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Journal of Chromatography B, 813 (2004) 175–180
JOURNAL OF CHROMATOGRAPHY B
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Sensitive analytical method for Topiramate in human serum by HPLC with pre-column fluorescent derivatization and its application in human pharmacokinetic studies

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Received 30 April 2004; accepted 17 September 2004

Abstract

A sensitive and specific high performance liquid chromatographic method for quantitation of topiramate in human serum was developed using HPLC with fluorescence labeling reagent. Topiramate was extracted from human serum by dichloromethane and derivatized by reaction with 9-fluorenylmethyl chloroformate (FMOC-Cl) in the presence of borate buffer. Analysis was performed on a CN column with sodium phosphate buffer (pH 2.2) containing 1 ml/l triethylamine and methanol (52:48 (v/v)) as mobile phase. Amantadine was used as internal standard. The standard curve was linear over the range 20–5000 ng/ml of topiramate in human serum. The mean intra-day precision was from 10.5% (low concentration) to 1.2% (high concentration) and the within-day precision from 1.5 to 12.5% determined on spiked samples. The accuracy of the method was 96.5–107.5% (intra-day) and 98.4–105% (inter-day). The limit of quantification was 20 ng/ml of serum. This method was used in a bioequivalence study after administration of 2 × 25 mg topiramate in 24 healthy volunteers.

Keywords: Reverse phase chromatography; HPLC; Topiramate; Serum; Bioequivalence study

1. Introduction

Topiramate (2,3,4,5-bis-*O*-(1-methyl)-[β]-D-fructopyranose sulfamate), is a new antiepileptic drug, which has been approved for adjunctive therapy in partial seizure with or without secondary generalization [1]. After oral administration of topiramate, its absorption is rapid with a bioavailability of about 80% and time to peak plasma drug concentration of 2–4 h [2]. The role of therapeutic drug monitoring in management of patients is still being determined and the exact relationship between blood concentration of topiramate and its toxicity is not yet established [3]. Thus, a reliable and simple method of analysis is needed to support clinical studies. The analysis of topiramate in blood is complicated, because the molecule has no ultraviolet, visible or fluorescence absorption and cannot be quantified by the more readily accessible technique such as high performance liquid chromatography (HPLC) with spectrophotometric or fluorescence detection. In in-vitro studies, however topiramate can be analyzed by HPLC using refractive index detector [4]. Several methods have been developed for determination of topiramate in plasma including capillary gas chromatography (GC) coupled to flame ionization (FID) [5] or nitrogen phosphorous detection (NPD) [6,7] and, more recently, by HPLC coupled to electrospray (ESP) mass spectrometry (MS). Although reproducible assay was achieved by early GC methods however, nonspecific detection and endogenous peaks in the chromatograms were problematic. Also topiramate is subject to thermal decomposition during GC analysis. Fluorescence polarization immunoassay (FPIA) which is used in routine therapeutic drug monitoring of topiramate is accurate and precise but may be prone to interference from metabolites [8,9]. In published LC–MS

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doi:10.1016/j.jchromb.2004.09.054

methods [9–13] the sensitivity and recovery are improved, but this procedure is expensive and the analytical method requires highly trained personnel. The limit of quantification (LOQ) in the LC–MS methods was about 200 ng/ml and in different GC techniques was 0.5–1 μg/ml, however in pharmacokinetic and bioequivalence studies more sensitive methods is needed. This is the first report for high performance liquid chromatographic determination of topiramate in human serum using derivatization and fluorescence detection. In the present study, a selective and very sensitive HPLC method with fluorescence detection for assay of topiramate in human serum is reported. FMOC-Cl has been shown to be suitable reagent for fluorescent labeling of both primary and secondary amines [14]. The derivatization of the amino group of topiramate using FMOC-Cl with analysis of the derivatives by reverse-phase HPLC is described in this paper. The method was applied to assay topiramate in serum samples obtained from a bioequivalence study carried out in 24 healthy volunteers after administration of 2 × 25 mg of the drug.

2. Experimental

2.1. Chemicals

Reference standards of topiramate (Johnson–Cilag) and amantadine hydrochloride (I.S.) (Sigma, St. Louis, MO, USA) were kindly donated by Exir pharmaceutical company (Lorestan, Iran). Methanol (HPLC grade), ethyl acetate, boric acid, potassium chloride, potassium hydroxide, potassium dihydrogen phosphate, triethylamine, phosphoric acid and glycine were purchased from Merck (Darmstadt, Germany). 9-Fluorenylmethyl chloroformate (FMOC) was obtained from Sigma (St. Louis, MO, USA). All reagents used were of analytical grade except methanol which was HPLC grade and were used without further purification. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Preparation of standard solutions

Stock solutions of topiramate and the I.S. were prepared by dissolving the compounds in acetonitrile at a concentration of 100 and 200 μg/ml respectively. Topiramate stock solution was further diluted with acetonitrile to obtain the different working solutions ranging from 20 ng/ml to 5.12 μg/ml. Plasma calibration samples were prepared using drug-free human serum and 100 μl of these solutions provided concentrations of 20, 40, 80, 160, 320, 640, 1280, 2560 and 5120 ng/ml. Borate buffer were 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 7.8 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 at least for 2 weeks.

2.3. Chromatography

The Shimadzu (Kyoto, Japan) HPLC system used in this study consisted of a liquid chromatographic pump Model LC-10AD which was used to deliver the mobile phase at a flow rate of 2 ml/min and equipped with a model RF-551 spectroflurometric detector operating at an excitations wavelength of 260 and emission wavelength of 315 nm. The HPLC column (150 mm × 6 mm i.d.) was packed with 5 μm particles of CN packing material (Shimpack-CLC-CN) and was maintained at 62 °C by a model Shimadzu CTO-10A column oven (Shimadzu). For quantitative calculations a Shimadzu C-R4A data module was employed. The mobile phase consisted of acetonitrile–0.05 M sodium phosphate buffer pH 2.2 (52/48 (v/v)) containing 1 ml/l triethylamine. The eluent was filtered through a 50 μm filter (Milipore, Bedford, MA, USA), and degassed (DGU-10A Shimadzu)

2.4. Sample preparation and derivatization

To 1 ml serum samples 100 μl of I.S., and 5 ml dichloromethane were added. After mixing for 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 at 35 °C. To the residue 125 μl FMOC-Cl (500 μg/ml in acetonitrile) and 25 μl of borate buffer (pH 7.8) were added and after brief mixing for 10 s on a vortex mixer the samples were kept at 50 °C for 15 min. The reaction was stopped by adding 10 μl glycine (0.1 M) and, after a further 1 min, a volume (20 μl) of the reaction mixture was injected in to the chromatograph.

2.5. Optimization of the derivatization conditions

The reaction of topiramate with FMOC-Cl was optimized using 10 μg/ml solution of topiramate in acetonitrile as a model. The reaction time, the proportion of acetonitrile-water, the pH of borate buffer, and the temperature of reaction were varied around the expected optimal values. Buffer solutions ranging from pH 6 to 10 were used to adjust the pH to the desired values. Various acetonitrile–water proportions, ranging from 1:1 to 18:1 were used to optimize the polarity of the reaction solution. The mixture was allowed to react in a water bath at temperature ranging from 30–80 °C. Different concentrations of FMOC-Cl ranging from 100–5000 μg/ml were tested to adjust the concentration of FMOC-Cl to the desired values. The reaction time was optimized by following the reaction for a total of 60 min. After derivatization, all solutions were chromatographed on the HPLC system described. Relative peak areas were measured to find the optimal conditions.

2.6. Calibration

Calibration curves were prepared by the analysis of 1 ml human blank serum samples spiked with 100 μl each of working standard topiramate solutions within the concentration

range of 20–5120 ng/ml. The samples were then submitted to the procedures of extraction, derivatization and chromatographic analysis described above. Calibration curves were obtained by linear least-squares regression analysis plotting of peak-area ratios (topiramate/I.S.) versus the topiramate concentrations.

2.7. Validation of the methods

The recovery of topiramate from serum was determined at the concentration ranges of 80, 320 and 1280 ng/ml by comparing peak areas obtained after derivatization of topiramate extracted from serum with peak areas obtained after derivatization of the same amounts of unextracted topiramate solutions in acetonitrile. Within and between days variations were determined by repeated analysis ($n = 6$) of different topiramate concentrations within the range of calibration curve in a single analytical run and in ten analytical run performed on 10 days. The presence of disturbing endogenous peaks was examined on 24 human serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the I.S. The limit of detection was defined as the concentration of drug giving a signal to noise ratio of 4:1. The lower limit of quantification was defined as the lowest serum concentration of topiramate quantified with a coefficient of variation of less than 20% (range recommended by the Conference Report on Bioanalytical Methods Validation [15]).

2.8. Application of the method

The developed method has been applied in randomized crossover bioequivalence study in which the concentration of topiramate was measured in different serum samples in 24 healthy volunteers following single oral doses (2×25 mg) of topiramate formulations developed by either Exir pharmaceutical company (test) or Topamax (Janssen–Cilag; Reference). The drugs were administrated under fasting conditions and blood samples were collected at 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24 and 48 h after drug administration. Serum was separated by centrifugation at 6000 rpm for 5 min and stored at −80 °C until assay.

3. Results

3.1. Optimization of the derivatization conditions

The derivatization reaction appeared to be highly dependent on pH of borate buffer, acetonitrile concentration in the reaction mixture and reaction temperature (Fig. 1). The optimal conditions were found to be: a pH of 7.8–8.5, a reaction temperature of 50 °C for 15 min and a reaction solution consisting of water–acetonitrile (1:5 (v/v)). Time course for the reaction of topiramate with FMOC-Cl in optimized conditions is presented in Fig. 2

3.2. Validation

3.2.1. Specificity and selectivity

Representative chromatograms of human blank serum spiked with I.S. and human blank serum spiked with topiramate (2560 ng/ml) and the I.S. are shown in Fig. 3A and B respectively. Topiramate and the I.S. were well resolved with good symmetry with respective retention times of 3.8 and 5.0 min. Endogenous components chromatographed within

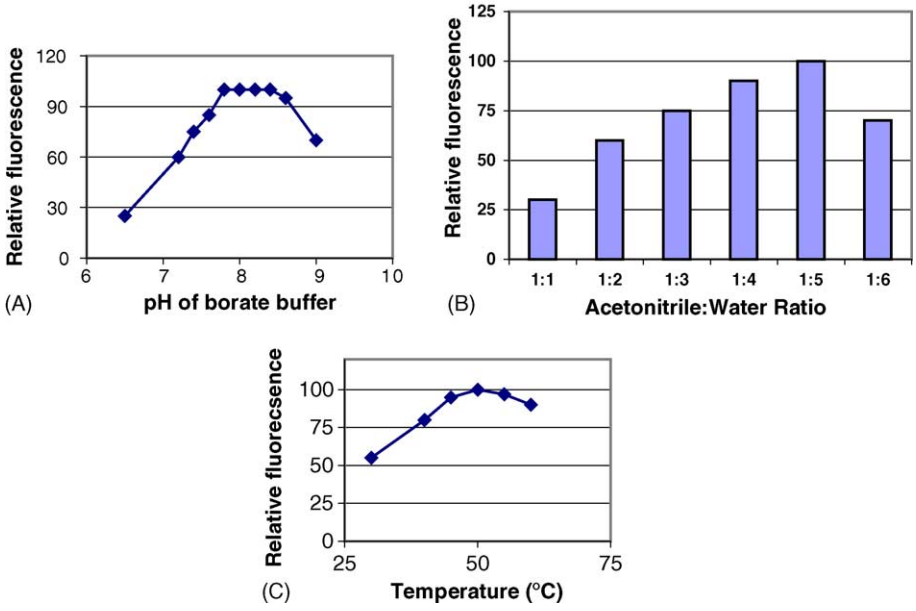

Fig. 1. Effects of (A) pH of borate buffer, (B) acetonitrile concentration and (C) reaction temperature on the reaction of topiramate with FMOC-Cl. Condition as in Section 2.

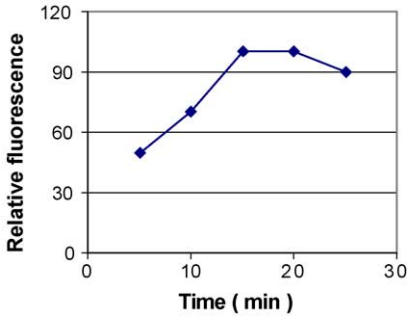

Fig. 2. Time course for the reaction of topiramate with FMOC-Cl. Topiramate (200 ng/ml) was derivatized with 125 μl of FMOC-Cl (500 μg/ml) and 25 μl the borate buffer (pH 7.8) in 50 °C.

2 min and no endogenous peaks from serum were found to interfere with the elution of the drug or I.S. Fig. 3C and D show the chromatograms of serum samples obtained at 3 and 48 h after a single oral dose of 2 × 25 mg topiramate from a healthy volunteers. Fluorescence response can be increased by increasing the amount of derivatization reagent. However, significant band-broadening was seen for a large excess of FMOC-Cl. Suitable responses for different concentration of topiramate were observed for 125 μl FMOC-Cl (500 μg/ml in acetonitrile).

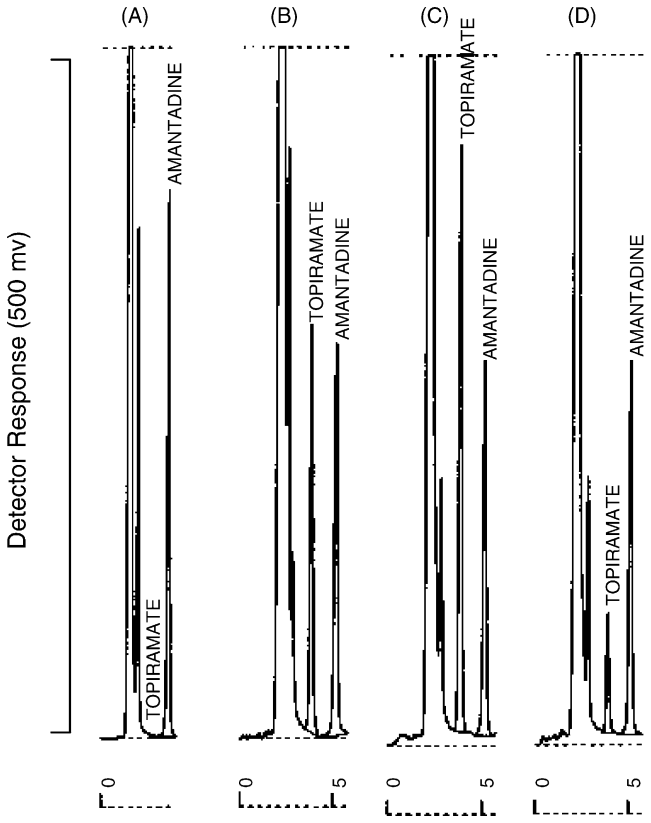

Fig. 3. Typical chromatograms obtained from an extract of (A) human blank serum (B) human blank serum spiked with 2560 ng/ml topiramate and the I.S. (C) and (D) serum samples from a volunteer 9 and 48 h after a single oral dose of 2 × 25 mg drug containing 3920 ng/ml and 690 ng/ml of topiramate, respectively.

3.2.2. Recovery, accuracy and precision

The recovery of topiramate and I.S. from serum was examined by extracting and derivatization of spiked serum samples comparing with peak areas obtained after derivatization of the same amounts of unextracted topiramate solutions in acetonitrile. The recoveries were found to be 98% for topiramate and 95% for the I.S. The within day and between day accuracy and precision values of the assay method are presented in Table 1. The coefficient variation values of both within day and between days were all less than 12% whereas the percentage error was less than 8%.

3.2.3. Limit of quantification, linearity and stability

The limit of detection was approximately 1 ng/ml at a signal to noise ratio of 4:1. Although peaks corresponding to the derivatives were detectable well above the instrument noise level from a 5 ng/ml topiramate in serum, because of the dif-

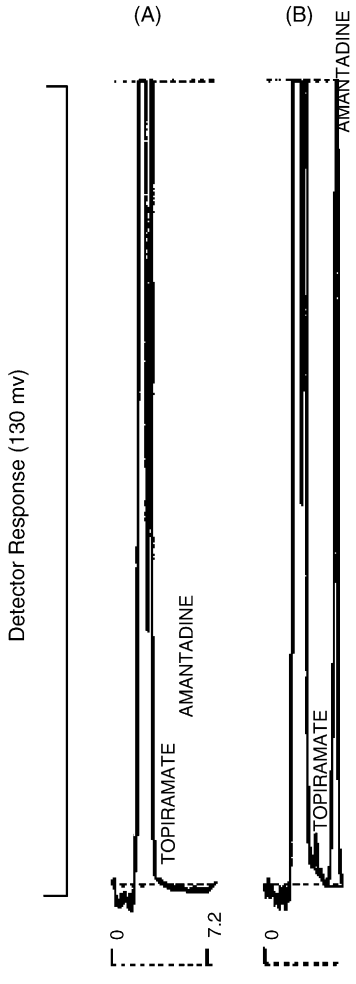

Fig. 4. Chromatograms obtained from an extract of (A) human blank serum and (B) human blank serum spiked with the I.S. and topiramate at LOQ (20 ng/ml) on the same scale.

Table 1
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
Within-day ($n = 6$)			
20	20.9 ± 1.73	8.3	+4.7[a]
80	81.4 ± 2.6	3.2	+1.75[a]
320	323.7 ± 9.7	3.0	+1.2[a]
640	646.2 ± 16.1	2.5	+1.0[a]
1280	1290.0 ± 15.7	1.2	+0.9[a]
5120	5183.3 ± 58.5	1.1	+1.2[a]
Between-day ($n = 10$)			
20	21.0 ± l.97	9.36	+5.1[b]
80	81.7 ± 2.79	3.42	+2.1[b]
320	324.0 ± 12	3.71	+1.3[b]
640	646.7 ± 19.7	3.1	+1.1[b]
1280	1292.5 ± 18.1	1.4	+1[b]
5120	5179.2 ± 65.2	1.3	+1.2[b]

Percentage difference = [(mean concentration found−known concentration)/known concentration] × 100.
[a] Percentage difference.
[b] %Mean deviation.

ficulty of measuring peaks accurately on a steeply soloing baseline at such low level, the practical limit of quantification corresponding with a coefficient of variation of less than 20% was 20 ng/ml. Chromatograms of human blank serum and human blank serum spiked with the I.S. and topiramate at LOQ (20 ng/ml) on the same scale are shown in the Fig. 4A and B, respectively. The standard calibration curves were linear over the concentration ranges of 20–5120 ng/ml ($y = 0.0381x + 6.147$) with a coefficient of 0.9974. 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. After 60 days the concentration of topiramate in serum stored at −40 °C were found to be 101% from the initial value.

4. Discussion

9-Fluorenylmethyl chloroformate (FMOC-Cl) has been shown to be a suitable reagent for the fluorescent labeling of both primary and secondary amines, which makes it a suitable reagent for detection of amino acids. [12]. In a similar way, it is also capable of forming stable ester-bonded derivatives with hydroxyl groups.

In this manuscript for the first time the development and validation of a sensitive and precise HPLC-method for determination of topiramate, using this derivatization technique, is described.

In order to extract of topiramate from the serum various extraction procedures including protein precipitation methods and liquid-liquid extraction were investigated. Direct protein precipitation with acetonitrile gave low recovery with high background noise. Extraction efficiency of different solvents including ethyl acetate, hexane, diethyl ether, dichloromethane and chloroform were compared. Ethyl acetate and dichloromethane gave the best recovery for topiramate and the I.S., however, deterioration of amantadine was occurred during evaporation of ethyl acetate using stream of nitrogen. Thus dichloromethane is selected as extracting solvent. The conditions of reaction between FMOC-Cl and topiramate were optimized. Due to the insolubility of FMOC-Cl and derivatization products in water, it was necessary to apply a reaction solution with high content of acetonitrile (more than 80% (v/v)) and maximal yields of the derivatives were obtained following reaction in solution consisting of water–acetonitrile (1:5 (v/v)). In lower concentrations the fluorescence response was reduced and excess of acetonitrile (more than 85%) resulted in buffer precipitation. Optimal reaction of topiramate with FMOC-Cl occurred at pH 7.8–8.5 and was complete after 15min in temperature of 50 °C. A sufficiently high concentration of FMOC-Cl was required for the reaction to proceed efficiently. In practice this was limited at 500 μg/ml because FMOC-Cl concentration above this increased the reagent excess and had a deleterious effect on the determination of low level of topiramate and significant band-broadening was seen for a large excess of FMOC-Cl. The analyte peaks could be separated further from a reagent excess by increasing slightly the proportion of buffer in the mobile phase, but at the expense of longer analysis time. Alternatively it may be possible to remove the most of reagent excess with an amino acid such as glycine and direct injection of reaction products (FMOC–glycine being eluted in the first part of the chromatogram).

A number of drugs with secondary or primary amines or hydroxyl groups (eg. etidronate, alendronate, gentamicin, amikacin, and different macrolide antibiotics) were tested but rendered unsuitable because of low recovery or inappropriate retention time. Amantadine was selected as internal standard because of its high recovery, suitable retention time and stability of its derivatized product. The pH dependence of the reaction was consistent with that reported for amino acids [12] but complete derivatization

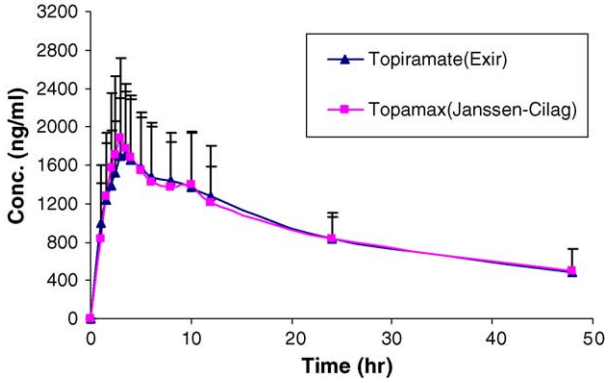

Fig. 5. Mean serum concentrations vs. time profiles of topiramate for test and reference preparations in 24 human volunteers after administration of a single 2 × 25 mg oral dose.

of topiramate required a longer time. Different analytical columns (C8, CN, phenyl and TMS) were tested and considering the resolution of the drug from both endogenous peaks and the I.S. the Shimpack CLC-CN was selected. Commonly used benzodiazepines (including; Diazepam, Oxazepam, Lorazepam, Flurazepam, Temazepam, Flunitrazepam, Chlordiazopoxide, Clonazepam, and Alprazolam) and antiepileptics (including; Phenytoin, Carbamazepine, Phenobarbital, Lamotrigine, Zonisomide, Primidone and Vigabatrin) checked for interference with topiramate assay and none of these drugs react with FMOC under above conditions.

4.1. Application of the method and conclusions

The method has been applied for determination of the drug in serum following single oral administration of 2 × 25 mg of two different topiramate preparations in 24 normal subjects in randomized crossover bioequivalence study. Fig. 5 shows a typical serum concentration–time profile for both formulations and resulted pharmacokinetic parameters are summarized in Table 2.

In conclusion, the current method appeared to be precise, rapid and sensitive for the determination of topiramate in human serum in pharmacokinetic studies in healthy volunteers.

Table 2
Mean (S.D.) pharmacokinetic parameters of topiramate for test and reference preparations in 24 human volunteers after administration of a single 2 × 25 mg oral dose

Parameter	Topamax (Jansssen–Cilag) (n = 24)	Topiramate (Exir) (n = 24)
T_{max} (h)	3.8 (2.1)	4.5 (2.6)
C_{max} (ng/ml)	2121 (682)	1920 (641)
AUC 0–48 (mgh/ml)	41457 (13077)	43240 (13951)
AUC 0–∝ (ngb/ml)	68455 (31391)	62707 (24275)
$T_{1/2}$ (h)	31.2 (14.9)	27.1 (9.2)

T_{max} = Time to maximum concentration, C_{max} = maximum concentration, AUC = area under the concentration time curve, $T_{1/2}$ = elimination half life, n = no. of volunteers.

Acknowledgments

This work was supported by Exir and Arya Pharmaceutical Companies and in part by Kermanshah University of Medical Sciences.

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