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A novel high sensitivity HPLC assay for topiramate, using 4-chloro-7-nitrobenzofurazan as pre-column fluorescence derivatizing agent

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Abstract

A new, sensitive and simple high-performance liquid chromatographic method for analysis of topiramate, an antiepileptic agent, using 4-chloro-7-nitrobenzofurazan as pre-column derivatization agent is described. Following liquid–liquid extraction of topiramate and an internal standard (amlodipine) from human serum, derivatization of the drugs was performed by the labeling agent in the presence of dichloromethane, methanol, acetonitrile and borate buffer (0.05 M; pH 10.6). A mixture of sodium phosphate buffer (0.05 M; pH 2.4): methanol (35:65 v/v) was eluted as mobile phase and chromatographic separation was achieved using a Shimpack CLC-C18 (150 × 4.6 mm) column. In this method the limit of quantification of 0.01 μg/mL was obtained and the procedure was validated over the concentration range of 0.01 to 12.8 μg/mL. No interferences were found from commonly co-administrated antiepileptic drugs including phenytoin, phenobarbital carbamazepine, lamotrigine, zonisamide, primidone, gabapentin, vigabatrin, and ethosuximide. The analysis performance was carried-out in terms of specificity, sensitivity, linearity, precision, accuracy and stability and the method was shown to be accurate, with intra-day and inter-day accuracy from −3.4 to 10% and precise, with intra-day and inter-day precision from 1.1 to 18%.

Keywords: Reverse phase chromatography; Topiramate; Serum; 4-Chloro-7-nitrobenzofurazan; NBD-Cl

1. Introduction

Topiramate (Fig. 1A), a sulfamate-substituted monosaccharide (2,3:4,5-bis-*O*-(-1-methyl)-[beta]-D-fructopyranose sulfamate) is a new second generation antiepileptic agent. The drug is structurally different from other anticonvulsants and has been approved in partial and generalized tonic-clonic seizure. Topiramate is currently being investigated for its effects in several neurologic disorders. After oral administration, topiramate is rapidly absorbed with time to peak plasma concentration of about 2 h and bioavailability of 80%. It is not extensively metabolized and no active metabolite has been identified for the drug, however, in patients receiving enzyme inducer antiepileptics (e.g. carbamazepine) up to 50% a dose may undergo metabolism [1]. Although an exact relationship between topiramate blood concentrations and its efficacy has not been established yet, clinical and pharmacokinetic studies of the drug need simple and reliable analytical methods. Topiramate has no ultraviolet, visible or fluorescence absorption and available methods for analysis of the drug in biological fluids consisted of gas chromatography (GC) coupled with flame ionization (FID) [2] or nitrogen phosphorous detection (NPD) [3,4], fluorescence polarization immunoassay [5,6] and LC–MS [7–11]. The sensitivity of analysis in published LC–MS (0.2 μg/mL) and GC (0.5–1 μg/mL) methods is not enough to measure drug levels obtained in human single dose studies More recently analysis of the drug in human plasma following derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) using fluorescence [12] or UV [13] detection have been reported by our laboratory. 4-Chloro-7-nitrobenzofurazan (NBD-Cl; Fig. 1B) is a suitable labeling agent which reacts with both primary and secondary amines. Stable adducts with absorption maximum at

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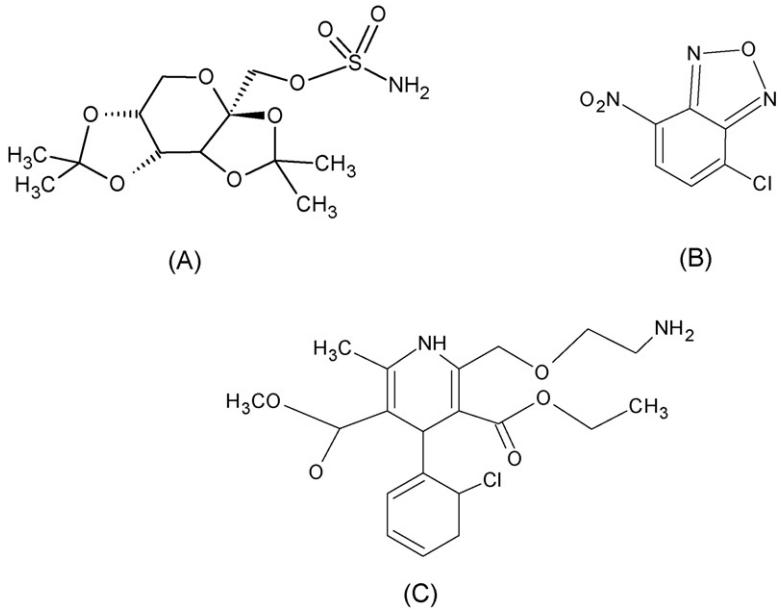

Fig. 1. Chemical structures of (A) Topiramate;(B) NBD-Cl; and (C) amlodipine.

the visible region, low background noise and higher signal/noise ratio are provided by this agent. However, in our knowledge there is not any report about its application in derivatization of sulfamates. In the present paper a novel HPLC method with limit of quantification (LOQ) of 0.01 μg/mL, is described for the analysis of topiramate, a sulfamate derivative in human serum using NBD-Cl as pre-column fluorescence labeling agent.

2. Experimental

2.1. Chemicals and solutions

Topiramate (purity 99.1%) was from Dr. Reddy's pharmaceutical company (Hyderabad, India) Amlodipine (I.S.; Fig. 1C) was from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade (except methanol which was HPLC grade) and were purchased from Merck (Darmstadt, Germany). Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England). Stock solutions of topiramate (1000 μg/mL) and amlodipine (500 μg/mL) were prepared in methanol and acetonitrile, respectively. A working standard solution of the I.S. (50 μg/mL) was prepared in acetonitrile and working standards of the drug (0.1–128 μg/mL) were prepared by serial dilution of the stock in methanol. A 10 mg/mL solution of NBD-Cl was prepared in a mixture of methanol–acetonitrile (1:1 v/v). A borate buffer (0.05 M) was prepared in water and adjusted to pH 10.6 with 0.05 M potassium hydroxide solution. All solutions were stored at 4 °C and were stable for at least 3 weeks.

2.2. Instrumentation and 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-C_{18} column (Shimadzu, Kyoto, Japan; 150 × 4.6 mm I.D., 5 μm) which was protected by a Shimpack G-C18 guard column (1 cm × 4.0 mm I.D., 5 μm particle size). Isocratic elution of mobile phase was performed with 0.05 M sodium phosphate buffer (pH 2.4 adjusted with *o*-phosphoric acid) – methanol (35:65 v/v). The column oven temperature was set at 60 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 2.2 mL/min with backpressure of 1.5 kPa.

For preparation of calibration plots, blank pooled human serum was used. 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 1.0 mL of drug-free human serum using an automatic pipettor (Eppendorf Research, 200–1000 μL). Calibration plots (weighted regression line) were obtained by linear least-squares regression analysis plotting of peak-area ratios (topiramate/I.S.) versus the topiramate concentrations.

2.3. Sample preparation and derivatization

A 1.0 mL aliquot of each blank, calibration or unknown human serum samples were transferred into disposable glass tubes (100 × 16 mm), using an automatic pipettor (Eppendorf Research, 200–1000 μL). After addition of 100 μL of the I.S. and 5 mL dichloromethane and brief mixing for 30 s on a vortex mixer, the samples were centrifuged for 5 min at 6000 × *g*. The organic phase was then removed and evaporated to dryness under stream of nitrogen at 50 °C. To the residue 100 μL of the NBD-Cl solution, 100 μL dichloromethane and 25 μL of the borate

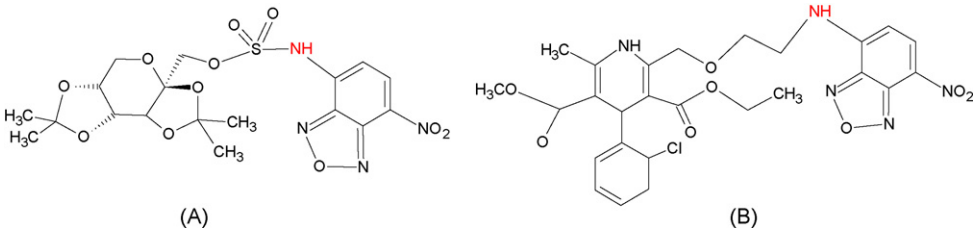

Fig. 2. Derivatization reaction of NBD-Cl with (A) topiramate and (B) amlodipine (I.S.).

buffer were added and after brief mixing for 10 s on a vortex mixer, the samples were kept at 60 °C for 10 min. The NBD-Cl derivatives were then analyzed by injection of a 20 μL volume of the reaction mixture onto the chromatographic column.

2.4. Optimization of the derivatization conditions

Solutions of 0.01, 1 and 10 μg/mL of topiramate were used to optimize derivatization of the drug with NBD-Cl, while the I.S. was reacted with the reagent at the concentration of 50 μg/mL. Concentrations of the NBD-Cl solutions in the range of 0.5–50 mg/mL and pH of the buffer solutions ranging from 6 to 12 were tested to obtain optimal conditions for derivatization. The polarity of the reaction solution was optimized using various organic solvents–water proportions, ranging from 1:1 to 10:1, the reaction was allowed to proceed in a water bath at temperature ranging from 40 to 80 °C. Different organic solvents including acetone, ethyl acetate, dichloromethane, acetonitrile and chloroform were used to increase the yield of the reaction.

2.5. Validation of the methods

Serum samples obtained from healthy volunteers were used for method validation and linearity studies. Average recoveries of the extraction procedure for both topiramate and the I.S. were estimated by comparing the peak areas obtained from derivatization of an extracted spiked sample blank with those obtained after derivatization of the same amounts of un-extracted solutions in acetonitrile. 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-administered drugs with topiramate. 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 ten 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 and LOQ was estimated as the lowest serum concentration of the drug quantified with a coefficient of variation of less than 20%.

3. Results

3.1. Reaction of topiramate with the reagent

Topiramate and the I.S. react with the labeling agent in alkaline medium (Fig. 2A and B, respectively) and the reaction efficiently proceeds in the presence of dichloromethane. The optimal conditions were found to be: a NBD-Cl solution of 10 mg/mL, a borate buffer with pH of 10.6, a reaction temperature of 60 °C for 10 min and a reaction medium consisting of the buffer–acetonitrile–methanol and dichloromethane (1:2:2:4 v/v).

3.2. Specificity and selectivity

Topiramate and the I.S. gave well resolved peaks with retention times of 4.4 and 7.8 min, respectively. Blank human serum was consistently free of endogenous peaks at the retention times of the drugs. Representative chromatograms of drug-free human serum containing the I.S., human blank serum spiked with topiramate (0.02 μg/mL) and the I.S. are shown in Fig. 3A and B, respectively. Fig. 3C shows the chromatograms of human serum obtained 4 h after a single oral dose of 100 mg topiramate from a healthy volunteer. The results of the selectivity study showed that there were no interfering peaks from any of the following drugs: phenytoin, phenobarbital carbamazepine, lamotrigine, zonisamide, primidone, vigabatrin, gabapentin, ethosuximide, clonazepam, propranolol, etidronate, gentamicin, ciprofloxacin, fluconazole, erythromycin, cefalexin, ceftriaxone, diazepam, alprazolam, oxazepam, lorazepam, flurazepam, chlordiazepoxide, baclofen, theophylline, acetaminophen, naproxen, diclofenac and codeine. Gabapentin, vigabatrin, propranolol, baclofen, reacted with the NBD-Cl and eluted at 2.7, 2.9, 10.5 and 14 min, respectively.

3.3. Sensitivity, linearity and stability

The LOD was approximately 0.003 μg/mL and LOQ was 0.01 μg/mL. The standard calibration plots were linear over the concentration ranges of 0.01–12.8 μg/mL using least-squares regression analysis. The correlation coefficients for calibration plots were equal to or better than 0.9972. Intra and inter-day reproducibility were determined for calibration plots prepared on the same day ($n = 4$) and different days ($n = 10$) using pooled serum sample and the same stock solutions. Results are given in Table 1.

Stock solutions of topiramate and I.S. were stable for at least 60 days when stored at 4 °C. Derivatized solutions were found to be stable (>95%) for about 12 h if the samples were refrigerated (4 °C). The concentrations of topiramate in serum stored at −80 °C for 60 days and following three freeze–thaw cycles were found to be 99 ± 2% from the initial values.

Table 1
Assay linearity for determination of topiramate in human serum by the HPLC method

	Correlation coefficient of the linear regression analysis[a] ($r \pm$ SD)	Slope (b) (mean ± SD)	Intercept (a) (mean ± SD)
Inter-day reproducibility ($n = 6$)	0.9972 ± 0.0023	0.236 ± 0.0115 (C.V. = 4.9%)	1.352 ± 0.1625 (C.V. = 12.5%)
Intra-day reproducibility ($n = 10$)	0.9975 ± 0.0025	0.234 ± 0.0121 (C.V. = 5.2%)	1.411 ± 0.1713 (C.V. = 12.1%)

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

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

Known concentration (μg/mL)	Concentration found (mean ± SD)	Coefficient of variation (%)	Accuracy (%mean deviation)[a]
Intra-day ($n = 6$)			
0.01	0.011 ± 0.0019	17.9	8.3
0.08	0.081 ± 0.010	12.6	1.1
0.32	0.312 ± 0.018	5.6	−2.6
1.28	1.31 ± 0.043	3.3	2.3
5.12	5.175 ± 0.070	1.3	1.1
10.24	10.390 ± 0.112	1.1	1.5

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

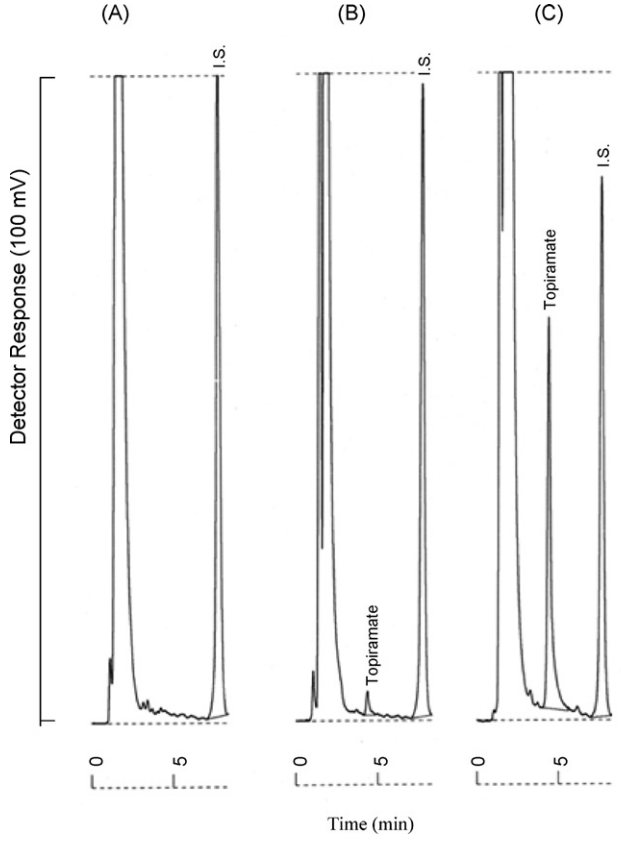

Fig. 3. Typical chromatograms obtained from an extract of (A) drug-free human serum containing the I.S.; (B) human blank serum spiked with topiramate (0.02 μg/mL) and the I.S. (C) human serum obtained at 4 h after a single oral dose of 100 mg topiramate from a healthy volunteer containing 0.315 μg/mL of the drug. (A and B ATEN = 2$\hat{A}$; 16 mV/full scale, C ATEN = 2$\hat{S}$; 32 mV/full scale).

3.4. Recovery, accuracy and precision

The mean extraction efficacies of topiramate and the I.S. from serum were found to be 99 ± 2% and 100 ± 2%, respectively. The intra- and inter-day accuracy and precision values of the assay method are presented in Tables 2 and 3, respectively. The coefficient of variation values of both inter- and intra-day analysis were from 1.1 to 18% whereas accuracy, which has been calculated as a percentage of the real concentration, never deviated from 100% by more than 10%.

4. Discussion

NBD-Cl is suitable labeling agent for reaction with both primary and secondary amines. Also, it has been frequently used for analysis of pharmaceutical agents, however, this is the first report for using of this reagent in quantitation of topiramate in human serum. In our previously published method for analysis of the drug using FMOC-Cl and fluorescence detection, LOQ of 0.02 μg/mL was obtained. Unlike the FMOC-Cl, the resultant derivatives with the NBD-Cl can be analyzed in the visible region with very low background noise, thus sensitivity of the NBD-Cl derivatives is expected to be higher than the FMOC-Cl adducts. In the present study however, using different concentrations of the reagent, different proportions of organic-aqueous phases, different buffer solution with various pH range and different incubation time, failed to provide better sensitivity than the FMOC-Cl method and apparently desired derivatization reaction did not efficiently proceed under these conditions. Topiramate is a sulfamate derivative and in comparison with the amines it has different electronic distribution, thus, it is expected to be more difficult to label. The reactions of NBD-Cl with amines have been found to take place within an aqueous-organic phase system and in our previously published method for derivatization and analysis of gabapentin

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

Known concentration (μg/mL)	Concentration found (mean ± SD)	Coefficient of variation (%)	Accuracy (%mean deviation)[a]
Inter-day ($n = 10$)			
0.01	0.011 ± 0.0021	18.2	10
0.08	0.078 ± 0.011	14	−3.1
0.32	0.309 ± 0.019	6.3	−3.4
1.28	1.30 ± 0.044	3.4	2
5.12	5.183 ± 0.067	1.3	1.2
10.24	10.35 ± 0.127	1.2	1.4

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

[14] by the reagent, sensitivity of the method was significantly improved when both methanol and acetonitrile was used as organic phase in the reaction mixture. Thus, effect of different organic solvents on the speed and yield of the reaction was tested and a significantly improved LOQ (0.01 μg/mL) was obtained when dichloromethane was added to the reaction mixture. It seems, the reaction is effectively proceeding in the presence sufficient amounts of dichloromethane. The stability of the derivative also allows NBD-Cl adducts 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 can be further improved. Different analytical columns (C_8, CN, phenyl, ODS and TMS) were tested and considering the resolution of the drug from both endogenous peaks and the I.S. the Shimpack CLC-ODS was selected. A number of drugs with secondary or primary amines (e.g. amantadine, amlodipine, baclofen, propranolol and glucosamine) were tested and amlodipine was selected as internal standard because of its suitable recovery and retention time. Hydroxylation, hydrolysis and glucuronidation are main metabolite pathways of topiramate and in total six inactive metabolites have been identified for the drug. In normal subjects metabolite fraction of the drug is low however, this fraction may increase in epileptic patients receiving enzyme inducer anticonvulsants (e.g. phenytoin, carbamazepine and phenobarbital) [1]. Although there are no data on the plasma concentrations of these metabolites, it is possible that the labeling agent reacting with amino group of the drug can react with the metabolites too. Due to difficulty in obtaining of standards, possible interference of the metabolites was not studied.

In conclusion a simple method using NBD-Cl as pre-column labeling agent was described in this paper. As resultant derivative with the reagent can be analyzed in the visible region with very low background noise, comparing to our previously published methods for analysis of the drug, the sensitivity was improved and limit of quantification of 0.01 μg/mL obtained.

Acknowledgments

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