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Short communication

Determination of gabapentin in human plasma and urine by high-performance liquid chromatography with UV–vis detection

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Abstract

A simple and reliable high-performance liquid chromatographic (HPLC) method with UV–vis detection has been developed and validated for the determination of gabapentin (GBP) in human plasma and urine. The clean up of the sample was carried out by solid-phase extraction with C18-cartridge. After the clean up procedure, the samples were pre-column derivatizated with 1,2-naphthoquinone-4-sulphonic acid sodium salt (NQS). A chromatographic separation was achieved on a C18 column with a mobile phase consisting of acetonitrile and 10 mM orthophosphoric acid (pH 2.5) with isocratic elution (35:65). Baclofen was used as an internal standard (I.S.). The method developed for GBP was linear over the concentration range of 0.05–5.0 μg/ml and 0.1–10.0 μg/ml for plasma and urine, respectively. The method is precise (relative standard deviation, R.S.D. <4.05%) and accurate (relative mean error, RME <0.15%); mean absolute recoveries were 72.21% for plasma and 72.73% for urine.

Keywords: Gabapentin; NQS; Biological fluids; HPLC

1. Introduction

Gabapentin (GBP) (1-(aminomethyl)cyclohexaneacetic acid) (Fig. 1A), is a new antiepileptic drug which is a structural analogue of neurotransmitter γ-aminobutyric acid (GABA). GBP, unlike GABA, has a cyclohexane molecule system and is able to penetrate through blood–brain barrier. GBP is used for the treatment of partial onset seizures with or without secondary generalized tonic-clonic convulsions in clinical practice. After oral administration, GBP is well absorbed and reaches maximal plasma concentrations within 2–3 h. The elimination half-life of the drug is 5–7 h after a single oral dose of 200–400 mg. GBP is not metabolized and mainly excreted by kidney. The drug does not bind plasma proteins. Pharmacokinetics of GBP is not affected by foods and other drugs [1,2].

Several pharmacokinetic or therapeutic drug monitoring studies have been reported for the determination of GBP in human biological fluids. For gas chromatography (GC), different detection methods are reported such as flame ionization [3,4] and mass spectrometry (MS) [5]. These methods require derivatization of GBP to improve the volatility and to avoid column interactions. High-performance liquid chromatography (HPLC) is also used for this purpose with spectrophotometric [6–9] and spectrofluorimetric [10–17] detections. 2,4,6-Trinitrobenzene-sulphonic acid (TNBS) [6–8] and phenylisothiocyanate (PITC) [9] are used in spectrophotometric detection while *o*-phthaldialdeyde (OPA) [10–17] is used in spectrofluorimetric detection as derivatizing reagents. Methods with tandem MS detection systems, where there is no need for derivatization have also been reported [18,19].

In the developed capillary electrophoresis (CE) methods; fluorescamine [20] and 6-carboxyfluorescein succinimidyl ester [21] are used to obtain fluorophore.

In the present study, an HPLC method with UV–vis detection is described for the determination of GBP in plasma and urine. The procedure is based on the off-line derivatization of the drug with 1,2-naphthoquinone-4-sulphonic acid sodium salt (NQS), the color labeling reagent for primary and secondary amines. NQS has been used in HPLC analyses for both off-line [22,23] and on-line derivatizations [24–26]. These methods usually employ UV–vis [22,23,25] and fluorimetric [24,26] detections, however electrometric detection [27] has also been used. In this study, NQS derivatization has provided the sensitivity required for the pharmacokinetic study of GBP.

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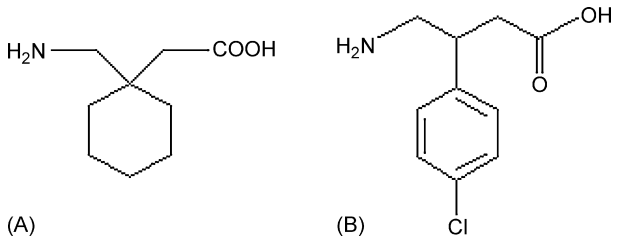

Fig. 1. Chemical structure of GBP (A) and I.S. (B).

2. Experimental

2.1. Chemicals and reagents

GBP and its capsules (Neurontin®, 300 mg gabapentin per capsule) were kindly provided by Pfizer (Istanbul, Turkey). The internal standard (I.S.), baclofen was provided from Novartis (Istanbul, Turkey). NQS was purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, chloroform and all other chemicals and solvents were obtained from Merck (Darmstadt, Germany). Water was deionized and purified by a Milli-Q water purification system from Millipore (Bedford, MA, USA). C18-cartridges (100 mg, 1.0 ml) were purchased from Alltech (IL-USA). Blood plasma was obtained from healthy human volunteers and collected into tubes treated with disodium EDTA as anticoagulant. Plasma and urine samples were stored at approximately −20 °C until they were analyzed.

2.2. Preparation of stock solutions

The stock solution of GBP (1 mg/ml) was prepared and diluted with water to give standard solutions of 0.5–50.0 μg/ml. Standard calibration samples were prepared daily by spiking 1 ml of drug-free human plasma or urine (diluted with water to a ratio of 1:1) with 0.1 ml of appropriate GBP standard solutions to achieve final concentrations of 0.05–5.0 μg/ml for plasma and 0.1–10.0 μg/ml for urine.

Baclofen (I.S.) (β-(aminomethyl)-4-chlorobenzenepropanoic acid) (Fig. 1B) and GBP can be determined simultaneously by HPLC with a good separation and baclofen has a similar retention time to GBP, therefore, baclofen was chosen as I.S. in this study. The working solution of I.S. was prepared by dissolving in water to obtain a concentration of 10 μg/ml.

The reagent solution was freshly prepared in water at 3 mg/ml concentration for the analysis of plasma and urine samples.

Borate buffer was prepared by dissolving 0.620 g of boric acid and 0.750 g of potassium chloride in 100 ml water. The pH level was adjusted to 8.5 with 0.1 M sodium hydroxide solution and the volume was made up to 200 ml with water.

2.3. HPLC system

A Shimadzu (Kyoto, Japan) LC 10 liquid chromatograph consisted of a LC 10 AT solvent delivery system, a Rheodyn injection system with a loop of 20 μl and a CTO 10 A column oven was used at room temperature. SPD 10 A spectrophotometric detector was set at 458 nm. Separation was performed on a Phenomenex C18-column, 5 μm (250 mm × 4.6 mm i.d.) with a guard column (4.0 mm × 3.0 mm i.d.) packed with the same material.

The mobile phase consisting of acetonitrile −10 mM orthophosphoric acid (pH 2.5) (35:65) was delivered as an isocratic elution at a flow rate of 1 ml/min. Before use the mobile phase was degassed by an ultrasonic bath and filtered by a Millipore vacuum filter system equipped with a 0.45 μm HV filter. The data were collected and analyzed via the automation system software.

2.4. Sample preparation and derivatization

Blood samples were collected into the tubes containing disodium EDTA and centrifuged at 4500 × *g* for 10 min. A 1.0 ml of the resultant plasma and urine samples were spiked with 0.1 ml of GBP, 0.1 ml of internal standard and 0.5 ml 1 M NaH_2PO_4 solutions and mixed on a vortex mixer (urine samples were diluted with water to a ratio of 1:1). Samples were applied to the C18-cartridge, which was preconditioned with methanol (4 ml) followed by water (2 ml) and then 1 M NaH_2PO_4 (2 ml). After loading the sample, the cartridge was washed with 0.1 M NaH_2PO_4 (1 ml), 0.1 M HCl (2 ml) and then dichloromethane (1 ml). After the cartridge was dried applying vacuum for 5 min, samples were eluted with methanol (1 ml) and the eluate was evaporated to dryness at 50 °C on a block heater, under nitrogen. The residue was dissolved with 0.5 ml of borate buffer (pH 8.5) and 0.2 ml of NQS solutions the sample was kept at 60 °C for 20 min. Then the mixture was extracted with 2 × 2.5 ml of chloroform after cooling and acidifying with 0.5 ml of 0.1 M HCl. A 4 ml aliquot of the organic phase was evaporated to dryness. The residue was dissolved with 0.1 ml of mobile phase and the solution was injected into the HPLC system.

2.5. Validation

2.5.1. Specificity

Preparation of plasma and urine samples were processed by this solid-phase extraction procedure and samples are chromatographed to determine to which extent endogenous plasma and urine components may contribute to the peak interference at retention time of analyt and internal standard.

2.5.2. Linearity

The linearity of the method was evaluated by a calibration curve in the range of 0.5–50 μg/ml of the drug ($n = 5$). Drug-free plasma and urine were spiked GBP standard solutions to achieve final concentrations of 0.05, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μg/ml for plasma and 0.1, 0.4, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 μg/ml for urine samples. The samples were assayed using the method described above. Calibration graphs were prepared by plotting the peak area ratios of GBP to I.S. versus the drug concentrations with least-squares linear regression analysis.

The quality control (QC) samples were separately prepared in blank plasma at the concentrations of 0.05, 0.5 and 5.0 μg/ml for plasma and 0.1, 1.0 and 10.0 μg/ml for urine, respectively.

2.5.3. Recovery

Absolute recoveries of GBP at three QC levels (0.05, 0.5 and 5.0 μg/ml for plasma and 0.1, 1.0 and 10.0 μg/ml for urine) ($n = 5$) were measured by comparing the peak area of the drug obtained from the plasma with peak area obtained by the direct injection of pure aqueous drug standard. The mean recovery of the drug at three QC levels (0.05, 0.5 and 5.0 μg/ml for plasma and 0.1, 1.0 and 10.0 μg/ml for urine) was calculated by comparing the concentration obtained from the drug supplemented plasma to the actually added concentration.

2.5.4. Precision and accuracy

Intra-day and inter-day precision and accuracy were determined in plasma and urine samples by determining QC samples at three concentration levels (0.05, 0.5 and 5.0 μg/ml for plasma and 0.1, 1.0 and 10.0 μg/ml for urine). For intra-day assay precision and accuracy, six replicates of samples at each concentration were assayed all at once within day. The inter-day assay precision and accuracy was determined by analyzing samples on five different days. Six replicates at each concentration were assayed per day.

2.5.5. Sensitivity

The sensitivity was evaluated by the lower limit of quantitation (LOQ), the lowest concentration of the plasma and urine spiked with GBP in the calibration curve. The limit of detection (LOD) was determined as the lowest concentration, which gives a signal-to-noise ratio of 3 for GBP.

2.5.6. Stability

The stability of GBP and I.S. standard solutions were tested at several storage conditions (room temperature for 2 weeks and 4 °C for 1 month). The stability of GBP-NQ derivative in the extraction solvent was determined at 4 °C.

The freeze-thaw stability of GBP in plasma and urine samples was evaluated over three freeze-thaw cycles. Stability control plasma and urine samples in triplicate at the levels of 0.05, 0.5 and 5.0 μg/ml for plasma and 0.1, 1.0 and 10.0 μg/ml for urine were immediately frozen at −20 °C, and thawed at room temperature three consecutive times. After that, the samples were processed and assayed. The stability of GBP in spiked plasma and urine samples stored at room temperature for 24 h and −20 °C for 2 weeks was evaluated as well. Long-term stability was assessed using samples stored at −20 °C over a period of 8 weeks.

2.6. Pharmacokinetic study

This developed method was applied to investigate the plasma profile of GBP in healthy male volunteer (aged 34 years, weighing 80 kg), after an oral administration of 300 mg GBP (single dose Neurontin®, 300 mg/capsule). Venous blood samples (5 ml) were collected into the tubes containing disodium EDTA at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 18.0, 24.0, 36.0 and 48.0 h after dose administration. Blood samples were centrifuged at 4500 × *g* for 10 min and the plasma was separated and kept frozen at −20 °C until analysis. Urine samples were also collected at intervals for up to 72 h and stored at −20 °C until analysis. Collected urine samples were diluted between 1:50 and 1:500 with water, depending on its concentration before analysis.

3. Results

3.1. Specificity and separation

It was determined that solid-phase extraction process was necessary at the sample preparation procedure. Different solid-phase systems were tried at this purpose and C_{18} cartridges were chosen. By this way GBP was adequately separated from closely eluting endogenous amino acids existing in the biological samples. Following this procedure, the samples were derivatizated with NQS reagent. Extraction of the NQ-derivates from reaction mixtures with chloroform (liquid–liquid extraction) minimized the extraction of the excess of NQS and its degradation products. The unextracted amino acids with C18-cartridges were also cleaned up by this step.

To obtain sharper and symmetrical peak, acidic mobile phase system used with acetonitrile at 25 °C. The retention times of GBP – and internal standard – NQ derivatives were 12.33 and 13.64 min, respectively and the total run time of analysis was 15 min. Representative chromatograms of (A) drug-free plasma, (B) the plasma spiked with GBP (3.0 μg/ml) and internal standard (1.0 μg/ml), (C) the plasma obtained at 2.5 h after a single dose of 300 mg GBP, (D) drug-free urine, (E) the urine spiked with GBP (1 μg/ml) and internal standard (1.0 μg/ml) and (F) the urine obtained at 10 h after a single dose of 300 mg GBP were given in Fig. 2. There is no interference in the chromatogram of drug-free plasma and urine.

Commonly prescribed antiepileptic drugs (carbamazapine and its epoxide and hydroxyl metabolites, valproic acid, pirimidone, phenobarbital, clonazepam, ethosuximide, lamotrigine, vigabatrin) were analysed for possible interference. No interference was observed because they did not react with NQS except vigabatrin, which was derivatized but showed no interference peak under the chromatographic conditions.

3.2. Calibration and linearity

Calibration curves were linear over the range 0.05–5.0 μg/ml for plasma and 0.1–10.0 μg/ml for urine. The regression equations were as follows:

$A = 0.6791C - 0.0134$ ($r^2 = 0.9995$), for plasma and $A = 0.5598C + 0.0143$ ($r^2 = 0.9995$), for urine samples, where A is the peak area ratios ($A_{GBP}/A_{I.S.}$) and C is the concentration of GBP (μg/ml).

3.3. Recovery

As shown in the Table 1, the mean absolute recoveries of GBP were of 72.21% for plasma and 72.73% for urine. The mean relative recoveries of GBP were of 71.11% for plasma and 72.73% for urine. The mean recovery of the internal standard was

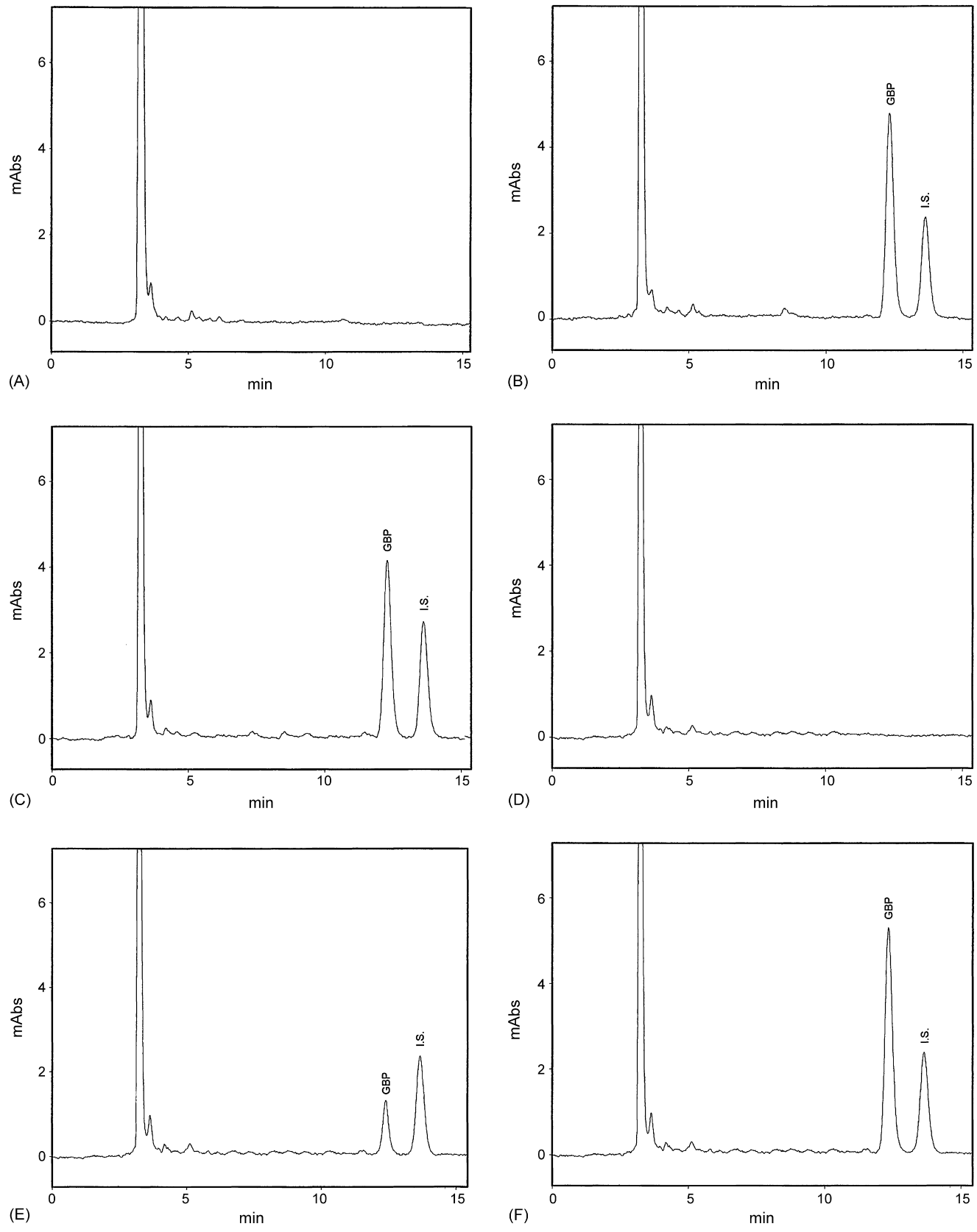

Fig. 2. Representative chromatograms of (A) drug-free plasma, (B) the plasma spiked with GBP (3.0 μg/ml) and internal standard (1.0 μg/ml), (C) the plasma obtained at 2.5 h after a single oral dose of 300 mg GBP, (D) drug-free urine, (E) the urine spiked with GBP (1 μg/ml) and internal standard (1.0 μg/ml) and (F) the urine obtained at 10 h after a single oral dose of 300 mg GBP.

Table 1
Absolute and relative recovery of GBP from plasma and urine ($n = 5$)

Sample	Concentration (μg/ml)		Recovery (%)	R.S.D. (%)
	Added	Found (mean ± S.D.)		
Absolute				
Plasma	0.05	0.0349 ± 0.0023	69.80	6.59
	0.50	0.4033 ± 0.0149	80.66	3.70
	5.00	3.3080 ± 0.1376	66.16	4.16
Urine	0.10	0.0758 ± 0.0053	75.80	6.99
	1.00	0.7878 ± 0.0266	78.78	3.38
	10.0	6.3611 ± 0.1845	63.61	2.90
Relative				
Plasma	0.05	0.0344 ± 0.0025	68.80	6.44
	0.50	0.3936 ± 0.0189	78.73	3.30
	5.00	3.2903 ± 0.1461	65.81	4.38
Urine	0.10	0.0741 ± 0.0052	74.07	6.46
	1.00	0.7799 ± 0.0427	77.99	3.83
	10.0	6.3312 ± 0.2260	63.11	3.01

found to be 72.11% and 71.22% for plasma and urine. The results in Table 1 show no clear relationship between concentration and recovery.

3.4. Precision and accuracy

The values of precision and accuracy of GBP are summarized in Table 2. Intra-day and inter-day relative standard deviation (R.S.D.) values were found within 2.01% and 4.05% for plasma and 0.79% and 3.24% for urine, respectively. The results were determined analysing the samples spiked with GBP at three different concentrations. Accuracy of the method expressed as relative mean error (RME) was below 0.15%.

3.5. Sensitivity

The limit of quantitation values for each sample were accepted as the lowest concentration on the calibration curves for 0.05 μg/ml and 0.1 μg/ml that the plasma and urine, respectively. Under the experimental conditions, the lower limit of detection values were 0.02 μg/ml for plasma and 0.05 μg/ml for urine, with a signal to noise ratio 3.

Table 2
Intra-day and inter-day precision and accuracy of GBP in plasma and urine ($n = 6$)

Sample	Concentration (μg/ml)		R.S.D. (%)	R.M.E. (%)
	Added	Found (mean ± S.D.)		
Plasma				
Intra-day	0.05	0.0501 ± 0.0014	2.69	0.15
	0.50	0.4855 ± 0.0166	3.42	−2.91
	5.00	4.7776 ± 0.1507	3.16	−4.45
Inter-day	0.05	0.0501 ± 0.0014	3.02	−0.88
	0.50	0.4953 ± 0.0200	4.05	−0.95
	5.00	4.8768 ± 0.0981	2.01	−2.46
Urine				
Intra-day	0.10	0.0995 ± 0.0015	1.46	−0.55
	1.00	0.9834 ± 0.0275	2.80	−1.66
	10.0	9.9877 ± 0.0784	0.79	−0.12
Inter-day	0.10	0.0978 ± 0.0016	1.59	−2.22
	1.00	0.9800 ± 0.0317	3.24	−2.00
	10.0	9.8332 ± 0.1727	1.76	−1.67

3.6. Stability

The derivative of GBP-NQ was stable in this solvent for at least 48 h at 4 °C in the dark. The stability of stock solutions of GBP in water was checked and proved to be stable for at least 1 month at 4 °C. The stock solution of I.S. in water was stable for 2 weeks at 4 °C.

The stabilities of drug and I.S. in a biological fluid are affected by the chemical properties of drug and I.S., the storage conditions, the matrix effects. The stability of GBP under various conditions is described in Table 3. Under all conditions tested, GBP was stable with detected concentrations of at least 83.64% for plasma, 84.61% for urine samples of the initial concentration.

3.7. Pharmacokinetic study

To check the clinical applicability of the method, the pharmacokinetic parameters of GBP was investigated in a healthy male volunteer after a single oral administration of 300 mg of the drug. The concentration-time profile is shown in Fig. 3. The results of analysis revealed a maximum plasma concentration (C_{max}) of 2.256 μg/ml in plasma, which was reached at 3 h (t_{max}). The elimination half-life ($t_{1/2}$) and the area under the

Table 3
Stability of GBP in plasma and urine

Treatment	Recovery (mean ± S.D.) (%)					
	Plasma concentration (μg/ml)			Urine concentration (μg/ml)		
	0.05	0.50	5.00	0.10	1.00	10.00
Three freeze-thaw cycles	96.40 ± 0.0022	97.33 ± 0.0306	98.47 ± 0.1124	95.93 ± 0.0054	98.67 ± 0.0404	99.63 ± 0.0153
Stored at RT for 24 h[a]	83.64 ± 0.0014	82.00 ± 0.0265	83.60 ± 0.2138	85.21 ± 0.0018	84.67 ± 0.0473	84.03 ± 0.2730
Stored at −20 °C for 2 weeks	91.49 ± 0.0153	90.67 ± 0.0153	90.33 ± 0.2875	92.95 ± 0.0037	92.33 ± 0.0153	93.83 ± 0.4980
Stored at −20 °C for 8 weeks	84.00 ± 0.0037	84.00 ± 0.0265	85.33 ± 0.3266	84.61 ± 0.0045	85.67 ± 0.0058	85.60 ± 0.3500

[a] RT, room temperature.

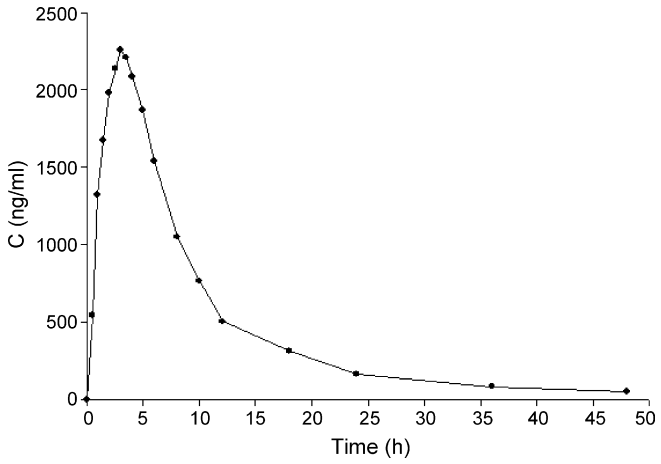

Fig. 3. Plasma GBP concentration-time profile for a healthy human male volunteer who took a single oral dose of GBP, 300 mg.

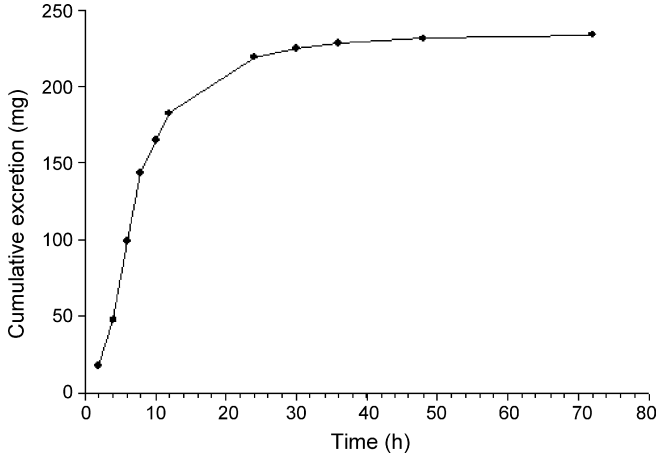

Fig. 4. Cumulative excretion of GBP in urine of a healthy human male volunteer after a single oral dose of GBP, 300 mg.

curve ($AUC_{0–48h}$) were calculated as 4.09 h and 22.12 μg h/ml, respectively. The cumulative urinary excretion of GBP is shown in Fig. 4. It appears that approximately 78% of the administered drug was excreted unchanged, within 72 h after oral administration. Pharmacokinetics results obtained using this method are agreement with those of the studies reported previously [1,28].

4. Discussion

GBP shows weak absorption band in UV range. UV-spectrophotometric methods sensitivity is not enough for the determination of GBP in biological samples. Attachments of chromophoric group to GBP increases the sensitivity of its detection. For this reason, NQS was chosen as a chromagenic derivatization reagent. NQ derivative of GBP in the extraction solvent was stable for at least 48 h at 4 °C.

In this study, UV–vis detector was preferred to fluorescence detector because the level of GBP in biological fluids is sufficient for detection by UV–vis detector. Moreover, this detector is commonly available in a laboratory and not expensive as the fluorescence detector. In addition, the used reagent is cheaper than fluorimetric reagents. During method development, it became evident that gabapentin and internal standard were very sensitive to matrix effects during the derivatization process in plasma and urine. Sample preparation techniques, such as liquid–liquid and solid-phase extraction were used in order to minimise matrix suppression effects.

When compared to some HPLC studies carried out with fluorescence detection [10–12,14–16] (LOD values of these studies were in the range of 0.050–0.85 μg/ml) the sensitivity of the proposed method is low enough (0.02 μg/ml) to use it for drug monitoring of GBP. However, there is a HPLC-fluorescence detection method [13] with a detection limit lower (0.01 μg/ml) than this method in term of sensitivity. When compared to OPA derivatization, the presented method offers an advantage, because NQS derivatization provides highly stable derivatives extractable into an organic phase. Among the HPLC analyses, there are four reports [6–9] involving UV–vis detection after TNBS [6–8] and PITC [9] derivatizations. Among these methods [6,7], minimal explanation of the method's validation data forms the major drawback. PITC method [9] is a simple process yet PITC reagent degrades when it is in contact with water. For this reason, this procedure has a handicap in term of reagent. The extraction residue is free from water before the addition of reagent and PITC should be stored in an airtight container and exposure to air should be minimized during its use.

5. Conclusion

As mentioned in introduction part, the GC method [4] lack selectivity because of multiple background peaks produced during the derivatization of coextracted endogenous compounds and aminoacids. The same method was also developed for pharmacokinetic study but it has not enough sensitivity for this purpose. LC-MS [18,19] techniques were developed for biological sample studies. In terms of specificity and selectivity MS detection is superior to UV methods and the required time for the analysis is relatively shorter. However, MS detection is not readily applicable for many researchers since they generally require expensive devices.

The developed method is relatively simple and rapid to perform, requires solid-phase extraction and one step derivatization prior to chromatography. The method showed high selectivity, precision and accuracy for the use in pharmacokinetic study and therapeutic monitoring of GBP.

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