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Rapid and Specific Approach for Direct Measurement of Topiramate in Human Plasma by LC-MS/MS: Application for Bioequivalence Study

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Abstract

A rapid liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantification of topiramate in heparinized human plasma. The plasma samples were prepared by solid phase extraction (SPE) method without drying and then reconstitution. Topiramate and the topiramate d12 (Internal Standard IS) were chromatographed on a Betasil C18 column at a flow rate of 0.5 ml/min. The total run time was 1.80 min. An electrospray ionization interface was selected for ionization of analyte and IS. The mass transition [M-H] ions used for detection were m/z 338.10→78.20 for topiramate, m/z 350.40→90.10 for IS. The method was linear in the concentration range of 10–4200 ng/ml with r ≥0.9982. Recovery of topiramate and IS ranged from 78.20 to 87.74%. The validated method has been successfully used to analyze human plasma samples for application in 100 mg fasted pharmacokinetic studies.

Keywords: Human plasma; Topiramate; Selected reaction monitoring; LC-MS/MS

Introduction

Topiramate (2,3:4,5-bis-*O*-(1-methylethylidene)-β-D-fructopyranose sulfamate) is a new sulfamate-substituted monosaccharide drug approved as add-on therapy in patients with partial epilepsy, with or without secondary generalized seizures (Dichter and Broddie, 1996). Topiramate is rapidly and well absorbed from the gastrointestinal tract, with time to peak plasma drug concentration of 2-4 h. The oral bioavailability is (>80%) and the plasma elimination half-life is around 20 h in healthy volunteers (Dichter and Broddie, 1996; Perucca and Bialer, 1996). More than 60% of dose of topiramate eliminated unchanged by the renal route, and by different metabolic pathways for the most of the remaining absorbed fraction. In patients treated with AED (ant-epileptic drug) inducers of cytochrome P450 metabolism, such as carbamazepine phenobarbital and phenytoin, metabolic elimination possibly becomes the major determinant of topiramate disposition and elimination of unchanged drug into urine is reduced to only about 30% (Dichter and Broddie, 1996; Perucca and Bialer, 1996).

Several chromatographic methods with HPLC-UV (Bahrami et al., 2005), GC (Wolf et al., 2000; Riffitts et al., 1999; Holland et al., 1988; Tang et al., 2000; Gidal and Lensmeyer, 1999) and LC-MS/MS (Chen and Carvey, 1999; Chen and Carvey, 2001; Britzi et al., 2003; Park et al., 2008; Contin et al., 2001; la Marca et al., 2008; Christensen et al., 2002; Goswami et al., 2009) have been developed to measure topiramate in biological fluids. These techniques were inadequate for pharmacokinetic studies due to the need for large volumes of biological samples; the long chromatography runs time, high injection volumes and low sensitivity. Most of the methods (Bahrami et al., 2005; Wolf et al., 2000; Riffitts et al., 1999; Holland et al., 1988; Tang et al., 2000; Chen and Carvey, 1999; Chen and Carvey, 2001; Britzi et al., 2003) requires laborious extraction procedure like liquid-liquid extraction involving time-consuming and error prone solvent evaporation and reconstitution steps and long chromatographic run time. Methods (Gidal and Lensmeyer, 1999; Contin et al., 2001; Park et al., 2008) involve protein precipitation extraction technique. Even though these techniques are inexpensive, it may give ESI source contamination and matrix effect after consistent number of injection because of sample muck. (Goswami et al., 2009) developed an LC-MS/MS based method for determination for topiramate in human plasma. The method was enough sensitive (10 ng/ml). However, the chromatographic run time was long (2.5 min); requires laborious SPE extraction steps involving time-consuming and error prone elution solvent evaporation and reconstitution steps. The linearity range for topiramate was restricted (10-2045 ng/ml). Therefore, it was necessary to develop a simple, specific, rapid and sensitive analytical method for the quantification of topiramate in human plasma. All comparative methods are presented in (Table 1).

This paper describes the development and validation of an LC-MS/MS method for the quantification of topiramate in human plasma, which reduces sample preparation and analysis time relative to other commonly employed techniques (LLE) and has a limit of quantitation (LOQ) 10 ng/ml. Topiramate d12 was used as an internal standard to control assay drift during clinical study sample analysis.

Materials and Methods

Chemicals and reagents

Pharmaceutical grade of topiramate was supplied by Torrent Pharmaceuticals (Ahmedabad, India) and IS was supplied by BDS Synthesis (Wellington, New Zealand) used without further purification and certified to contain >98% and >97%, respectively. The chemical structures of these compounds are as shown in (Figure 1). Organic

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Comparison of analytical methods developed for estimation of topiramate in biological matrix.							
Sr. No.	Processing volume	Extraction procedures	Evaporation and Reconstitution	Analytical run time (Min)	LOQ (ng/ml)	Detection technique	Reference
1	NA	LLE and derivatization	Done	NA	40	HPLC-UV	1
2	0.1 ml plasma	LLE	Done	NA	2000	LC-MS-MS	2
3	0.5 ml plasma	PPT	Not required	4	250	LC-MS-MS	3
4	0.1 ml plasma	NA	NA	NA	20	LC-MS-MS	4
5	0.1 ml plasma	LLE	Done	NA	2000	LC-MS-MS	5
6	0.1 ml plasma	LLE	Done	6	2000	LC-MS-MS	6
7	NA	NA	NA	NA	16.6	LC-MS-MS	8
8	0.5 ml plasma and blood	PPT	Not required	5	3000	GC-FID	9
9	0.3 ml plasma	SPE	Done	2.5	10.4	LC-MS-MS	10
10	0.5 ml plasma	LLE	Done	>6.0	100	GC-FID	12
11	NA	PPT	Done	2.5	20	LC-MS-MS	13
12	NA	LLE	Done	>5.32	500	GC-NPD	15
13	NA	LLE	Done	>7.2	1000	GC-NPD	16
14	0.5 ml in Serum	LLE	Done	>4.0	2500	GC-NPD	17
15	0.1 ml plasma	SPE	Not required	1.8	10	LC-MS-MS	Present method
LLE: Liquid-Liquid extraction; PPT: Protein precipitation; NA: Not Available							

Table 1: Comparison of analytical methods developed for estimation of topiramate in biological matrix

solvents used where of gradient grade and were purchased from (Ranbaxy, Delhi, India). Water was prepared from Milli Q gradient water purification system (Massachusetts, USA). *Ortho*-phosphoric acid and ammonia, suprapur® grade, were procured from Merck (Germany), Ammonium acetate used for mobile phase preparation was of molecular biology tested, procured from Sigma-Aldrich (Germany). The solid phase extraction (SPE) cartridge HLB 1 cm^3 (30 mg) was from Waters (Massachusetts, USA). Control human plasma was procured from Clinical Research Department of Prathama Lab. (Ahmedabad, India) and was stored below -70° C.

Instrumentation

Triple quadrupole mass spectrometer used was TSQ quantum, manufactured by Thermo Finnigan (Thermo-Electron Corporation, San Jose, CA, USA), while the HPLC modules were Shimadzu. Chromatographic separation was carried out on Shimadzu HPLC with Betasil, C18 (100 mm x 3 mm i.d., 3.0µm particle size column) obtained from Thermo-Electron Corporation (Waltham, MA, USA).

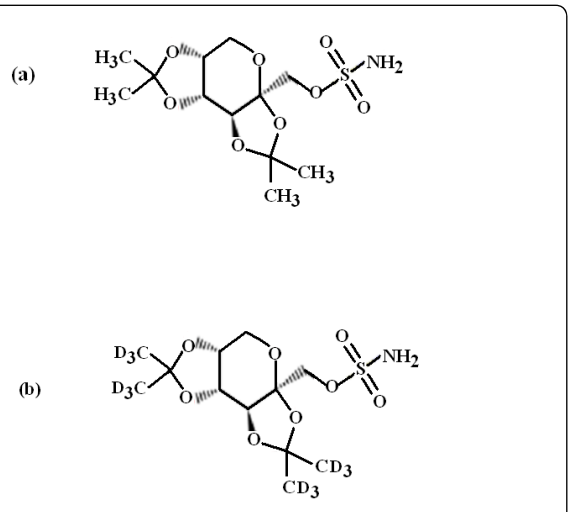

Figure 1: Chemical structure of topiramate (a) and IS (b).

Chromatographic and ESI-MS/MS detection conditions

The HPLC analysis was performed on Shimadzu prominence pump operating at a flow rate of 0.5 ml/min; autosampler tray temperature was set at 10°C. The mobile phase consists of methanol: 2mM ammonium formate pH adjusted to 8.0 with ammonia (95:5, v/v).

A mass spectrometer was equipped with an electrospray ionization (ESI) ion source. The mass spectrometer was set in negative ionization, selected reaction monitoring (SRM) mode. The Ion Spray conditions for topiramate and IS were as follows: collision gas pressure 1.5 mTorr, sheath gas 40.0 (arb), auxiliary gas 20.0 (arb), capillary temperature 350.0°C, ion spray voltage (IS) 3500.0 V, tube lens offset and collision energy (CE) applied for topiramate was 63 V and 42 V, for IS was 92 V and 44 V, respectively. The MS/MS transition selected to monitor topiramate was m/z 338.10 to a product ion at m/z 78.20 as. The internal standard IS was monitored using the transition from 350.40 to a product ion at m/z 90.10. The deprotonated molecules were fragmented using argon as the collision gas.

Quantitation

The LCquan software, version-2.5.6 software provided a standard method for the quantitative calculations. The peak areas for all the SRMs were automatically integrated and the area ratios (topiramate/IS) of the calibration were used to generate a linear regression analysis with weighting factor of $1/X^2$.

Standard solutions

Stock solutions of topiramate and IS, were prepared in methanol at free base concentration of 2.5 and 1.0 mg/mL, respectively.

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Secondary and working standard solutions were prepared from stock solutions by dilution with water. These diluted working standard solutions were used to prepare the calibration curve and quality control (QC) samples in human plasma.

A nine-point standard calibration curve for topiramate was prepared by spiking the blank plasma with appropriate amount of topiramate. The calibration curves were ranged from 10 to 4200 ng/ml. QC samples for topiramate were prepared in three concentration levels: 30 ng/ml LQC; 1500 ng/ml MQC and 3000 ng/ml HQC, in a manner similar to preparation of standard solutions from the stocks solution.

CS and QC sample preparation

Calibration standard (CS) samples were prepared just prior to extraction by spiking 95 µL blank human plasma with topiramate to give concentrations of 10, 20, 200, 400, 800, 1600, 2400, 3200, and 4200 ng/ml. Quality control (QC) samples were prepared at concentration of 30, 1500 and 3000 ng/ml.

A 0.1 ml (CS, QC and volunteers plasma) samples were mixed with 25 µl of IS working solution (500 ng/ml of IS). 775 µL of 0.05 M sodium hydroxide was added to it. The sample mixture was loaded into an Oasis HLB (1 cm^3/30mg), an extraction cartridge that was pre-conditioned with 1 ml methanol followed by 1 ml water. The extraction cartridge was washed with 2 ml water followed by 1.0 ml 5% methanol. Topiramate and IS were eluted with 0.8 ml of methanol; 3.0 µl of the eluate was injected into LC-MS/MS system.

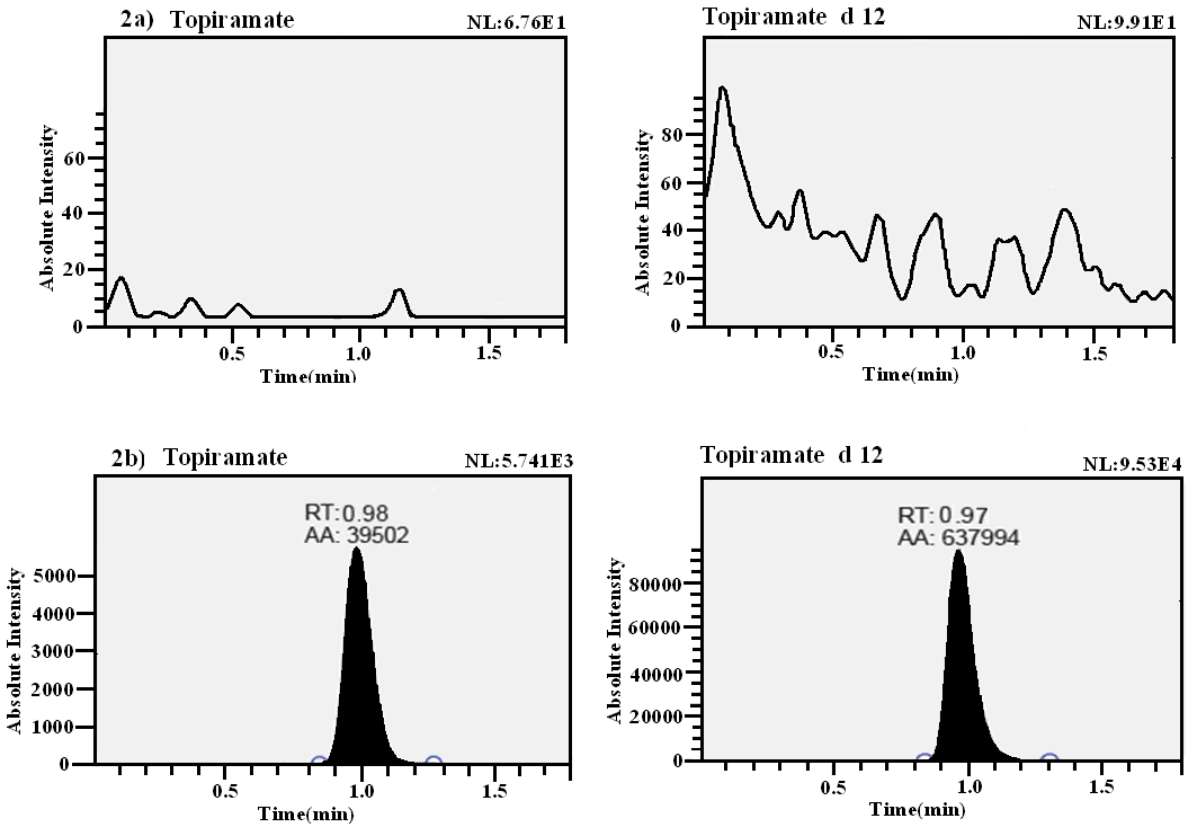
Results and Discussion

Method development

The aim of this method was to develop and validate (FDA. Guidance for Industry: Bioanalytical Method Validation et al., 2001) a simple, rapid, sensitive and high throughput method for the extraction and quantitation of topiramate in clinical studies. Topiramate lost the proton in a basic mobile phase and produced a deprotonated precursor ion at m/z 338.10. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were evaluated to get better response of analytes. It was found that the best signal was achieved with ESI negative ion mode. A product ion m/z *78.20* of topiramate was monitored, which gave better sensitivity and selectivity.

Further optimization in chromatography conditions resulted in improvement in signal and reduction in run time. It is observed that increase in buffer pH from 3.0 to 8.0 resulted in improved response and peak symmetry. Use of Betasil C18 (100 mm x 3 mm i.d., 3.0µm particle size) column enabled use of 0.5 ml/min flow rate, which resulted in run time as low as 1.8 min with better peak symmetry and signal of analytes. The column oven temperature was optimized to 45°C in order to get symmetry of analyte peaks.

In order to achieve cleanliness in extract, solid-phase extraction was optimized for extraction of analyte from plasma. Both analytes showed good retention when eluted with basic conditioned on cartridges. It was observed that washing the solvent with 5% methanol strength resulted in reduced interference without losing



Figuure 2: Representative chromatograms for topiramate (a) Extracted blank plasma; (b) Extracted 10.0 ng/ml of topiramate.

Summary of calibration curves for topiramate with backcalulated concentrations									
Linearity	Topiramate, concentration in ng/ml								
	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9
	10	20	200	400	800	1600	2400	3200	4200
1	10.010	20.542	200.010	400.254	800.277	1623.210	2425.210	3254.354	4,245.550
2	10.254	20.005	213.214	394.521	789.351	1652.012	2435.021	3215.221	4,221.584
3	11.001	21.254	198.542	420.124	812.254	1623.120	2432.214	3200.124	4,121.897
4	9.854	20.023	197.586	398.542	800.001	1599.257	2465.263	3242.214	4,212.541
5	10.005	19.258	200.012	400.235	789.210	1624.258	2432.123	3245.125	4,255.654
Mean	10.225	20.216	201.873	402.735	798.219	1624.371	2437.966	3231.408	4211.445
% Nominal conc.	102.25	101.08	100.94	100.68	99.78	101.52	101.58	100.98	100.27
SD	0.457	0.739	6.423	9.998	9.542	18.693	15.682	22.760	53.010
%CV	4.47	3.65	3.18	2.48	1.20	1.15	0.64	0.70	1.26
CS = Calibration Standard									
Conc. = Concentration									

Table 2: Summary of calibration curves for topiramate with backcalulated concentrations.

Intra and Inter accuracy and precision for topiramate									
Topiramate intra assay precision and accuracy						Topiramate inter assay precision and accuracy			
Quality control samples	Conc. added (ng/ml)	Mean conc. found (ng/ml) (a)	SD	Precision % CV	Accuracy (%)	Mean conc. found (ng/ml) (b)	SD	Precision % CV	Accuracy (%)
LLOQ	10	9.870	1.018	10.32	98.70	9.923	1.271	12.81	99.23
LQC	30	30.907	0.691	2.24	103.02	30.705	1.470	4.79	102.35
MQC	1500	1503.451	83.393	5.55	100.23	1489.480	104.147	6.99	99.30
HQC	3000	2994.612	83.271	2.78	99.82	3093.498	49.904	1.61	103.12
(a) = mean of six replicates									
(b) = mean of thirty replicates									
Conc. = Concentration									

Table 3: Intra and Inter accuracy and precision for topiramate.

Stability results of topiramate				
Stability experiments	Storage conditions	Mean comparison sample conc. found (ng/ml)	Mean stability sample conc. found (ng/ml)	% Mean change at quality control level
Bench top	Room temperature (28 hr)	30.213	31.221	LQC 3.34
		3089.321	3045.651	HQC -1.41
Process (extracted sample)	Auto sampler (72 hr)	31.254	29.965	LQC -4.12
		3154.231	3170.112	HQC 0.50
Freeze and thaw stability in plasma	After 4th FT cycle at -70°C	30.012	31.111	LQC 3.66
		3089.685	3045.365	HQC -1.43
Long term stability in plasma	For 72 days at-70° C	30.224	31.231	LQC 3.33
		1512.321	1552.632	MQC 2.67
		3055.321	3001.285	HQC -1.77
LQC = 30 ng/ml, MQC = 1500 ng/ml, HQC = 3000 ng/ml				

Table 4: Stability results of topiramate.

recovery of analytes. In order to eliminate the time-consuming and error-prone solvent evaporation and reconstitution steps for concentration of samples after elution with methanol, the elution volume of methanol was reduced to 0.8 ml to concentrate the sample in elution solvent. The optimized detection and sample extraction chromatography are enabled to reduce processing and analysis time without compromising the sensitivity. Sample volume injected 3.0 µl to avoid the column backpressure and ESI source contamination during clinical study sample analysis.

Bioanalytical method validation

Specificity: The specificity of the method was investigated by comparing chromatograms obtained from six different sources of plasma. The representative chromatograms shown in (Figure 2) indicates that there was no interference of analytes and IS from endogenous substances in plasma. The area observed at the retention time of topiramate was much less than 20% that of the LLOQ (10 ng/ml) area (Figure 2).

Calibration curve: The calibration curves were appeared linear and were well described by least squares lines. A weighting factor of 1/concentration i.e. $1/x^2$ was chosen to achieve homogeneity of variance for topiramate. The accuracy and precision values observed for the back calculated concentrations of five linearity from CS-1 to CS-9 are presented in (Table 2). The correlation coefficient were ≥0.9982 (n=5) for topiramate.

Recovery: Recovery of topiramate was calculated by comparing the peak area of the analyte from extracted plasma standard with that obtained from unextracted standard at the same concentration for QC samples. The percent mean recovery for topiramate was observed 78.20. The mean recovery of IS was 87.74% at concentration 500 ng/ml.

Precision and accuracy: The intra-assay precision and accuracy were calculated in six replicates analyses for topiramate at four concentration level viz. LLOQ (10 ng/ml), LQC, (30 ng/ml), MQC, (1500

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ng/ml) and HQC, (3000 ng/ml) each on the same analytical run. Inter-assay precision and accuracy was calculated after repeated analysis in three different analytical runs. The results are given in (Table 3).

Matrix effect: Matrix effects were investigated for six different samples of plasma comprising four lots of normal control heparinzed plasma, one lot of lipemic plasma, and one lot of haemolyzed plasma. Three samples each at the LQC and HQC levels were prepared from different lots of plasma (i.e. a total of 36 QC samples) and checked for accuracy, to see whether the matrix affected the back-calculated value of the nominal concentrations for these different plasma samples. The results obtained were well within the acceptable limit of ±15%, which clearly proves that elution of endogenous matrix peaks in the dead volume time does not affect the pattern of elution of topiramate and IS, respectively.

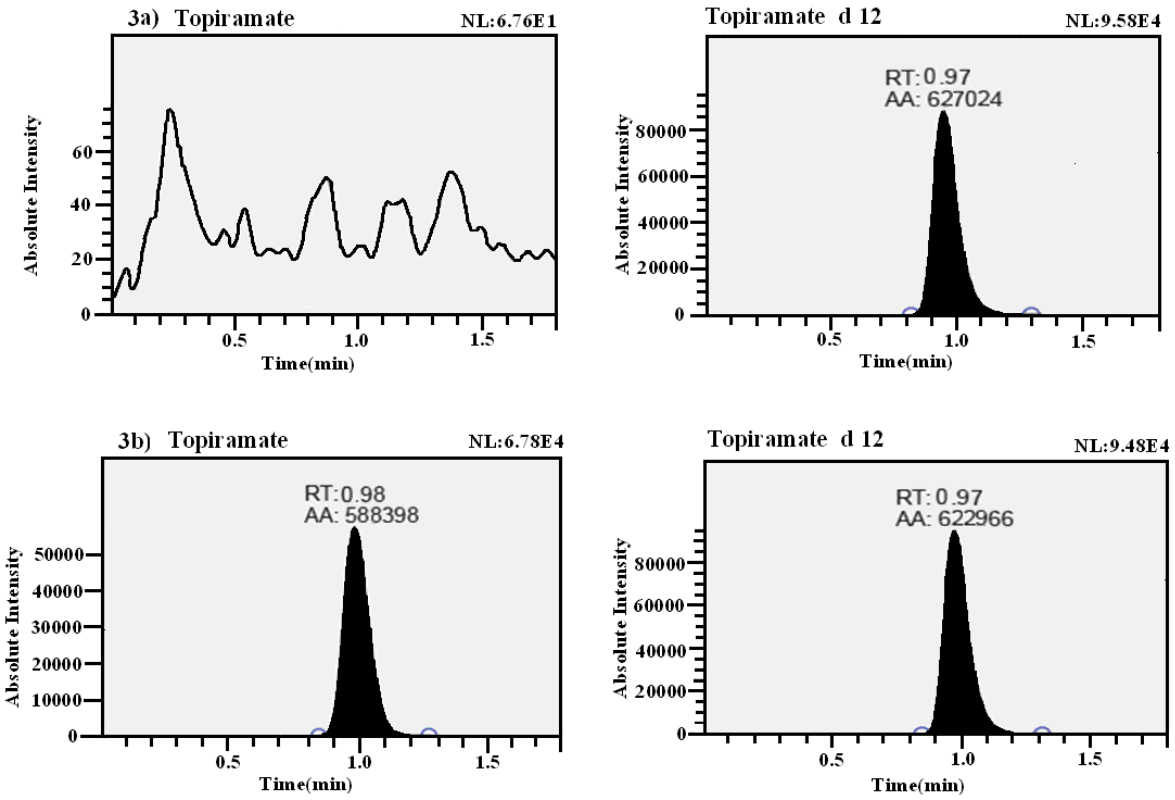
Stability: Exhaustive experiments were performed to assess the stability of topiramate in stock solution and in plasma samples under different conditions simulating the conditions occurring during analysis of study samples—room-temperature stability, extracted sample stability (process stability), freeze–thaw stability, and long term stability of plasma samples. The results obtained were well within acceptable limits. IS stock solution was also found to be stable.

Stock solutions of topiramate and IS were stable at room temperature for 20 h and at 2–8°C for 28 days. Topiramate in control human plasma were stable for 28 h at room temperature. Both the analytes in extracted plasma samples were stable for 72 h in an autosampler at 10°C. Topiramate found stable at least four freeze–thaw cycles. Topiramate spiked plasma samples stored at -70°C to test long-term stability were stable for at least 72 days. Percentage changes of concentration in these stability experiments are listed in (Table 4).

Bioequivalence study and application

The design of study comprised an open randomized, two period, two sequence, replicate, crossover, comparative evaluation of relative bioavailability of test formulation of topiramate with reference (100 mg Topamax ®, tablets) in 18 healthy adult human subjects under fasting condition. All the subjects were informed of the aim and risk involved in the study and written consent was obtained. Ethics committee approved the study protocol. Health check up for all subjects was done by general physical examination, ECG and laboratory tests like hematology, biochemistry and urine examination. They were orally administered a single dose of test and reference formulation after recommended with 240 ml of water. Blood samples were collected in vacutainers containing heparin before collection of each time point's administration of drug. Plasma samples were collected 0.00, 0.17, 0.33, 0.50, 0.66, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 8.00, 12.00, 16.00, 20.00, 24.00, 48.00, 72.00, 96.00, 120.00, 144.00 and 168.00h after administration of a single oral dose of a 100-mg tablet to 18 male volunteers in each phase. Blood samples were centrifuged at 3500 rpm for 10 min and plasma was separated, stored at -70°C until use.

Application of method: The proposed method was applied to the determination of topiramate in plasma samples from ongoing projects for the development of an immediate release formulation. A total of 1102 human plasma samples from 18 male volunteers were analyzed along with CS and QC samples. Total 460 samples were analyzed per day. No interference peak was found in pre-



Figuure 3: Representative chromatograms for topiramate (a) Extracted pre-dose volunteer sample; (b) Extracted volunteer sample at 2 h.

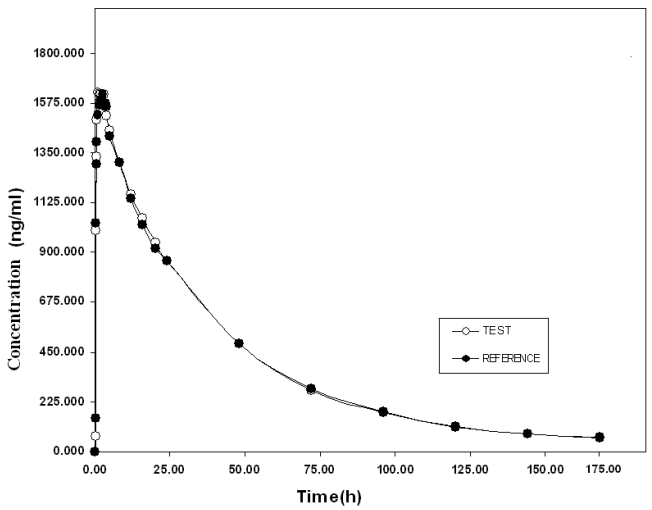


Figure 4: Mean plasma concentration topiramate time profile in 18 healthy volunteers after 100 mg oral dose.

dose samples for all volunteers. The concentration of topiramate in volunteer samples observed at 2.0 h after dosing (Figure 3). The mean topiramate plasma concentration-time profile following a 100 mg oral dose of topiramate to human subjects is shown in (Figure 4).

Conclusion

The objective of this work was to develop a simple, specific, rugged and a high throughput method for estimation of topiramate in human plasma. The advantage of using an SPE technique in the present work is due to (i) minimize the sample extraction time, (ii) present method has been used only 100 µl of human plasma compare to reported methods and hence to reduce the amount of blood withdrawn from volunteers during study, iii) proposed method is applicable for 25, 50, 100 and 200 mg bioequivalence studies, (iv) it gives cleaner and consistent extraction with minimum matrix effect, (v) because of rapid sample preparation technology and short chromatographic run time, large numbers of pharmacokinetic samples can be analyzed.

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