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Short communication

High performance liquid chromatographic determination of topiramate in human serum using UV detection

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Abstract

Topiramate has no ultraviolet, visible or fluorescence absorption. Analysis of the drug in human serum has been reported by high performance liquid chromatography (HPLC) with either mass detector or fluorescence detection after precolumn derivatization using 9-fluorenylmethyl chloroformate as fluorescent labeling agent. This study was aimed to validate derivatization and analysis of topiramate in human serum with HPLC using UV detection. The drug was extracted from human serum by liquid–liquid extraction and subjected to derivatization with 9-fluorenylmethyl chloroformate. Analysis was performed on a phenyl column using of spectrophotometer detection operated at wavelength of 264 nm. A mixture of phosphate buffer (0.05 M) containing triethylamine (1 ml/l, v/v; pH 2.3) and methanol (28:72, v/v) at a flow rate of 2.5 ml/min was used as mobile phase. No interference was found with endogenous substances. Validity of the method was studied and the method was precise and accurate with a linearity range from 40 ng/ml to 40 μg/ml. The limit of quantification was 40 ng/ml of serum. The correlation coefficient between HPLC methods using fluorescence and UV detections was studied and found to be 0.992
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1. Introduction

Topiramate (2,3:4,5-bis-*O*-(-1-methyl)-[beta]-D-fructopyranose sulfamate), a novel antiepileptic drug, is distinct in structure from other antiepileptic drugs. The drug has been approved as adjunctive therapy in partial seizure [1]. It has rapid and extensive absorption after oral administration with time to peak plasma drug concentration of 2–4 h [2]. Although the exact relationship between blood concentration of topiramate and its toxicity is not yet established and the role of therapeutic drug monitoring in management of patients is still being determined [3], a reliable and simple method of analysis is needed to support clinical and pharmacokinetic studies of the drug. Topiramate has no ultraviolet, visible or fluorescence absorption and published methods for analysis of the drug in biological fluids consisted of gas chromatography (GC) coupled with flame ionization (FID) [4] or nitrogen phosphorous detection (NPD) [5,6], fluorescence polarization immunoassay[7,8] and LC–MS [8–13]. Gas chromatographic methods are limited by thermal decomposition of the drug during GC analysis and fluorescence polarization immunoassay methods are prone to interference from metabolites. Published LC–MS methods are expensive and need to highly trained personnel. Furthermore, the reported sensitivity of assay in both LC–MS (200 ng/ml) and GC (0.5–1 μg/ml) methods are not enough for human pharmacokinetic studies using low dosages. 9-Fluorenylmethyl chloroformate (FMOC-Cl) has been shown to be suitable reagent for detection of both primary and secondary amines. We recently reported the precolumn derivatization and analysis of topiramate in human serum using FMOC-Cl as fluorescent labeling agent and HPLC with fluorescence detection [14]. Although

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FMOC-Cl has frequently been used as fluorescence labeling agent in different analytical methods, this study was aimed to develop a validated method for analysis of topiramate using derivatization with FMOC-Cl and HPLC-UV detection.

2. Experimental

2.1. Chemicals

Topiramate (analytical grade) was from Janssen-Cilag Institute research and donated by Exir pharmaceutical company (Tehran, Iran). Amantadine hydrochloride (I.S.) and FMOC-Cl were obtained from Sigma (St. Louis, MO, USA). Methanol, dichloromethane, boric acid, potassium chloride, potassium hydroxide, potassium dihydrogen phosphate, triethylamine, phosphoric acid and glycine 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).

2.2. Preparation of solutions

Stock solutions of topiramate (2000 μg/ml) and the I.S. (200 μg/ml) were prepared by dissolving the drugs in acetonitrile and distilled water, respectively. Topiramate stock solution was further diluted to working solutions ranging from 400 ng/ml to 400 μg/ml. A borate buffer was prepared by dissolving 0.625 g of boric acid and 0.750 g of potassium chloride in 100 ml water and adjusting the pH to 7.7 with 0.2 M potassium hydroxide solution. A 500 μg/ml solution of FMOC-Cl was prepared in acetonitrile. Stock solution of glycine (4 mg/ml) was prepared in water. All solutions were stored at 4 °C and were stable for at least 30 days.

2.3. Absorption spectra

For measurement of absorption curve of derivatized topiramate the stock solution of 200 μg/ml topiramate in acetonitrile was used. A 25 μl of this solution was subjected to the derivatization and analysis with HPLC using fluorescence detection which has previously been described [14]. A fraction covering the retention time range (3.2–4.5 min) where derivatized topiramate elutes (but not excess of FMOC-Cl and endogenous peaks) was collected and subjected to absorption spectra measurement using the mobile phase as the blank with a Uvikon 933 double beam UV–vis spectrophotometer (Italy).

2.4. Instrumentation

The HPLC system used consisted of two pumps of Shimadzu LC-10A solvent delivery system, a system controller (SCL 10AD), a variable wavelength UV–vis spectrophotometric detector (SPD-10A), a spectroflurometric detector (RF-551), a rheodyne injection valve with a 20 μl filling loop, a column oven (CTO-10A) set at 62 °C, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan.

2.4.1. Sample preparation and derivatization

Topiramate and the I.S. were extracted from human serum as described in detail elsewhere [14]. Briefly To 1 ml serum samples containing 100 μl of I.S 5 ml dichloromethane was added. After mixing (30 s on a vortex mixer) and centrifugation (5 min at 6000 × *g*), the organic phase was removed and evaporated to dryness under stream of nitrogen. To the residue 125 μl FMOC-Cl (500 μg/ml in acetonitrile) and 25 μl of borate buffer (pH 7.7) were added and after mixing for 10 s the samples were kept at 50 °C for 15 min. The reaction was stopped by adding of 10 μl glycine (4 mg/ml) and after 1 min, 20 μl of the reaction mixture was injected in to the chromatograph.

2.4.2. Analysis conditions

For the analysis of the drug using HPLC with UV detector a Shim-pack G-phenyl precolumn (4.0 mm i.d. × 1 cm) and a reverse phase phenyl column (150 mm × 6 mm ID) which was packed with 5 μm particles (Shimpack, CLC–phenyl), were used. A mixture of phosphate buffer (0.05 M) containing triethylamine (1 ml/l, v/v) adjusted to pH 2.3 with *o*-phosphoric acid and methanol (28:72, v/v) were pumped at flow rate of 2.5 ml/min with backpressure of 150 kg/fcm^2 as mobile phase. The eluent was filtered through a 50 μm filter (Milipore, Bedford, MA, USA) and degassed before use. The detection was performed at the wavelength of 264 nm. The analysis conditions for measuring of the drug using HPLC with fluorescence detection have previously been described [14].

2.5. Calibration

Calibration curves samples were prepared within the concentration range of 40 ng/ml to 40 μg/ml. In disposable glass tubes (16 mm × 100 mm) 100 μl each of working standard topiramate solutions were evaporated under a gentle stream of nitrogen at 50 °C. The residue was reconstituted in 1 ml drug-free human serum. The samples were then submitted to the procedures of extraction, derivatization and chromatographic analysis described above. Calibration curves (weighted regression line) were obtained by linear least-squares regression analysis plotting of peak-area ratios (topiramate/I.S.) versus the topiramate concentrations using the S.D. as weight factor.

2.6. Validation of the methods

Serum samples obtained from healthy volunteers were used for method validation and linearity studies. 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 from the addition of the I.S. The selectivity of the assay was evaluated by analysis of a group of potentially co-administrated drugs with topiramate. Within-day variation was measured by assessing the different controls in replicates of six. Between-days variation was based on repeated analysis of the same concentration controls in ten analytical run performed on different days using the same stock solutions and plasma batches. The limit of detection (LOD) was defined as the concentration of drug giving a signal to noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest serum concentration of topiramate quantified with a coefficient of variation of less than 20%. Different samples ($n = 20$), between the concentration range of 40 and 5120 ng/ml were run in duplicate in HPLC using UV and fluorescence detections and the correlation studied between both methods using regression analysis based on the least squares method.

3. Results and discussion

3.1. Validation

3.1.1. Specificity and selectivity

The resulting profile of absorption curve for optimal wavelength of derivatized topiramate has been shown in Fig. 1. The compound shows sufficient absorption in the UV region, with maximum absorption at around 264 nm and can be monitored with a UV detector. Representative chromatograms of drug-free human serum, human blank serum spiked with topiramate (40 ng/ml) and the I.S. are shown in Fig. 2A and B, respectively. Topiramate and the I.S. were well resolved with good symmetry with respective retention times of 3.5 and 6.6 min, respectively. Endogenous components chromatographed within 2.8 min and no endogenous peaks from serum were found to interfere with the elution

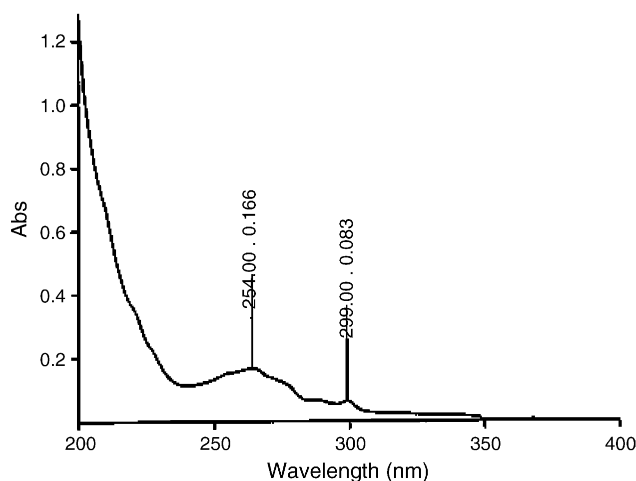

Fig. 1. The profile of absorption curve of derivatized topiramate.

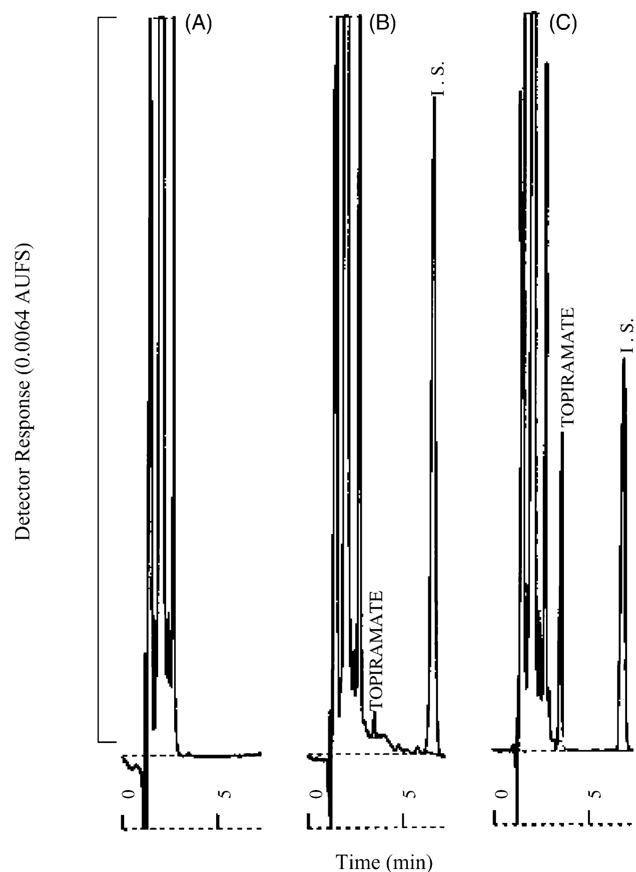

Fig. 2. Typical chromatograms obtained from an extract of (A) human blank serum (B) human blank serum samples spiked with 40 ng/ml topiramate and the I.S. and (C) serum samples obtained at 5 h after a single oral dose of 100 mg topiramate from a healthy volunteer containing 2375 ng/ml of the drug, respectively.

of the drug or I.S. Fig. 2C shows the chromatogram of human serum sample obtained at 5 h after a single oral dose of 100 mg topiramate from a healthy volunteer. For the selectivity study the following drugs were tested: phenytoin, phenobarbital carbamazepine, lamotrigine, zonisamide, primidone, vigabatrin ethosuximide, clonazepam, acetaminophen, naproxen, diclofenac, codeine, diazepam, alprazolam oxazepam, lorazepam, flurazepam, temazepam, flunitrazepam, chlordiazopoxide, theophylline and caffeine. The results showed that other than codeine which dose not appeared up to 10 min, other substances are eluted at less than 2.5 min under described conditions and none of them gave interference with the analysis of topiramate or the I.S.

3.1.2. Sensitivity, linearity, accuracy, precision and stability

In the our previously described method for the analysis of topiramate in human serum using HPLC with the fluorescence detection the LOD of 1 ng/ml and LOQ of 20 ng/ml were obtained. Although the signal intensity in fluorescence detection is higher than the UV (about nine-fold) however, due to lower background noise in applied UV wavelength, the

Table 1
Assay linearity for determination of topiramate 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 ($n = 6$)	0.9984 ± 0.0023 (C.V. = 0.4%)	0.0333 ± 0.00405 (C.V. = 4.8%)	2.1682 ± 0.3165 (C.V. = 12.8%)
Intra-day reproducibility ($n = 10$)	0.9978 ± 0.0028 (C.V. = 0.48%)	0.0318 ± 0.0095 (C.V. = 7.8%)	2.1243 ± 0.3568 (C.V. = 12.3%)

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

Table 2
Within-day precision and accuracy for determination of topiramate in human serum by the HPLC method

Known concentration (ng/ml)	Concentration found (mean ± S.D)	Coefficient of variation (%)	Accuracy (%)[a]
Within-day ($n = 6$)			
40	41.1 ± 1.25	8.2	103.5
80	80.9 ± 3.1	5.2	102.2
320	324.3 ± 8.4	2.8	100.9
640	647.5 ± 18.7	2.3	103.2
1280	1295 ± 18.7	1.8	102.9
2560	2578 ± 31.2	1.5	101.8

[a] Accuracy has been calculated as a percentage of the real concentration.

Table 3
Between-day precision and accuracy for determination of topiramate in human serum by the HPLC method

Known concentration (ng/ml)	Concentration found (mean ± S.D)	Coefficient of variation (%)	Accuracy (%)[a]
Between-day ($n = 10$)			
40	41.4 ± 1.36	9.3	102.3
80	81.5 ± 3.15	4.8	103.8
320	325.0 ± 8.8	4.1	102.5
640	651.2 ± 21.3	4.2	102.9
1280	1295.5 ± 14.3	1.7	101.5
2560	2581 ± 28.5	1.7	102.0

[a] Accuracy has been calculated as a percentage of the real concentration.

LOD was approximately 5 ng/ml at a signal to noise ratio of 3:1 and the practical LOQ corresponding with a coefficient of variation of less than 20% was 40 ng/ml in the present study. The standard calibration curves were linear over the concentration ranges of 40 ng/ml to 40 μg/ml ($y = 0.0333x + 2.1682$). The correlation coefficients for calibration curves were equal to or better than 0.9984. Intra-assay reproducibility was determined for calibration curves prepared the same day in replicate ($n = 6$) and on different days ($n = 10$) using pooled serum sample and the same stock solutions. Results are given in Table 1. The within day and between days accuracy and precision values of the assay method are presented in Tables 2 and 3, respectively. The coefficient variation values of both within day and between days were all less than 14.3% and accuracy which has been calculated as a percentage of the real concentration never deviated from 100% by more than 4%. The correlation coefficient between HPLC methods using fluorescence and UV detections was 0.992.

Stock solutions of topiramate and amantadine were stable at least for 30 days when stored at 4 °C. Derivatized solutions were found to be stable (>95%) for 12 h if the samples were kept at 4 °C using sample cooler. The concentrations of topiramate in serum stored at −80 °C for 30 days and following two freeze-thaw cycles were found to be 100 ± 2% from the initial values.

In conclusion the present method is simple, accurate and precise and although less sensitivity is obtained comparing to the fluorescence detection, the LOQ of the new method is better than both LC mass and GC methods and is enough for pharmacokinetic and clinical trial studies of topiramate. This method will be useful for analysis of the drug in human serum when the fluorescence detector is not available.

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