | 2.2 |
| Ondansetron | 1.000 | 0 | 0 | 0 | 0 | 0 |
| Ramosetron | 1.247 | 1.5 | 4.2 | 1.3 | 0.8 | 2.5 |

Table 7: The results obtained by the QAMS and ESM methods ($n = 3$).

| Compound | The QAMS methods | | The external standard methods | |
|---|---|---|---|---|
| | Accuracy (%) | RSD (%) | Accuracy (%) | RSD (%) |
| *The five 5-$HT_3$ receptor antagonists in injection dosage form* | | | | |
| Azasetron | 97.3 | 1.5 | 97.4 | 1.8 |
| Granisetron | 102.1 | 2.1 | 101.8 | 1.0 |
| Tropisetron | 101.7 | 0.9 | 102.1 | 1.6 |
| Ondansetron | 99.2 | 1.7 | 99.4 | 0.8 |
| Ramosetron | 101.3 | 2.4 | 100.9 | 1.4 |
| *The five 5-$HT_3$ receptor antagonists in infusion samples* | | | | |
| Azasetron | 94.9 | 1.3 | 95.1 | 1.5 |
| Granisetron | 100.3 | 0.8 | 99.8 | 0.7 |
| Tropisetron | 97.1 | 2.2 | 97.2 | 1.3 |
| Ondansetron | 103.6 | 1.9 | 103.8 | 1.2 |
| Ramosetron | 98.8 | 1.5 | 99.0 | 0.9 |

ranging from 0.3% to 4.2%) under different experimental conditions. These results ensure that the HPLC-QAMS method can be well applied to routine analysis.

*3.5. Sample Analysis.* The developed HPLC-based QAMS method and ESM analytical method were applied to determine the five 5-$HT_3$ receptor antagonists in their infusion samples and injection dosage form. The amounts of individual 5-$HT_3$ receptor antagonists in injection dosage form and in infusion samples were calculated, and the results are listed in Table 7. From the comparative analysis results, we can conclude that there was no significant difference between the two analytical methods (using a $T$-test, $p > 0.05$), and the RSD values were <2.5%. Meanwhile, the contents of individual 5-$HT_3$ receptor antagonists were also determined by HPLC methods described in the Chinese Pharmacopoeia (2015 Edition), and the results showed no significant difference between the above methods.

## 4. Conclusions

The developed HPLC-based QAMS and external standard analytical method are fast, selectively convenient, and sensitive to the simultaneous determination of ODT, GNT, TPT, AZT, and RMT in their injection dosage form and infusion samples. The comparative analysis results show no apparent distinction between the assay results of the two methods. The QAMS analysis method can provide reliable results, save reference materials, and shorten the analysis time. This method has great potential and can play an enhanced role in hospital-based quality control and quality assurance programs.

## Data Availability

The majority of the data used to support the findings of this study are included within the article. Other data are available from the corresponding author upon request.

## Conflicts of Interest

The authors have no conflicts of interest to declare.

## Authors' Contributions

Fuchao Chen and Baoxia Fang contributed equally to this work and should be considered equal first authors.

## Acknowledgments

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*Research Article*

# Extraction of Pregabalin in Urine Samples Using a Sulfonated Poly(ether ether ketone) Membrane

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In this work, a simple polymer-assisted microextraction technique was developed to determine pregabalin (an anticonvulsant drug) in the urine sample. A sulfonated poly(ether ether ketone) membrane was used as a sorbent for pregabalin extraction, and the extraction performance was compared with that of the conventional polydimethylsiloxane membrane. The extraction device is free moving and tumbles continuously throughout the stirred sample solution during extraction to enhance the extraction efficiency. The electrostatic interactions between the sulfonic-acid-functionalized polymeric membrane and the amine group in the pregabalin molecule facilitate higher preconcentration factor at a shorter extraction time. Optimizing conditions of the extraction method were investigated to obtain higher extraction efficiency. The developed method exhibited good linearity in the range of 0.05 to 2 $\mu$g/mL with a correlation of determination ($r^2$) 0.9998, acceptable limits of detection, limits of quantification, and preconcentration factor of 105-fold. The within-day and between-day precisions of pregabalin were lower than 7% relative standard deviations. Pregabalin was extracted from urine samples with recoveries of >92%, and no significant matrix effects were observed.

## 1. Introduction

Pregabalin is used as adjunctive therapy for partial seizures with or without secondary generalization in adults. Pregabalin (S-3-(aminomethyl)-5-methyl hexanoic acid) is a structural derivative of the inhibitory neurotransmitter aminobutyric acid. In recent research, pregabalin has been confirmed for adjunctive treatment of partial seizures to treat neuropathic pain from postherpetic neuralgia and diabetic neuropathy in adults in both the United States and Europe [1, 2]. The chemical structure of pregabalin is shown in Figure 1(a). Pregabalin is available under the trade name Lyrica (Pfizer, New York, NY) for use for treating epilepsy, diabetic neuropathy pain, postherpetic neuralgia [3], effective at fibromyalgia [4], and spinal cord injury [5].

The precise mode of action of pregabalin has not been fully elucidated. However, it does interact with the same binding site and has a similar pharmacologic profile as gabapentine (1-[aminomethyl] cyclohexane acetic acid) [6, 7]. Pregabalin is minimally metabolized and primarily excreted in urine as unchanged drugs, and studies in healthy volunteers indicate oral bioavailability to be approximately 90% [8]. It is available in 25 mg, 50 mg, 75 mg, 150 mg, and 300 mg capsules, and this variation allows it to be easier to prescribe when the medication is being introduced.

In clinic, dizziness and somnolence are the most frequently reported adverse events, with dizziness experienced by 29% of pregabalin-treated patients compared with 9% with placebo and somnolence experienced by 22% of pregabalin-treated patients. Decreased concentration, increased appetite and weight gain, dry mouth, and vomiting are other side effects [9].

On top of that, increasing slow-wave sleep in healthy volunteers is another side effect correlated with the sleep's restorative aspect.

A relatively uncomplicated and more cost-effective method such as spectrophotometry or spectrofluorometry is the basic requirement for routine analysis of the drug in bulk powder and pharmaceutical preparations, particularly in research laboratories and the pharmaceutical

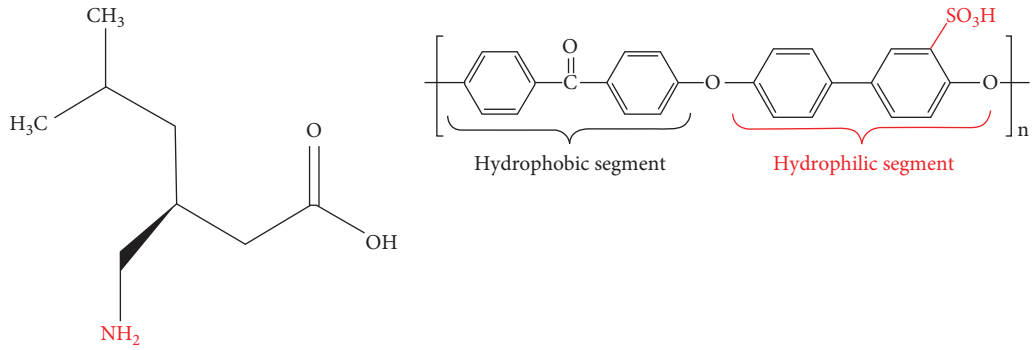


Figure 1: Chemical structure of (a) pregabalin and (b) SPEE.

industry. Literature review revealed that conventional direct analysis or liquid-liquid extraction methods were used to analyze pregabalin in tablets or biological matrices. After extraction, analyses were performed by gas chromatography-mass spectrophotometry (GC-MS), high-performance liquid chromatography (HPLC)-MS-MS [10, 11], HPLC [11–16], and fluorometry [17] without any derivatization.

However, to improve the direct analysis's sensitivity, various derivatization reagents were used; for example, cyclodextrins were added before capillary electrophoresis and nuclear magnetic resonance analysis [18]. Other chromogenic reagents such as 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and 7,7,8,8-tetracyanoquinodimethane (TCNQ) or 2,4-dinitrofluorobenzene and 2,3,5,6-tetrachloro-1,4-benzoquinone and ninhydrin were used for UV/visible spectrophotometry or spectrofluorometry analysis [2, 19–21].

The derivatization method requires additional tedious derivatization steps, which further dilute the analytes' concentrations [11, 22] and are challenging for trace-level quantitation. Unfortunately, the excess derivatization reagents often result in resolution and detection problems such as difficulty to separate a trace target derivative in the presence of a significant excess of an unreacted reagent. The excessive reagent might interfere with the chromatographic separation process [23].

It is well known that, in sorptive processes, higher extraction efficiencies are obtained when the analytes are in their nonionic form. Various sorptive extraction techniques using polydimethylsiloxane (PDMS) have been reported for the extraction drug samples in urine samples which include solid-phase microextraction [24] and stirrer bar sorptive microextraction (SBSE) [25, 26]. Recently, functionalized polymers were used to extract a wide range of organic compounds with high selectivity and enrichment [27]. The selective extraction mechanism of analytes was explained by various interactions, which includes chelation [28, 29], chiral recognition [30–32], ion-exchange through electrostatic interactions [33–35], creation of recognition sites for target analytes using molecularly imprinted polymers [36–38], and immunoaffinity through antibody-antigen interactions [39–42].

In this study, for the first time, we have developed polymer-assisted microextraction (PME) using a sulfonated poly(ether ether ketone) (SPEEK) functionalized polymer membrane as an adsorbent. After extraction, the extract was injected into HPLC (without any derivatization). The developed method required only small amounts of sample and solvent.

## 2. Materials

*2.1. Chemicals.* Sulfonated poly(ether ether ketone) (Fumatech, ion-exchange capacity = 1.6 mmole/g) and sodium hydroxide were purchased from Fluka (AG, Switzerland). Pregabalin (99.5%) was obtained from Symed Laboratories (AP, India). HPLC-grade organic solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The polydimethylsiloxane (PDMS) polymer comprising the Slygard 184 silicone elastomer and a curing agent was purchased from Dow Corning Corporation (Midland, MI, USA). Ultrapure water was used for the preparation of all standard solutions and mobile phase. Stock standard solution of pregabalin was prepared by dissolving 50 mg of pregabalin into 25 mL distilled water. Standard solutions of pregabalin (2.0, 1.5, 1, 0.5, 0.1, and 0.05 $\mu$g/mL) were prepared by subsequent dilution.

*2.2. Urine Samples.* Urine samples were collected from a healthy volunteer at the university campus. The samples were taken in amber glass bottles previously rinsed with methanol and ultrapure water, and the samples were stored in the dark at 4°C for a maximum of 48 hours. Before their analysis, urine samples were filtered using cellulose acetate membranes (0.45 $\mu$m pore size). In order to test the accuracy of the method, the drug-free urine samples were spiked with a known amount of pregabalin standard.

## 3. Instrument

The Waters HPLC system was used in the study with a $\mu$-Bondapak $C_{18}$ column (3.9 × 300 mm) (USA). The flow rate was adjusted to 1 mL/min, and the injection volume used was 10 $\mu$L with a retention time of 15 mins. A Waters 2996 Photodiode Array was used as the detector, and the

wavelength of detection was 210 nm. The mobile phase was prepared by dissolving 1.2 g of monobasic potassium phosphate in 940 mL water. Then, the pH of the resultant solution was adjusted to 6.90 (by using 5N NaOH solution), then 60 mL of acetonitrile was added, and the solution was degassed.

## 4. Membrane Preparation

In this study, sulfonated poly(ether ether ketone) (SPEEK), with the chemical structure shown in Figure 1(b), is used as the acid-functionalized polymer to prepare the corresponding acid-functionalized membrane.

Dry SPEEK polymer was dissolved in dimethylacetamide (DMAc) solvent to form an approximately 10 wt.% solution. Next, the solution was poured into a glass plate, the solvent was slowly evaporated, and the resulted membrane was peeled off by immersing in deionized water for a few hours. The residual solvent was then removed by drying the obtained membrane in a vacuum oven. The dried membrane was conditioned by soaking in 0.5 M $H_2SO_4$, washed with deionized water, and finally, dried at 60°C for 3 hours.

The attached sulfonic acid functionality on the poly(-ether ether ketone) backbone makes the polymer surface more hydrophilic. It provides robust, accessible acidic ion-exchange sites for possible interaction with analytes containing basic nitrogen functionality such as pregabalin, as illustrated in Figure 2. Moreover, the extraction performance of PME was compared for polymeric adsorbents using SPEEK and the commercial polydimethylsiloxane (PDMS) membrane.

## 5. Polymeric-Membrane-Assisted Microextraction (PME)

50 mg of the SPEEK membrane was placed into a 10 mL drug-free urine sample spiked with various pregabalin concentrations, and the sample was agitated with a stir bar for 2 min. Then, the polymeric sorbent was removed from the sample vial and wiped softly with tissue.

The pregabalin-extracted SPEEK membrane was desorbed in 500 $\mu$L of 0.1 M NaOH solution via ultrasonication for 2 min. Finally, the membrane was removed from the HPLC autosampler vial, and 10 $\mu$L of the extract was injected into HPLC. The SPEEK membrane was conditioned again with a large volume of NaOH solution for 10 min to check the carryover effect; no analyte was detected after the second desorption. This indicates that the membrane is suitable for subsequent extraction. To condition and regenerate the SPEEK membrane, the membrane was ultrasonicated with 50 mL of 0.5 M $H_2SO_4$ for 5 min and used for further extraction.

## 6. Results and Discussion

*6.1. Optimization of PME.* The extraction principle of PME is based on ion-pair partitioning of pregabalin with the SPEEK membrane. The ion-exchange extraction mechanism of SPEEK provides a convenient pathway to the simultaneous sampling, sample preparation, and preconcentration of pregabalin. The extraction parameters affecting PME, such as extraction time, desorption time, desorption solvent, and sample pH, were optimized. These are the factors that were considered to play an essential role in determining extraction efficiency.

*6.2. Selection of Polymeric Sorbent.* The extraction performance of PDMS and SPEEK membranes were tested at identical conditions (2 min extraction time, 0.1 M NaOH desorption solvent with 2 min desorption time). Compared with PDMS, SPEEK showed improved extraction ability for the pregabalin analyte, as shown in Figure 3. The enhanced extraction ability of the SPEEK membrane could be attributed to the hydrophilicity of the acid-functionalized polymer as well as the presence of electrostatic interactions between the grafted sulfonic acid moiety on the polymer backbone ($Pk_a < 1$) and the amine group of the pregabalin analyte ($Pk_a = 10.6$), as illustrated in Figure 2. The significant $Pk_a$ difference allows for fast and effective ionic crosslinking and successful electrostatic interaction between the SPEEK and the–$NH_2$ functionality on the pregabalin molecules. Hence, the SPEEK membrane was further selected as the functionalized extraction sorbent for method development.

*6.3. Extraction Time.* The extraction was monitored over the range of 2 to 20 min. Figure 4 shows the behavior of pregabalin under different extraction periods. The HPLC signals decreased to an extraction time of 2 min, and extraction efficiency remains approximately constant after 10 minutes. Faster mass transfer of the analyte was achieved due to the functionality of the SPEEK membrane. Thus, 2 min extraction time was selected for further analysis.

*6.4. Desorption Solvent.* After extraction, the SPEEK-containing analyte was desorbed with an organic solvent via ultrasonication. Two important factors should be considered before selecting a suitable solvent for desorption of the analyte from SPEEK: (i) the polymer should be insoluble in the desorbing solvent, and (ii) analytes must be soluble in the solvent.

In the present case, the SPEEK membrane with ion-exchange capacity (IEC = 1.6) is soluble in polar solvents such as methanol, ethanol, and acetonitrile. However, since the adsorption mechanism is based on electrostatic interactions and ion pair formation, the desorption solvent shall be able to break the complexed ion pairs by neutralization of the charged polymeric functionalities (–$SO_3H$/–$CO_2H$) as illustrated in Figure 2. Based on the abovementioned discussion, a 0.1 M NaOH solution was selected as the desorption solvent for pregabalin elution from the SPEEK membrane. Finally, NaOH extract was injected directly into the HPLC system.

*6.5. Desorption Time.* The effects of the desorption period (ultrasonication time using a 0.1 M NaOH solution as a desorption solvent) on the pregabalin extraction were studied.

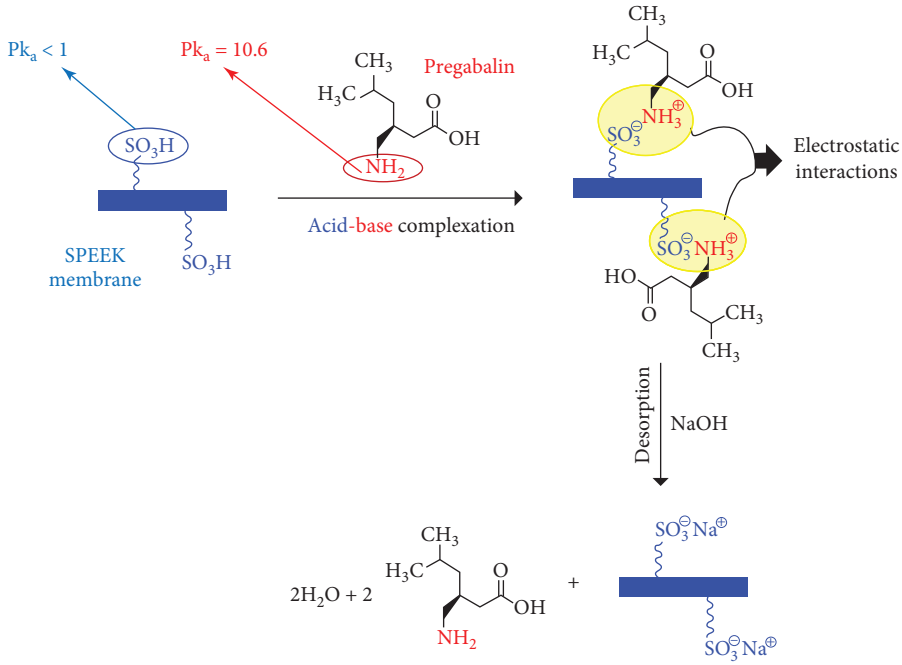


Figure 2: Illustration for the ion-exchange mechanism and the electrostatic interactions between the SPEEK membrane and pregabalin analyte.

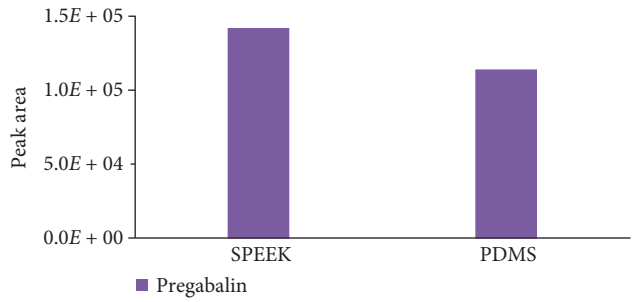


Figure 3: Comparison of SPEEK and PDMS as a sorbent. Extractions were performed at identical conditions.

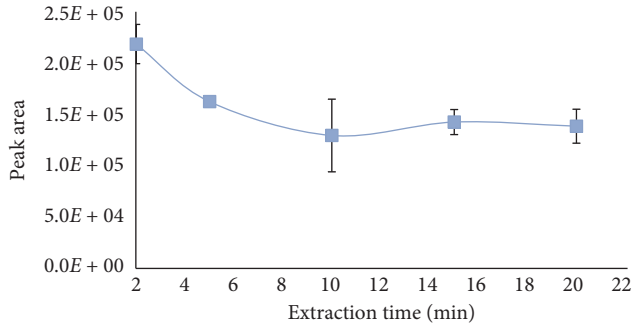


Figure 4: Extraction profile of pregabalin using PME.

According to the literature, the desorption time required using a solvent is usually no longer than 20 min, and mechanisms to facilitate the desorption process may be ultrasonication or agitation [43]. On this basis, desorption time between 2 and 20 min was investigated. Since the extraction process is based on an ion-exchange mechanism, the analyte was quickly desorbed in a shorter time (2 min). After each extraction and desorption, the SPEEK membrane was rinsed with acetone for 2 min to avoid any analyte carryover problem.

*6.6. Effect of Sample pH.* The effect of sample pH on the pregabalin's extraction efficiency was evaluated in the pH range of 2–12. The highest response was obtained at the lowest pH 2. The obtained highest response in the acidic environment could be attributed to the efficient ion pair electrostatic interactions between the SPEEK membrane and pregabalin analyte. In other words, when the medium becomes less acidic, the ion-exchange capacity of SPEEK polymer decreases, which means that the number of free acidic protons ($H^+$) decreases; thus, the number of free acidic sites available for interaction with the amine groups of pregabalin became less, which led to a slight reduction in the instrument response. Although the maximum obtained response at pH = 2 is about one order of magnitude higher than the lowest response at pH > 6, this observation supports the mechanism of electrostatic interaction and ion pair formation in the studied PME system. In comparison, when the medium became alkaline (pH > 7), the sulfonic acid groups neutralized and become negatively charged, which makes them inaccessible for the formation of ion pairs with basic (-$NH_2$) functional groups of the pregabalin analyte. Regardless, the obtained constant response in the alkaline pH range could be attributed to the improved surface hydrophilicity (Figure 1(b)) of the polymeric membrane due to the grafted polar functional groups (–$SO_3^-$). Hence, in the alkaline medium, the adsorption-desorption mechanism could be suggested due to both the pregabalin analyte and SPEEK membrane's hydrophilic nature.

Table 1: Quantitative parameter of PME.

| | Spiked | Detected Concentration (mg/L) ($n = 3$) | Recovery | Spiked | Detected Concentration (mg/L) ($n = 3$) | Recovery (%) |
|---|---|---|---|---|---|---|
| Pregabalin | 0.5 | 0.45 | 90.1 | 1.5 | 1.39 | 92.3 |

Table 2: Comparison of the methods reported in the literature for pregabalin.

| Method | SV (ml) | E T | LOD | %RSD | RR | Ref |
|---|---|---|---|---|---|---|
| LLE (manual) | 50 | 10 min | 4.8 ng/mL | 0.17 | 99.5–101 | [44] |
| LLE (ultrasonication) | 50 | ~15 min | 66.9 ng/mL | 0.068–0.167 | 97.12–98.86 | [13] |
| LLE (manual) | 15 | 3 min | 1 ng/mL | 11.4%. | 69.8–72% | [45] |
| PME | 20 | 2 min | 0.03 ng/mL | 6.8% | 90–92 | Current study |

SV: sample volume (mL). ET: extraction time (min). LOD: limit of detection (ng/mL). RSD: relative standard deviation (%). RR: relative recovery (%).

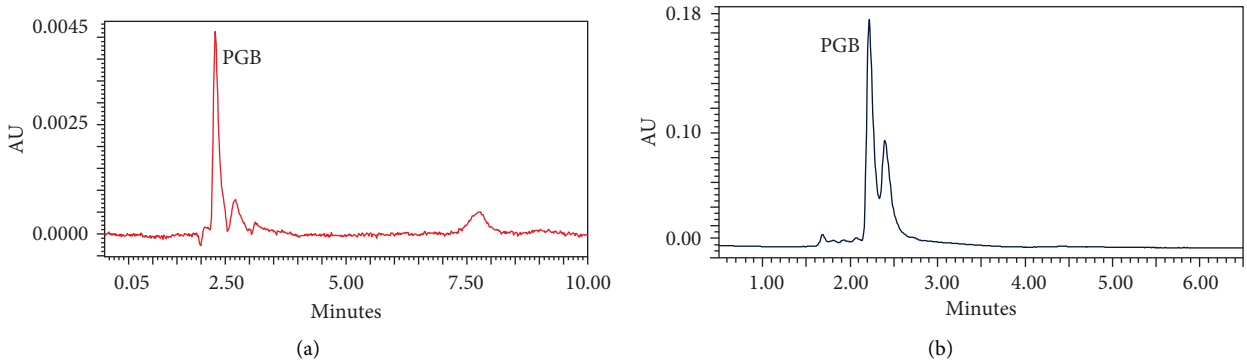


Figure 5: HPLC chromatogram of pregabalin (a) before extraction at 5 $\mu$L spiked sample and (b) after SPEEK extraction of drug-free urine sample spiked at 0.1 $\mu$g/mL.

*6.7. Effect of Salt Addition.* The addition of salt to the sample solution may decrease the polar analyte's solubility and increase the partition coefficient, and this process is called the salting-out effect. To assess the salting-out effect on the extraction of pregabalin from urine samples, various NaCl concentrations were added to urine samples in the range of 1–20% wt/vol. The addition of salt to the urine samples does not significantly improve the extraction (data not shown).

To illustrate, when NaCl is added in high concentration to the analyzed urine samples, $H^+$ protons of the sulfonic acid groups in SPEEK polymer are exchanged with $Na^+$ protons. As a result, HCl is liberated.

Hence, the sulfonic acid groups became neutralized by sodium counterions and unable to form ion pairs with basic functionalities ($-NH_2$). This result provided evidence for the critical role of the enhanced surface hydrophilicity of the SPEEK membrane, which facilitates the adsorption-desorption of hydrophilic analytes with polar functionalities such as pregabalin. Therefore, no salt was added to the urine samples in the reported experiments.

*6.8. Quantitative Analysis.* Based on the experiments discussed above, the optimal PME conditions were: as follows 50 mg of SPEEK was used, sample pH of 2, and an extraction time of 2 min. After the extraction, the analytes were desorbed using 500 $\mu$L of 0.1 M NaOH by ultrasonication for 2 min. 10 $\mu$L of the extract was injected into the HPLC system. To evaluate the PME technique, repeatability, linearity, and detection limits under the optimal extraction conditions were investigated. The repeatability in peak areas was studied for three replicate experiments (3 separate pieces of SPEEK). The relative standard deviations (RSDs) were lower than 7%. Extraction of pregabalin exhibited good linearity over the concentration range 0.05–2 $\mu$g/mL under optimum conditions. Coefficients of correlation ($r^2$) better than 0.999 were obtained. LOD and LOQ were calculated as 3.3 $\sigma$/s and 10 $\sigma$/s, respectively, as per International Conference on Harmonization (ICH) definitions, where $\sigma$ is the mean, standard deviation of replicate determination values under the same conditions as the sample analysis in the absence of the analyte (blank determination), and $s$ is the sensitivity, namely the slope of the calibration graphs. LOD values for pregabalin were 0.03 ng/mL and LOQ 0.09 ng/mL, respectively. The PME method obtained 105-fold enrichment.

The PME method was compared with already reported methods in the literature and summarized in Table 1. The results demonstrate the applicability of the method for routine trace-level analysis of pregabalin from a urine sample. The PME method's recovery was investigated with a healthy person's urine samples, and the samples were spiked with the known concentration of pregabalin, 0.5 and 1.5 $\mu$g/mL, respectively. The extraction recoveries were calculated

and reported in Table 2, and recoveries varied between 90 and 92%, respectively, which indicates that SPEEK polymer can successfully extract the pregabalin from urine samples more effectively. These results demonstrate the absence of significant matrix effects on the efficiency of PME. Figure 5 shows the HPLC chromatograms of (a) the pregabalin standard sample with a concentration of 5 $\mu$g/mL and (b) the SPEEK extract drug-free urine sample spiked with 0.1 $\mu$g/mL pregabalin.

## 7. Conclusions

In this work, a polymer-assisted microextraction coupled with HPLC is developed to rapidly determine the amount of pregabalin in urine samples. We reported the potential use of the acid-functionalized SPEEK as an ion-pair low-cost sorbent for selective sample enrichment of pregabalin from urine samples. The PME device was allowed to tumble freely in the sample solution to enhance the extraction efficiency. A small amount of low-cost sorbent, reusability of the sorbent up to 50 times without loss of analytes after consecutive extractions, and the extreme simplicity of the procedure are the main advantages of this technique. Also, the method permits the determination of analytes at low concentrations showing good performance over the commercial PDMS membrane. [46]

## Data Availability

The data used to support the findings of this study are included within the article as figures and tables.

## Ethical Approval

The urine samples were obtained from the University Medical Clinic with consent from a volunteer and the medical director.

## Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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