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Short communication

Sensitive high-performance liquid chromatographic quantitation of gabapentin in human serum using liquid–liquid extraction and pre-column derivatization with 9-fluorenylmethyl chloroformate

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Abstract

Most of the published methods for analysis of gabapentin, an antiepileptic agent, in human serum are based on the same approach, involving *o*-phthaldialdehyde derivatization of deproteinized serum samples. The present paper however, describes a new, simple and sensitive high-performance liquid chromatographic method for determination of gabapentin in human serum using liquid–liquid extraction and 9-fluorenylmethyl chloroformate (FMOC-Cl) as pre-column labeling agent. The drug and an internal standard (azithromycin) were extracted from serum by salting-out approach using a mixture of dichloromethane–2 propanol (1:1, v/v) as the extracting solvent. The extracted analytes were subjected to derivatization with FMOC-Cl in the presence of phosphate buffer (pH 7). A mobile phase consisting of methanol–0.05 M sodium phosphate buffer (73/27, v/v; pH of 3.9) containing 1 ml/l triethylamine was eluted and chromatographic separation was performed on a Shimpack CLC-C18 (150 mm × 4.6 mm) column. The standard curve was linear over the range of 0.03–20 μg/ml and limit of quantification was 0.03 μg/ml. The performance of analysis was studied and the validated method showed excellent performance in terms of selectivity, specificity, sensitivity, precision and accuracy. No interferences were found from commonly co-administered antiepileptic agents.
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1. Introduction

Gabapentin (GBP) is an antiepileptic drug and hydrophilic analogue of the γ-aminobutyric acid (GABA) which crosses the blood–brain barrier. It acts by increasing the GABA concentration in the brain and has been approved for treatment of partial seizure [1]. The drug is rapidly absorbed with time to peak plasma drug concentration of 2–3 h. GBP is not metabolized and the unchanged drug is directly excreted by the kidney with half-life of 5–8 h [1]. The role of therapeutic drug – monitoring 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. GBP has no significant ultraviolet, visible or fluorescence absorption and its quantitation in the biological fluids has been achieved using high-performance liquid chromatography (HPLC) with spectrophotometric [2] or fluorescence detections [3–11] following derivatization with 2,4,6-trinitrobenzene sulfonic acid [2], phenylisothiocyanate (PITC) [3], dansyl chloride [4], or *o*-phthalaldehyde (OPA) [5–11]. While the limit of quantifications (LOQ) of 1 μg/ml [2], 0.03 μg/ml [3], and the limit of detection of 0.3 μg/ml [4] have been reported using 2,4,6-trinitrobenzene sulfonic acid, PITC or dansyl chloride, respectively, different sensitivities (0.85 μg/ml [5], 1 μg/ml [6,11], 0.3 μg/ml [7], 0.1 μg/ml [8], 0.06 [9], and 0.281 μg/ml [10]) have been obtained with OPA as the fluorescent labeling agent. LOQs of 7.5 ng/ml and 2 μg/ml have also been reported using HPLC/MS [12] and GC/MS [13] techniques, respectively. From the published HPLC methods for analysis of GBP, quantification of the drug using OPA as labeling agent have frequently been reported. However, OPA derivative should be injected immediately after preparation, and the instability of the resulted adduct has hampered its use in pharmacokinetic studies and clinical drug monitoring. GBP is a polar

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amino acid and due to the marked hydrophilicity of the drug, sample preparation in the published methods involved protein precipitation [2,4–6,8–12] or solid phase extraction [3,7]. This paper describes the first report of liquid–liquid extraction and derivatization of GBP using 9-fluorenylmethyl chloroformate (FMOC-Cl) as the fluorescent labeling agent. As a highly fluorescent and stable adduct is produced using FMOC-Cl, the sensitivity and stability of our method has significantly been improved.

2. Experimental

2.1. Chemicals

Standard of GBP (purity 100.1%) was from Medichem (Barcelona, Spain) and kindly provided by Exir pharmaceutical company (Tehran, Iran). 9-Fluorenylmethyl chloroformate (FMOC) and azithromycin (I.S.) were from Sigma (St. Louis, MO, USA). Methanol (HPLC grade), dichloromethane, 2-propanol, sodium hydroxide, sodium dihydrogen phosphate, triethylamine, phosphoric acid, and glycine were purchased from Merck (Darmstadt, Germany). All reagents used were of analytical grade except methanol, which was of HPLC grade. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Apparatus

The chromatographic system which was used consisted of two high pressure pumps (LC-10AD), a column oven (CTO-10A), a spectroflurometric detector (RF-551) operated at an excitation and emission wavelengths of 260 and 315 nm, respectively, a degasser (DGU-3A), and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. A 7125i Rheodyne sample injector valve with 20 μl filling loop (Berkeley, CA, USA) was used. The analytical column was a Shimpack CLC-C18 (Shimadzu, Kyoto, Japan), 150 mm × 4.6 mm I.D., 5 μm particle size which was protected by a Shim-pack G-C18 guard column (1 cm × 4.0 mm I.D., 5 μm particle size). The mobile phase consisted of methanol–0.05 M sodium phosphate buffer (73/27, v/v) containing 1 ml/l triethylamine and was adjusted to a pH of 3.9 with o-phosphoric acid. The column oven temperature was set at 62 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 2.5 ml/min with a backpressure of 150 kg/cm^2.

2.3. Preparation of standard solutions

A stock solution of GBP (1000 μg/ml) was prepared in methanol. For working standards, the appropriate volumes of the stock solution were diluted with methanol to obtain different standards of the drug within the concentration range of 0.3–200 μg/ml. A working standard solution of the I.S. (200 μg/ml) was prepared in acetonitrile. A 500 μg/ml solution of FMOC-Cl was prepared in acetonitrile. A phosphate buffer (0.1 M) was prepared in water by adjusting the pH to 7.0 with 0.1 M sodium hydroxide solution. A stock solution of glycine

(4 mg/ml) was prepared in water. All solutions were stored at 4 °C and were stable for at least 4 weeks.

2.4. Standard curves, sample preparation and derivatization

Standard curves were constructed using pooled blank human serum. After evaporation of 100 μl from each working solution of the drug, under a gentle stream of nitrogen at 50 °C, the residues were reconstituted in 200 μl of drug-free human serum. In an Eppendorf tube, 200 μl of serum sample (blank, calibration or unknown), 100 μl of I.S., about 100 mg of sodium sulfate, and 1 ml of extracting solvent containing dichloromethane–2-propanol (1:1, v/v) were added. After a brief mixing for 10 s on a vortex mixer and centrifugation (3 min at 12,000 × g), the organic phase was removed and evaporated to dryness under a stream of nitrogen at 45 °C. To the residue, respective volumes of 100 and 25 μl of FMOC-Cl and phosphate buffer solutions were added and after a brief mixing, the samples were kept at 60 °C for 10 min. The reaction was stopped by adding 10 μl glycine (4 mg/ml) and, after 1 min, a volume (20 μl) of the reaction mixture was injected on to the chromatograph. Calibration curves (weighted regression line) were obtained by linear least-squares regression analysis plotting of peak-area ratios (GBP/I.S.) versus the drug concentrations.

2.5. Optimization of the derivatization conditions and method validation

The derivatization of GBP with FMOC-Cl was optimized using solutions of 0.5, 2 and 10 μg/ml of the drug, while the I.S. was reacted with the reagent at the concentration of 200 μg/ml. Concentrations of the FMOC-Cl solutions ranging from 100 to 2500 μg/ml, pH of the buffer solutions ranging from 6 to 11 and pH of the mobile phase ranging from 3 to 7 were tested to obtain the desired values. Various acetonitrile–water proportions, ranging from 1:1 to 10:1 were used to optimize the polarity of the reaction solution and the mixture was allowed to react in a water bath at a temperature ranging from 40 to 80 °C.

Average recoveries were measured using blank serum samples spiked with GBP and the I.S. at different concentrations (low: 0.03 μg/ml; medium: 2 μg/ml; and high: 10 μg/ml). These samples were analyzed in replicate as described and peak areas were compared with those obtained from derivatization of the same amounts of un-extracted samples. The specificity of the method was investigated by the analysis of human blank serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except for the addition of the I.S. The selectivity of the assay was evaluated by analysis of a group of potentially co-administered drugs with GBP. The limit of detection was defined as the concentration of drug giving a signal-to-noise ratio of 3:1. The lower limit of quantification was obtained as the lowest serum concentration of GBP quantified with a coefficient of variation of less than 20%. Within-day variation was measured by assessing the different controls in replicates of six. Between-day variation was based on the repeated analysis of the same concentration controls in 10 analytical run performed on different days.
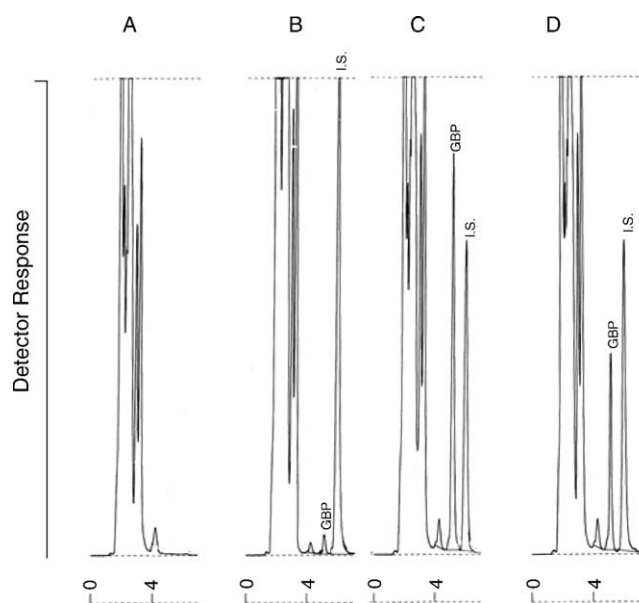
Fig. 1. Typical chromatograms obtained from an extract of (A) human blank serum, (B) human blank serum spiked with 0.06 μg/ml GBP and the I.S. and (C and D) serum samples obtained at 3 and 10 h after a single oral dose of 400 mg GBP from a healthy volunteer containing 6.1 and 3.0 μg/ml of the drug, respectively.

3. Results

3.1. Specificity and selectivity

The optimal conditions for reaction of GBP with the labeling agent were found to be a FMOC-Cl solution of 500 μg/ml, a buffer with pH of 7, a reaction temperature of 60 °C for 10 min, and a reaction medium consisting of the buffer–acetonitrile (1:4, v/v). Representative chromatograms of (A) human blank serum and (B) human blank serum spiked with GBP (0.06 μg/ml) and the I.S. are shown in Fig. 1. Endogenous components and excess of the reagent were chromatographed within 3.0 min and the peaks of analytes were well resolved from each other. The retention times for GBP and the I.S. were 5.1 and 6.1 min, respectively, with a total run time (injection-to-injection) of 8 min. Fig. 1C and D shows the chromatograms of serum samples obtained at 3 and 10 h after a single oral dose of 400 mg GBP (Neurontin, Parke-Davis) from a healthy adult volunteer,

respectively. The results of the selectivity study showed that the following drugs were not detected from the described analytical method: acetaminophen, naproxen, diclofenac, codeine, caffeine, phenytoin, phenobarbital carbamazepine, lamotrigine, zonisamide, topiramate, primidone, vigabatrin ethosuximide, clonazepam, diazepam, etidronate, gentamicin, ciprofloxacin, fluconazole, erythromycin, cefalexin, and ceftriaxone. Gentamicin, etidronate, topiramate, vigabatrin, and erythromycin reacted with the FMOC-Cl but did not give interference with the analysis of the I.S. or GBP.

3.1.1. Recovery, accuracy and precision

The recovery of GBP and the I.S. from serum was studied at low (0.03 μg/ml), medium (2 μg/ml), and high (10 μg/ml) concentrations and examined by extracting and derivatization of spiked serum samples, comparing with peak areas obtained after derivatization of the same amounts of un-extracted analytes solutions. The recoveries were found to be 90% for GBP and 97% for the I.S. The within- and between-day accuracy and precision values of the assay method are presented in Table 1. The coefficient variation values of both within- and between-day were all less than 15.2% whereas the percentage error was less than 7.7%.

3.1.2. Sensitivity, linearity and stability

The limits of detection and quantification were estimated to be 0.01 and 0.03 μg/ml, respectively using a volume of 200 μl serum sample and 20 μl injection. The standard calibration curves were linear over the concentration ranges of 0.03–20 μg/ml using a line-fit plot in regression analysis with a coefficient of 0.9932 and regression equation of $y = 0.0207x + 0.5629$. Intra- and inter-day reproducibility for calibration curves were determined on the same day in replicate ($n = 4$) and on different days ($n = 10$), respectively, using same-pooled serum sample. The intra-day average slope of the fitted straight lines was 0.0207 ± 0.0025 μg/ml (CV = 11.9%) and the mean intercept of the calibration curves was 0.5629 ± 0.0827 (CV = 14.7%). The corresponding mean ($\pm$S.D.) coefficient of the linear regression analysis was 0.9932 ± 0.0085 (CV = 0.85%). For calibration curves prepared on different days, the mean $\pm$ S.D. of results were as follows: slope = 0.0215 ± 0.0018 μg/ml (CV = 8.5%), coefficient of the linear regression analysis = 0.9928 ± 0.065 (CV = 0.67%) and

Table 1
Intra- and inter-day precision and accuracy for determination of GBP in human serum by the HPLC method

Known concentration (μg/ml)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (% mean deviation)
Inter-day			
0.03	0.028 ± 0.004	14.5	−7.7
0.2	0.185 ± 0.008	4.2	−7.7
2.0	1.98 ± 0.035	1.8	−0.6
20.0	20.7 ± 0.25	1.2	3.5
Intra-day			
0.03	0.027 ± 0.004	15.2	−7.2
0.2	0.190 ± 0.008	3.9	−4.5
2.0	2.08 ± 0.032	1.5	3.6
20.0	19.9 ± 0.23	1.2	−0.4

intercept = 0.6025 ± 0.0752 (CV = 12.5%). Stock solutions of GBP and azithromycin were stable for at least 30 days when stored at 4 °C. Derivatized solutions were found to be stable (>95%) for at least 12 h when the samples were kept at 4 °C using sample cooler. After 60 days maintenance of the serum at −80 °C and following three thaw–freeze cycles, the stability of the drug was found to be 101% from the initial value.

4. Discussion

Although several methods are now available for HPLC determination of GBP in human serum, however, this is the first report of liquid–liquid extraction and fluorescence detection of the drug using FMOC-Cl as the labeling agent. GBP is a polar drug and in previously published methods it has been extracted by either protein precipitation or solid phase extraction techniques. Precipitation of proteins with acetonitrile [2,4–6,11,12], methanol [8], perchloric acid [9,13], and trichloroacetic acid [10] was widely been used in sample preparation of the drug. However, dilution of the samples following protein precipitation with methanol or acetonitrile reduces the sensitivity of analysis. In the method described by Gauthier et al. [7] using C18 extraction column, LOQ of 0.01 μg/ml for 500 ml of serum sample was reported. By using the same extraction method with PITC as the labeling agent a sensitivity of 0.03 μg/ml was obtained by Zhu et al. [3]. Solid phase extraction is an expensive method and may not be available in some laboratories. Furthermore, because of the poor photo stability of PITC, special care is needed when it is used as the labeling agent. Thus, the extraction residue should be free of water, solution of PITC should be prepared daily, and usually dark place is needed for derivatization. The LOQ of 0.3 μg/ml for 50 μl injection and 50 μl of serum sample was reported by Krivanek et al. [4] using dansyl chloride as the fluorogenic agent. However, mixing of the drug with dansyl chloride in darkness medium for 20 min was needed in their method. In most of the published methods for determination of the drug, OPA has been used, but the fluorescent derivative produced by OPA is not stable, automated instrumentation is needed and these methods are difficult to apply to off-line techniques.

In our method, however, the reaction of the drug with FMOC-Cl is rapid, stable derivative is produced, the procedure is easy to perform, and more sensitivity is obtained. Liquid–liquid extractions of several polar agents using dichloromethane–2-propanol (1:1, v/v) have been reported in our laboratory [14,15]. However,

this mixture was not efficient for extraction of GBP from human serum. Thus, salting-out approach was tested and out of several chemical agents, sodium sulfate gave the best results. Hence, the drug was efficiently extracted using dichloromethane–2-propanol (1:1, v/v) as the extracting solvent in combination with the salting-out approach. The column temperature was set at 62 °C in our method (the temperature of the column can be raised up to 70 °C as recommended by the manufacturer); this temperature reduces the time of analysis, improves resolution and reduces the column backpressure.

In conclusion, a rapid, simple, and more sensitive method with the limit of quantification of 0.03 μg/ml for 200 μl of serum sample has been described in this paper. In the present method, simple liquid–liquid extraction and derivatization procedure have been used, the limit of quantification has been improved, and less time is needed for analysis of the drug.

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