A New Pre-column Derivatization Method for Determination of Gabapentin in Human Serum by HPLC Using UV Detection

Mohsen Amini[1], Mohammad-Reza Rouini[2], Alireza Asad-Paskeh[1], and **Abbas Shafiee[1,*]**

[1]Department of Medicinal Chemistry and Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences and [2]Biopharmaceutics and Pharmacokinetics Division, Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences

Abstract

A high-performance liquid chromatography method using UV detection for determination of gabapentin in human serum has been developed. Serum was deproteinized with acetonitrile, and supernatant was evaporated. Derivatization was performed using 4-nitrophenylisothiocyanate, and separation was achieved on a C_{18} column with detection at 335 nm. The method was linear in the 0.12–6.0 µg/mL range. No interference was found from endogenous amino acids. The method is suitable for routine therapeutic drug monitoring and for pharmacokinetic studies.

Introduction

Gabapentin (GBP) and some other γ-amino butyric acid inhibitors such as vigabatrin are gaining widespread acceptance in a number of seizure models (Figure 1). GBP is an oral anticonvulsant for the treatment of partial and generalized tonic-clonic seizures (1). The role of therapeutic drug monitoring (TDM) of GBP in management of epileptic patients is still being determined. However, a reliable and simple method of analysis is needed to support clinical and pharmacokinetic studies (16).

Numerous methods for the determination of GBP in human plasma have been published, including some high-performance liquid chromatographic (HPLC) methods (2–8), liquid chromatography–tandem mass spectrometry (9), gas chromatography (10), and electrophoresis (11). In the HPLC case, all published methods used derivatization technique for the assay of GBP because the drug has no suitable chromophor to be detected by UV or fluorescence detectors. Phenylisothiocyanate, dansyl chloride, 2,4,6-trinitrobenezensulfonic acid, *O*-phethaldehyde (OPA), and 9-flurenylmethylchloroformate (FMOC) were used as labeling agents for detection of GBP by fluorescent or UV detection. The use of *O*-phetaldehyde-3-mercaptopropionic acid derivatives has been described for determination of GBP in plasma by HPLC. They are only stable for 25 min, making them difficult to apply to offline derivatization (8).

However, some of these methods provide enough sensitivity for determination of GBP in plasma or serum at steady state level or 24 h after a single 400 mg dose of GBP (three half-lives), multi-step derivatization procedure, solid-phase cartridge clean-up sampling, or instability of GBP-derivatives have been limited to use them.

In continuation of our research work on a pre-column derivatization (13,14), herewith we describe HPLC conditions for determination of GBP after pre-column derivatization with 4-nitrophenylisothiocyanate and UV detection at 335 nm without using solid-phase extraction.

Experimental

Chemicals

HPLC-grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). HPLC-grade water was obtained by double distillation in glass and purified through a Milli-Q water purification system (Millipore, Bedford, MA). Water was

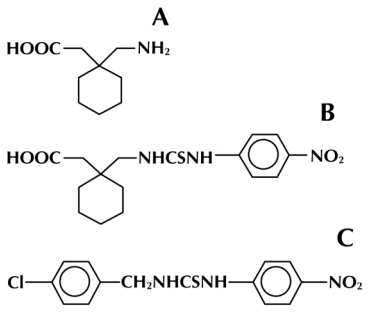

Figure 1. Structure of (A) GBP, (B) GBP-4-nitrophenylisothiocyanate derivatives, and (C) internal standard.

*Author to whom correspondence should be addressed: email ashafiee@ams.ac.ir.

filtered through 0.45-µm filter. Derivatization reagents, 4-nitrophenylisothiocyanate and 4-chlorobenzylamine, were purchased from Merck and were synthetic-grade. Standard of GBP was kindly provided by EXIR Pharmaceutical Company (Tehran, Iran). 1-Octanesulfonic acid sodium salt was purchased from Sigma Aldrich (St. Louis, MO). Drug-free serum was obtained from healthy volunteers and blood donors.

Purification of 4-nitrophenylisothiocyanate was carried out by vacuum sublimation as described previously (15). Internal standard (IS) was prepared as described in the "Sample and standard preparation" section.

Instrumentation and conditions

A Waters HPLC system (Milford, MA) was employed and consisted of a 510 pump, a Rheodyne injector equipped a 20-µL sample loop, and a 486 UV detector adjusted to 335 nm connected to a 746 data module integrator. The mobile phase consisted of a mixture of methanol and water (75:25). The pH of mobile phase was adjusted to 3.0 by adding of 0.5 mL H_3PO_4 (17% v/v) to 1 L of mobile phase. The mobile phase was prepared daily and delivered at a flow rate of 0.9 mL/min. Separation was achieved using a Teknokroma C_{18} column (5 µm, 0.46 × 15 cm) (Montreal, Canada). All chromatographic separations were performed at room temperature.

Sample and standard preparation

Internal standard solution

To 10 mmole of 4-chlorobenzylamine solution in 200 mL of acetonitrile and 2 mL triethylamine was added 20 mmole of 4-nitrophenylisothiocyanate over a 30 min period at 5°C. The solution was stirred at room temperature overnight, and the crystals were collected by vacuum filtration. Crystallization was carried out in acetonitrile (twice) to give 1-(4-chlorobenzyl)-3-(4-nitrophenyl)thiourea derivative, m.p. = 177–178°C, ^{1}HNMR (DMSO-d_6) δ: 4.75 (d, J = 4.8 Hz, 2H, CH_2), 7.39 (s, 4H, chlorophenyl), 7.82 (d, J = 9.2 Hz, 2H, nitrophenyl), 8.18 (d, J = 9.2 Hz, 2H, nitrophenyl), 8.66 (bs, 1H, NH), 10.21 (bs, 1H, NH); MS: *m/z* (%) 321 (M^+, 19), 180 (62), 150 (59), 140 (60), 127 (65), 125 (100), 122 (90), 106 (99), 90 (82), 88 (81), 63 (85), 50 (92).

The purity of IS was checked by HPLC according to the same conditions described for determination of GBP. IS solution was prepared in acetonitrile (250 µg/mL). A solution of sodium octanesulfonate (1.5 mg/mL) was prepared in water.

Sample preparation

To 1 mL serum was added IS solution (25 µL) and 0.1 mL sodium octanesulfonate solution in a regular glass tube. Acetonitrile (3 mL) was added to precipitate the protein. The capped tubes were vortex-mixed and centrifuged (2000 × *g* for 10 min). The supernatant was decanted and evaporated under airflow at 55°C to dryness. 50 µL of water and 300 µL of acetonitrile were added and mixed for 3 min for dissolving the residue. 250 µL of the solution was transferred to a 1-mL screw-vial. 50 µL of triethylamine solution in acetonitrile (2% v/v) and 50 µL of 4-nitrophenylisothiocyanate solution (200 µg/mL) in acetonitrile were added. The reaction took place at 45°C for 60 min. 25 µL of the solution was injected into HPLC system for analysis.

Standard preparation

Stock solutions of GBP was prepared in water and stored at 4°C. The working standard solution was prepared by diluting the stock solution with 10 volumes of water prior to use. Calibration serum was prepared by spiking blank human serum with GBP in six concentrations: 6.0, 3.0, 1.2, 0.6, 0.3, 0.12 µg/mL.

Calibration curve was constructed by plotting the measured peak area ratios of GBP to the IS versus concentrations of standard samples. The intra-day and inter-day precision of the method was determined by measuring three spiked standard serum samples (0.12, 0.6, 1.2 µg/mL, n = 5) on three separate days.

Results

Typical chromatograms of blank and spiked serum with IS and GBP are shown in Figure 2. Under chromatographic conditions described, IS and GBP were well-resolved and eluted at 10.70 and 9.30 min, respectively, following injection into HPLC. Optimization was achieved by monitoring varying mobile phase system, pH, and flow rate. The mean of recovery of GBP at three different concentrations (0.12, 0.3, and 0.6 µg/mL) was determined by comparing peak areas in extracted spiked human drug-free serum samples with those in standard solutions after derivatization of the same amounts of unextracted analyses solutions. The mean of recoveries were found to be 83% for GBP and 96% for IS (n = 3 for each concentration). The within- and between-day accuracy and precision values of the assay method are presented in Table I.

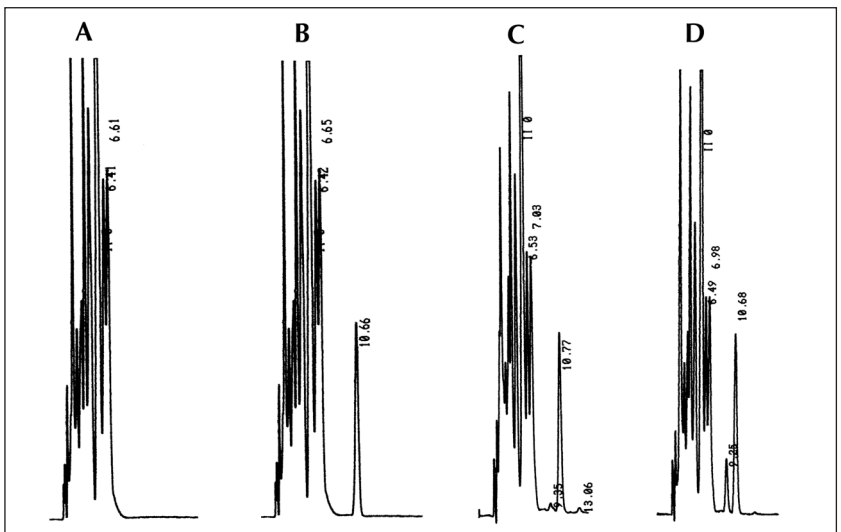

Figure 2. Typical chromatograms obtained from an extract of (A) human blank serum, (B) human blank serum spiked with 6.25 µg/mL (retention time 10.7 min) of internal standard and serum samples obtained at 1 and 24 h after a single oral dose of 400 mg GBP from a healthy volunteer containing (C) 170 ng/mL and (D) 1.8 µg/mL of the drug (retention time of GBP 9.3 min), respectively.

Calibration and sensitivity

The standard curve for GBP in different range of concentrations 0.12, 0.3, 0.6, 1.2, 3, and 6.0 µg/mL in serum was prepared. The calibration curve displayed a good linearity (r^2 = 0.9997) over the concentration range investigated. Typical calibration curve obtained in serum sample is described by $y = 0.143x + 0.0033$, where y is the peak-area ratio of GBP/IS and x is GBP concentration (µg/mL). Three calibration curves were investigated with a standard deviation of ± 7.65×10^{-3} (CV% = 5.4) for slope and a standard deviation of ± 2.1×10^{-4} (CV% = 6.4) for intercept. The limit of quantification of the method, defined as the minimum concentration that could be measured with a CV < 15%, was found to be 0.12 µg/mL. The limit of detection with a signal to noise ratio of 5:1 was 60 ng/mL.

Stability

Stability during repeated freeze-thaw cycles has been demonstrated (16). Stability of standard serum sample was evaluated at –20°C for two months and at room temperature for 24 h. Under the previous conditions, samples preserved their potency (> 96%). Stock solutions of GBP in water and IS in acetonitrile were stable for at least three weeks when stored at 4°C. Derivatized solutions were found to be stable (> 96%) for at least 48 h at room temperature for 2 weeks at 4°C. All the experiments for the evaluation of the stability were performed by HPLC with the same condition for assessment of GBP described in the text.

Application of the method

The method was applied to a study with more than 450 serum samples from human volunteers. Fifteen blood samples were drawn from each volunteer over a period of 24 h after an oral administration of 400 mg GBP. The study protocol was approved by Medical Ethics Committee of Tehran University of Medical Sciences. Figure 3 shows a characteristic mean concentration versus sampling time profile of GBP in human plasma from 12 volunteers.

Discussion

Gabapentin has no chromophor for UV absorbance and no inherent fluorescence property. Therefore, all of the published methods for determination of GBP in serum or plasma by HPLC were based on derivatization technique. For fluorescence detection, several reagents have been used. Several papers reported various amounts of LOQ (0.06–0.85 µg/mL) for determination of GBP when OPA was used as the fluorescent labeling agent (17). Instability of OPA-3-mercaptoalkenyl-GBP derivatives limits the use of the previous method in off-line pre-column derivatization (8). The handling of another method is not easy since they involved a multi-step derivatization with 2,4,6-trinitrobenzenesulfonic acid. Furthermore, a high LOQ (1 µg/mL) for this method was reported (7). Two new fluorescent labeling agents have been recently reported (17,18) for assessment of GBP with fluorescent detection via derivatization with FMOC or 4-chloro-7-nitrobenzofurazan. In the most routine assessment of GBP such as TDM, high sensitivity of the method is not needed because the concentration of GBP in steady state is much more than the LOQ in the reported methods by fluorescence detection. On the other hand, in the bioequivalence study usually the assessment of drug was extended up to three times the half-life. For a single 400 mg dose of GBP, the concentration is expected to be around > 200 ng/mL after 24 h (≅ three times the half-life).

In this regard, study with a simpler method using a general detector, UV, was considered. HPLC–UV detection for the determination of GBP in human plasma was reported (8), which GBP was labeled with phenylisothiocyanate. A 0.03 µg/mL of LOQ in plasma was reported using solid-phase extraction cartridge. An anhydrous condition for derivatization was mentioned to form GBP-PTIS.

4-Nitrophenylisothiocyanate was used to label the amino acids and UV detection (15), in which a stable thio-carbamyl derivative of amino acid which was formed.

In the present study a method was developed to determine GBP in serum with no need of solid phase clean-up sampling. Our method has enough sensitivity for TDM study or bioavailability or bioequivalence study till three half-lives. Anhydrous condition was not needed to perform derivatization. Optimization of the reaction was studied to provide high yield of the reaction. It was found that the pH of reaction media was very

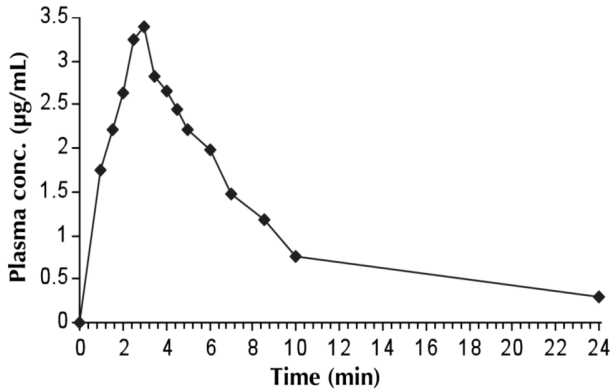

Figure 3. Mean serum concentrations-time profiles of GBP in 12 human volunteers after administration of single 400 mg oral dose.

Table I. Accuracy and Precision in Spiked Serum*

Conc. Added (µg/mL)	Conc. found (mean ± SD)	CV (%)	Error (%)
Intra-day (n = 5)			
0.12	0.13 ± 0.015	11.53	8.33
0.60	0.62 ± 0.034	5.48	3.33
3.00	2.98 ± 0.091	3.08	–0.67
Inter-day (n = 3)			
0.12	0.14 ± 0.020	14.28	16.67
0.60	0.63 ± 0.043	6.82	5.00
3.00	3.06 ± 0.111	3.62	2.00

* n = 15, five sets for three days.

critical to percentage of the yield. In general, the reaction took place in alkaline media. Several buffered solutions, such as phosphate and borate in pH range of 7–12, were used. None of them have enough efficacies to raise the yield of derivatization comparing to triethylamin (TEA).

The best condition was obtained with 50 µL of TEA solution in acetonitrile (2% v/v) to 250 µL of the mixture of solution before derivatization. Amounts less than 50 µL decreased the yield, and more than 50 µL resulted to fast destruction of derivatization reagent. Limiting the reagent concentration to 200 µg/mL reduced the peaks with retention time of 2–6 min that is related to the decomposition product of derivatization reagent or unknown endogenous compounds. However, with < 200 µg/mL reagent, peak intensity of GBP-thiourea derivative was reduced. The optimum time and temperature for the reaction were also studied. A temperature of 45°C was recognized as the optimum temperature because increasing the temperature did not enhance the yield. In addition, the reducing of the temperature decreased the yield. It was observed that the reaction was completed after 15 min at 45°C. However, the extension of time reaction resulted in reduction of the matrix effect resolution. A peak with retention time 8.79 min is related to the excess of derivatization reagents that could disappear with continuing of reaction time to 60 min. One phenomenon related to the low recovery of GBP was referred to hydrophilicity of GBP (8). Usage of octanesulfonic acid improved the recovery. Our experiment showed that the recovery without adding of octansulfonic acid was significantly low.

Several ISs were studied for the assay. Baclofen, which was previously used by Zhu, has short retention time. In our mobile phase, interference with several unknown peaks was observed. Several *N*-alkyl tryptophanes were synthesized, and *N*-propyltryptophane was used as IS in an experiment. Although the retention time of its thiourea derivatives was suitable, its lower reactivity with derivatization reagent in comparison to GBP limits its usage.

4-Chlorobenzylamine reacted with 4-nitrophenylisothiocyanat very quickly, but some artifact peaks were observed probably due to its high reactivity. Then, it was preferred to synthesize thiourea–IS and used it after derivatization. A good linearity was obtained in the presence of the last compound. Therefore, 1-(4-chlorobenzyl)-3-(4-nitrophenyl) thiourea was chosen as IS.

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