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Sensitive microanalysis of gabapentin by high-performance liquid chromatography in human serum using pre-column derivatization with 4-chloro-7-nitrobenzofurazan: Application to a bioequivalence study

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Abstract

Most of the published methods for analysis of gabapentin, an antiepileptic agent, in human serum require automated *o*-phthalaldehyde derivatization of the drug and immediate injection of the unstable derivatives formed. A new, very sensitive and simple high-performance liquid chromatographic method for quantitation of the drug in human serum using 4-chloro-7-nitrobenzofurazan (NBD-Cl) as a fluorescent labeling agent is presented. In this method the sensitivity was significantly improved and the limit of quantification of 0.002 μg/ml was obtained using 100 μl serum sample and 10 μl injection. However, the LOQ can be improved by increasing the sampling volume. The procedure involved protein precipitation of serum by acetonitrile followed by derivatization with NBD-Cl. Amlodipine was used as internal standard and chromatographic separation was performed on a Shimpack CLC-C18 (150 mm × 4.6 mm) column. The fluorescence derivative of the drug was monitored at excitation and emission wavelengths of 470 and 537 nm, respectively. A mobile phase consisting of methanol and sodium phosphate buffer (0.05 M; pH 2.5) containing 1 ml/l triethylamine (65:35, v/v) was used. The calibration curve was linear over the concentration range of 0.002–15 μg/ml. No interferences were found from commonly co-administrated antiepileptic drugs. The method was applied in a randomized cross-over bioequivalence study of two different gabapentin preparations in 24 healthy volunteers.

Keywords: HPLC; Gabapentin; Serum; 4-Chloro-7-nitrobenzofurazan; NBD-Cl

1. Introduction

Gabapentin (GBP) is a structural analogue of the inhibitory transmitter γ-aminobutyric acid (GABA) which has been approved for treatment of partial seizure. The exact mechanism of action of the drug is not fully understood however, it may be related to enhance the activity of GABA in the brain. Absorption of the drug is rapid with time to peak plasma concentration of 2–3 h. GBP is not metabolized in the liver 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 unknown however; a reliable and simple method of analysis is needed to support clinical and pharmacokinetic studies. Several analytical methods including high-performance liquid chromatography (HPLC) [2–13], gas chromatography coupled with mass spectrometry [14] as well as capillary electrophoresis [15,16] have been published for determination of the drug in biological fluids. As GBP has no significant ultraviolet, visible or fluorescence absorption, analysis of the drug have been achieved following its derivatization to produce a chromophore, detectable by fluorescence or UV detectors. Although different labeling agents including 2,4,6-trinitrobenzene sulfonic acid [2], phenylisothiocyanate (PITC) [3], dansyl chloride [4] or *o*-phthalaldehyde (OPA) [5–11] have been applied for assay of GBP, however, OPA has been used in the most of the published papers. Due to instability of the derivative formed with OPA, automated procedure is needed and the derivative should be injected immediately after preparation. Also the PITC and dansyl derivatives are limited by their poor photo stability. While limit of quantifications (LOQ) of 1 μg/ml [2], 0.03 μg/ml [3], and limit of detection of 0.3 μg/ml [4] have been reported using 2,4,6-trinitrobenzene

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sulfonic acid, PITC or dansyl chloride, respectively, different sensitivity (0.85 μg/ml [5], 1 μg/ml [6,11], 0.3 μg/ml [7], 0.1 μg/ml [8], 0.06 [9], 0.281 μg/ml [10]) have been obtained with OPA. LOQs of 7.5 ng/ml [12], 50 ng/ml [13] and 2 μg/ml have also been reported using the HPLC/MS and GC/MS techniques, respectively.

4-Chloro-7-nitrobenzofurazan (NBD-Cl) is a suitable labeling agent which reacts with both primary and secondary amines. In our knowledge the present paper describes first report of determination of GBP in human serum using HPLC after pre-column derivatization with NBD-Cl. In contrast to OPA, the derivatives formed by NBD-Cl are stable with absorption maximum at the visible region, hence low background noises are provided and higher signal/noise ratio is obtained. Therefore, the method presented here which has been approved in a bioequivalence study of two different GBP preparations, is very sensitive with LOQ of 0.002 μg/ml using 100 μl serum sample and 10 μl injection.

2. Experimental

2.1. Chemicals and standard solutions

GBP (purity 100.1%) was from Medichem (Barcelona, Spain) and kindly provided by Exir pharmaceutical company (Tehran, Iran). Amlodipine (I.S.) was from Sigma (St. Louis, MO, USA). Methanol (HPLC grade), sodium hydroxide, sodium dihydrogen phosphate, triethylamine, phosphoric acid and NBD-Cl were purchased from Merck (Darmstadt, Germany). All reagents used were of analytical grade except methanol which was HPLC grade. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England). A stock solution of GBP (1000 μg/ml) was prepared in methanol. Working standards of the drug (0.02–150 μg/ml) were prepared by serial dilution of the stock solution in methanol. A working standard solution of the I.S. (400 μg/ml) was prepared in a mixture of methanol–acetonitrile (1:9, v/v). A 2.5 mg/ml solution of NBD-Cl was prepared in a mixture of methanol–acetonitrile (1:1, v/v). A phosphate buffer (0.05 M) was prepared in water and adjusted to pH 11.0 with 0.05 M sodium hydroxide solution. A stock solution of glycine (20 mg/ml) was prepared in water. All solutions were stored at 4 °C and were stable for at least 3 weeks.

2.2. Sample preparation and derivatization

In an Eppendorf tube 100 μl serum samples (blank, calibration or unknown), 100 μl of I.S. and 400 μl acetonitrile were added. After brief mixing for 10 s on a vortex mixer and centrifugation for 3 min at 12,000 × *g*, the liquid phase was removed and evaporated to dryness under stream of nitrogen at 50 °C. The residue was dissolved in 100 μl of NBD-Cl solution and after addition of the phosphate buffer (25 μl), and brief mixing for 10 s on a vortex mixer, the samples were kept at 60 °C for 10 min. The reaction was stopped by adding 40 μl glycine and, after a further 1 min, 10 μl of the reaction mixture was injected onto the chromatographic column.

2.3. Chromatographic conditions

The chromatographic system used consisted of two high pressure pumps (LC-10AD), a column oven (CTO-10A), a spectroflurometric detector (RF-551) operated at excitation and emission wavelengths of 470 and 537 nm, respectively, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu (Kyoto, Japan). Separation was performed on a Shimpack CLC-C18 column (Shimadzu, Kyoto, Japan; 150 mm × 4.6 mm I.D., 5 μm) which was protected by a Shim-pack G-C18 guard column (1 cm × 4.0 mm I.D., 5 μm particle size). Isocratic elution was performed with methanol–0.05 M sodium phosphate buffer (65:35, v/v) containing 1 ml/l triethylamine adjusted to a pH of 2.5 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 2.2 ml/min with backpressure of 15 MPa.

Standard curves were obtained using pooled blank human serum. After evaporation of 100 μl from each working solutions of the drug, under a gentle stream of nitrogen at 50 °C, the residues were reconstituted in 100 μl of drug-free human serum. 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.

2.4. Optimization of the derivatization conditions

Solutions of 0.005, 1 and 10 μg/ml of the drug were used to optimize derivatization of GBP with NBD-Cl while, the I.S. was reacted with the reagent at the concentration of 400 μg/ml. Concentrations of the NBD-Cl solutions ranging from 0.5 to 10 mg/ml, pH of the buffer solutions ranging from 6 to 12 and pH of the mobile phase ranging from 2.2 to 7 were tested to obtain optimal conditions for analysis. Various organic solvents–water proportions, ranging from 1:1 to 10:1 were used to optimize the polarity of the reaction solution and the mixtures was allowed to react in a water bath at temperature ranging from 40 to 80 °C.

2.5. Method validation

Average recoveries of the extraction procedure for both GBP and the I.S. were estimated by comparing the peak areas obtained from derivatization of an extracted spiked sample blank, with those obtained from derivatization of the similarly treated standard. The limit of detection was defined as a peak height that produces three times of baseline noise. The lower limit of quantification (LOQ) was estimated as the lowest concentration that could be quantified with a coefficient of variation of less than 20%. Inter-day variation was measured by assessing the different controls in replicates of six. Intra-day variation was based on repeated analysis of the same concentration controls in ten analytical runs performed on different days. To examine the possible interferences of endogenous compounds, 24 human serum samples from different volunteers, were extracted and analyzed during validation studies. These samples were pre-

treated according to the sample preparation procedure, except from the addition of the I.S. The selectivity of the assay was verified by checking for interferences with a group of drugs that might be co-administered.

2.6. Application of the method

The present analytical method was validated in a randomized crossover bioequivalence study. The study protocol was approved by Medical Ethics Committee of the University. Twenty-four healthy volunteers were divided in two groups. They received a single oral dose of 400 mg GBP from either Exir (Tehran, Iran) or Park-Davis (Neurontin; UK) pharmaceutical companies after an overnight fasting. All the subjects were asked to refrain from food or water consumption for 3 h after drug administration. After a two weeks wash-out period the subjects were crossed-over. The blood samples were collected at suitable intervals up to 24 h after drug administration. Pharmacokinetic parameters were calculated and compared using a paired Student's-test. Statistical significance was defined at the level of $p < 0.05$.

3. Results

3.1. Derivatization

NBD-Cl reacts with GBP in alkaline medium and the derivatization is complete within 10 min at 60 °C. In our method the optimal conditions for reaction of GBP with the labeling agent were found to be: a NBD-Cl solution of 2.5 mg/ml, a buffer with pH of 11, a reaction temperature of 60 °C for 10 min and a reaction medium consisting of buffer–acetonitrile:methanol (1:2:2, v/v).

3.2. Chromatography

The method showed excellent chromatographic specificity with no endogenous serum interference at the retention times of GBP and the I.S. In Fig. 1, chromatograms of (A) human blank serum and (B) human blank serum spiked with GBP (0.002 μg/ml) and the I.S. are presented. Endogenous components and excess of the reagent were chromatographed within 2.5 min and the peaks of the analytes were well resolved from

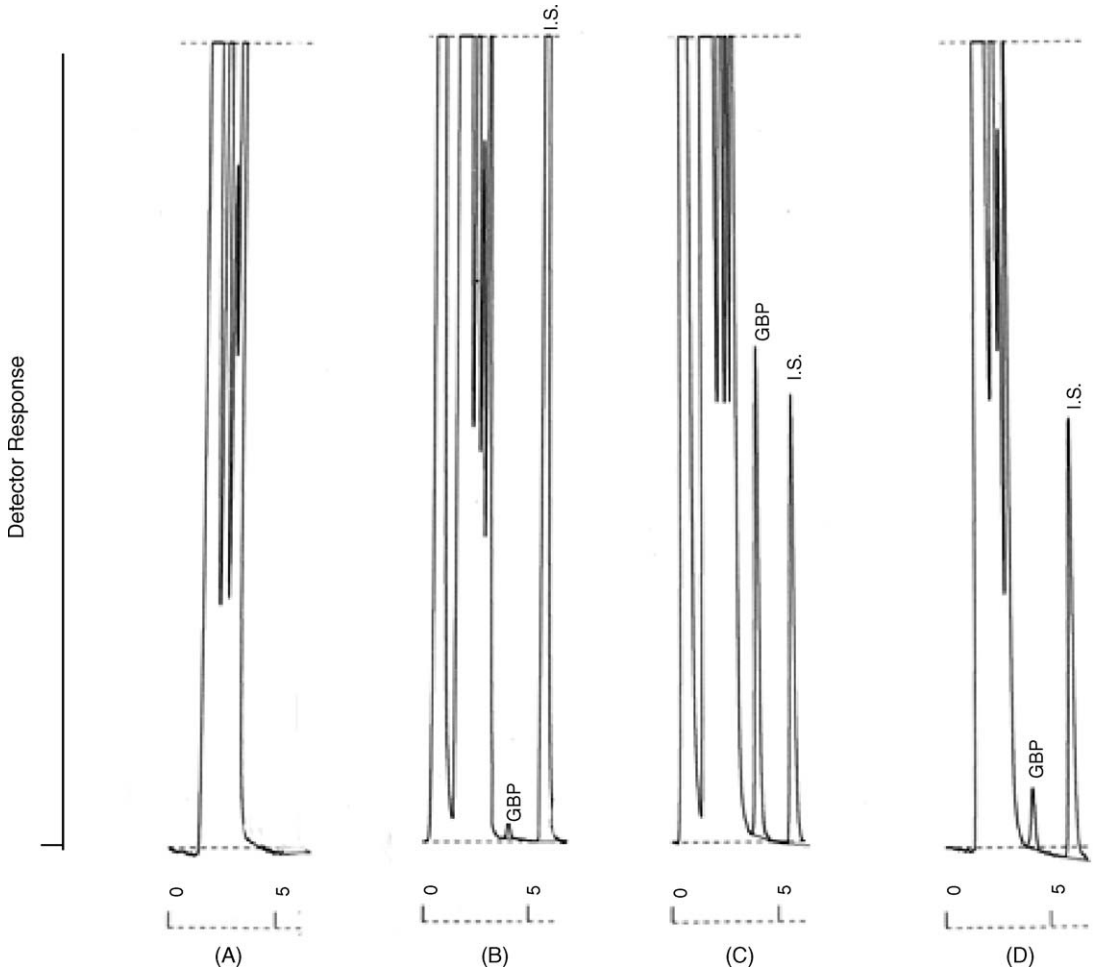

Fig. 1. Typical chromatograms obtained from an extract of (A) human blank serum (B) human blank serum spiked with 0.002 μg/ml GBP and the I.S, and (C and D) serum samples obtained at 3 and 24 h after a single oral dose of 400 mg GBP from a healthy volunteer containing 3.1 and 0.38 μg/ml of the drug, respectively (A and B ATEN = 2^4, 16 mv/full scale; C and D ATEN = 2^8, 256 mv/full scale).

Table 1
Assay linearity for analysis of GBP in human serum by the HPLC method

	Correlation coefficient of the linear regression analysis[a] ($r \pm$ S.D.)	Slope (b) (mean ± S.D.)	Intercept (a) (mean ± S.D.)
Inter-day reproducibility ($n = 4$)	0.998 ± 0.0016 (CV = 0.16%)	35.241 ± 1.77 (CV = 5.0%)	1.2242 ± 0.2201 (CV = 17.9%)
Intra-day reproducibility ($n = 10$)	0.997 ± 0.0021 (CV = 0.21%)	34.235 ± 2.06 (CV = 6.0%)	1.4420 ± 0.2512 (CV = 17.4%)

r, correlation coefficient.
[a] Linear weighted regression, formula: $y = bx + a$.

each other. The retention times for GBP and the I.S. were 3.8 and 5.2 min, respectively. Fig. 1C and D shows the chromatograms of serum samples obtained at 3 and 24 h after a single oral dose of 400 mg GBP from a healthy volunteer, respectively. The results of the selectivity study showed that there were no interfering peaks from any of the following drugs: acetaminophen, naproxen, diclofenac, codeine, caffeine, phenytoin, phenobarbital, carbamazepine, lamotrigine, zonisamide, topiramate, primidone, vigabatrin, ethosuximide, clonazepam, diazepam, propranolol, etidronate, gentamicin, ciprofloxacin, fluconazole, erythromycin, cefalexin, and ceftriaxone. Topiramate, propranolol, and vigabatrin reacted with the NBD-Cl but did not give interference with the analysis of GBP or the I.S.

3.2.1. Linearity and sensitivity

The calibration curve was linear over the concentration ranges of 0.002–15 μg/ml using line-fit plot in regression analysis and the limits of detection and quantification were estimated to be 300 pg/ml and 0.002 μg/ml, respectively using a volume of 100 μl sample and 10 μl injection. Correlation coefficient values greater than 0.997 were routinely obtained. 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 results have been shown in Table 1.

3.2.2. Recovery, accuracy, precision and stability

The recoveries of GBP and the I.S. were estimated by comparing peak areas in extracted spiked human drug-free serum samples, with those in standard solutions and were found to be 100% for both GBP and the I.S. The inter- and intra-day accuracy and precision values of the assay method are presented in Table 2. The coefficient variation values of both inter- and intra-day analysis were less than 14.2% whereas the percentage error was less than 8.3%. Stock solutions of GBP and amlodipine were stable for at least 21 days when stored at 4 °C. Derivatized solutions were found to be stable (>95%) for at least 24 h if the samples were kept at sample cooler (4 °C). Stability of the drug was found to be 101% from the initial value, after 60 days maintenance of the serum at −80 °C and following three thaw–freeze cycles.

Table 2
Inter- and intra-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			
0.002	0.0021 ± 0.0003	14.1	5
0.1	0.105 ± 0.01	10	5
2.0	2.04 ± 0.11	4.9	2.5
15.0	15.2 ± 0.36	2.3	1.4
Intra-day			
0.002	0.0022 ± 0.0003	14.2	8.3
0.1	0.107 ± 0.012	10.5	7.3
2.0	2.06 ± 0.13	6.0	3.3
15.0	15.4 ± 0.4	2.2	2.4

Accuracy has been calculated as a percentage of the nominal concentration.

4. Discussion

Most of the published methods for analysis of GBP in human serum, in which OPA has been used, require automated instrumentation or immediate injection of unstable derivatives. The present method however, provides stable derivatives and improved sensitivity relative to previously published techniques. In our knowledge this is the first report of the HPLC analysis of GBP with fluorescence detection using NBD-Cl as the labeling agent. NBD-Cl readily reacts with both primary and secondary amines under alkaline conditions. The resultant derivatives can be analyzed in the visible region with very low background noise. The reactions of NBD-Cl with amines have been found to take place within an aqueous–organic phase system. A phosphate buffer with pH 11 and an equal volume mixture of methanol and acetonitrile gave the optimum yield of derivative. Thus, the labeling agent was dissolved in the mixture of methanol–acetonitrile (1:1, v/v). A sufficiently high concentration of NBD-Cl was required for the reaction to proceed efficiently. However, excess of the reagent had a deleterious effect on the determination of low level of GBP. Glycine was used in our method to remove the reagent excess and increase the reproducibility of the reaction. Unlike the I.S. the retention behavior of GBP was pH dependent. A mobile phase with pH 2.5 was used in our method with the retention time of the drug increased by further reduction of the pH. As separation of GBP from endogenous peaks and reagent excess was complete at this pH, a mobile phase with pH 2.5 was selected. A 100 μl serum sample was extracted in our method and although the sensitivity was improved by increasing the sample volume (e.g. 200 μl serum samples) however, using this volume of sample and at concentrations of more than 10 μg/ml of the drug, the fluorescence signal of the resulting derivative goes up too high. Thus, the obtained standard curve was linear over the concentrations of 0.0005–10 μg/ml when 200 μl serum sample was used.

Table 3
Mean (S.D.) pharmacokinetic parameters of two GBP preparations after single oral administration of 400 mg in 24 human volunteers

Parameter\preparation	Test	Reference	Mean ratio test/reference (CV)	P-value[a]	90% confidence intervals
C_{max} (μg/ml)	3.19 (1.0)	3.23 (1.1)	1.05 (0.31	NS	94–116
AUC0-24 (μg/ml)	28.55 (9.5)	27.98 (9.0)	1.07 (0.26)	NS	98–116
AUC0-∞ (μg/ml)	33.49 (10.4)	32.49 (11.5)	1.10 (0.30)	NS	100–120
$T_{1/2}$ (h)	8.35 (1.5)	8.01 (1.4)	1.07 (0.25)	NS	95–115

T_{max}, time to maximum concentration; C_{max}, maximum concentration; AUC, area under the concentration–time curve; $T_{1/2}$, elimination half-life.
[a] NS, no significant difference ($P < 0.05$).

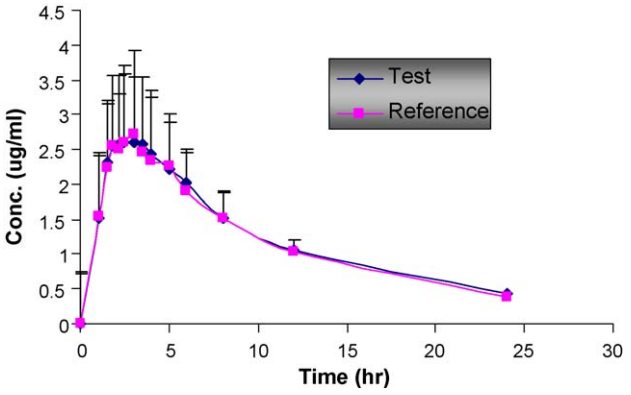

Fig. 2. Mean serum concentrations–time profiles of GBP for two preparations in 24 human volunteers after administration of a single 400 mg oral dose.

The stability of the derivative also allows NBD-Cl derivatives to be extracted using ethyl acetate, after dilution of the reaction mixture with water. In this case the residue can be reconstituted in smaller volume of an organic solvent (e.g. methanol) and the sensitivity is further improved.

5. Application of the method and conclusions

The present method has successfully been used for the determination of the drug in a randomized cross-over bioequivalence study following single oral administration of two different GBP preparations in 24 healthy volunteers. Typical serum concentration–time profiles and the resulted pharmacokinetic parameters of the drug have been shown in Fig. 2 and Table 3, respectively.

In conclusion, a simple, accurate, and specific HPLC method using pre-column derivatization with NBD-Cl and fluorescence detection has been described for the determination of GBP in human serum. In this new method which has been demonstrated to be suitable for use in pharmacokinetic studies, higher sensitivity is obtained, no automated instrument is needed and more simple procedure for derivatization is applied.

Acknowledgments

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