QUANTITATIVE ANALYSIS OF TOPIRAMATE IN HUMAN PLASMA USING LC-MS/MS AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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ABSTRACT

A simple, sensitive and reliable liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the quantification of topiramate in human plasma. The liquid-liquid extraction was used to extract topiramate from human plasma. Chromatographic separation was achieved on Gemini C₁₈ (150 mm x 4.6 mm, 5µm) column with the mobile phase composed of acetonitrile: 2 mM ammonium acetate (85:15, v/v) at a flow-rate of 0.5 mL/min. The method was validated over the linearity range 15 – 3000 ng/mL with 0.2 mL of human plasma using niclosamide as an internal standard (IS). The precursor to product ion transition was monitored at m/z 338.20 → 78.20 and m/z 325.20 → 171.20 for topiramate and IS in negative mode, respectively. This method confirmed precision, accuracy and extraction recoveries obtained for topiramate were consistent and reproducible. Topiramate was found to be stable throughout the freeze-thaw cycles, bench-top and post-operative stability studies. The method was successfully applied to epileptic patients to monitor the plasma drug concentration versus time profile. The inter variability in pharmacokinetic parameters shows the need for individual monitoring of topiramate plasma profile by the accurate, specific and sensitive bioanalytical method.

KEYWORDS: Bioanalytical method; Human plasma; LC-MS/MS; Pharmacokinetic study; Topiramate.
1. INTRODUCTION

Epilepsy is a chronic non-communicable disorder of the brain that affects people of all ages. Approximately 50 million people worldwide have epilepsy, making it one of the most common neurological diseases globally.[1] Topiramate (2,3:4,5-Bis-O-(1-methylethylidene)-beta-D-fructopyranose sulfamate) is a second generation anti-epileptic drug widely used drug alone or in combination for the treatment of epilepsy. It is useful for several types of partial-onset and generalized-onset seizures and is therefore considered a broad-spectrum agent.[2] It is also effective as a prophylactic against a migraine headache.[3] Topiramate is rapidly absorbed after oral administration, shows little (10-20%) binding to plasma protein. It is mainly excreted unchanged in the urine and by diverse metabolic pathways. Food slows the absorption but there is no significant effect.[4-5] Topiramate level monitoring in blood has been playing important role in treatment of epilepsy to determine the optimum therapeutic dose for an individual patient.

The topiramate does not have UV-Vis absorption or emit fluorescence and could not quantify by readily accessible techniques, nevertheless, a variety of analytical methods have been reported for quantitative determination of topiramate. It often employs derivatization methods followed by separation and/or detection. The efforts have been established to quantify topiramate by high performance liquid chromatography (HPLC) combined with different detector viz. indirect UV[6], fluorescence,[7-12] refractive index[13] and chemiluminescent nitrogen detection[14] in pharmaceutical formulations as well as from biological fluids. Further, spectrofluorimetric method[15], gas chromatography-mass spectrometry (GC-MS)[16] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods were also reported to investigate topiramate in various biological matrices such as in human plasma[17-22], serum[23], dried blood spots[24] and in combination with other drugs in human plasma[25-28] and serum[29-30] also. Moreover, Yin et al. discussed the comparative assessment of reported methods thoroughly for topiramate and sets many reported methods to have relatively long analytical run time, large sample volumes, and complex sample preparation. Additionally, Yin et al. were developed LC-MS/MS method to determine anti-epileptic drugs in human plasma using protein precipitation extraction technique (PPT).[28] Studies have shown that PPT could not remove phospholipid as compared to liquid-liquid extraction (LLE) presented in plasma which may be affecting the results. Hence, sometimes PPT was considered as less clean extraction or crude extraction technology from plasma.[31]
This method was demonstrated to provide sensitive, precise and accurate LC-MS/MS method for the quantification of topiramate in human plasma. This method allows simple and fast sample preparation using LLE technique and shorter runtime. The method was applied to a four epileptic patient to monitor topiramate concentration in plasma and to study pharmacokinetic behavior.

2. MATERIAL AND METHODS

2.1. Chemical and reagents
Topiramate and niclosamide (IS) were the generous gifts from Torrent Pharmaceutical Ltd., and Ami Lifescience Pvt. Ltd., India, respectively. HPLC grade acetonitrile, methanol and tert-butyl methyl ether were purchased from Spectrochem Pvt. Ltd., India. Ultrapure water was used throughout the study from MilliQ-Elix system, Millipore Pvt. Ltd., India. All other chemicals used in this study were analytical grade and used without further purification. The control human plasma with K$_3$EDTA anticoagulant was obtained from Rajkot voluntary blood bank and research center, Rajkot, Gujarat, India.

2.2. Instrumentation and analytical conditions
The LC-MS/MS system consisted of a LC-20AD solvent delivery system, a DGU-20A5R vacuum degasser, SIL-20AC autosampler and CTO-20AC thermostated column oven connected by an electrospray ionization (ESI) interface and coupled with a triple quadrupole mass spectrometer LCMS-8030 (Shimadzu Corporation, Kyoto, Japan). Data acquisition and processing were performed using Lab solution software (version 5.53) from Shimadzu Corporation, Japan.

The chromatographic separation was carried out on a Gemini C$_{18}$ (150 x 4.6 mm, 5μm) analytical column placed in thermostated column oven maintained at 40 °C. The mobile phase consisted of acetonitrile: 2mM ammonium acetate in the proportion of 85:15, v/v at a flow rate of 0.500 mL/min. The autosampler temperature was kept at 4°C and 10 μL sample volume was injected into the system. Analytical run time was 7 min.

In mass, the ionization and detection were performed by infusing a solution containing 500 ng/mL of topiramate and IS. The operating conditions were set as follows; the interface temperature and ion spray voltage fixed at 350 °C and 4.5 kV respectively. Nitrogen was used as nebulizing gas (3 L/min) and drying gas (15 L/min). With argon as collision gas, multiple reaction monitoring (MRM) mode was applied to the ionic transition of m/z 338.20
Thummar et al. World Journal of Pharmacy and Pharmaceutical Sciences

→ 78.20 for topiramate and m/z 325.20 → 171.20 for IS in negative ionization mode. The optimized collision energies 30eV were used for both. The dwell time was set to 100 ms.

2.3. Preparation of Standard and quality control (QC) Samples
The stock solutions of topiramate (1000 µg/mL) and IS (1000 µg/mL) were prepared in methanol. The stock solution was further serially diluted using a mixture of methanol and water in the ratio of (50:50, v/v) to get series of working standard solution having a concentration of 60000, 30000, 15000, 6000, 3000, 1500, 600 and 300 ng/mL. From that calibration curves were prepared by 5% spiking (10µL) of the working standard solutions of topiramate into blank plasma (190µL) to achieve a concentration of 3000, 1500, 750, 300, 150, 75, 30 and 15 ng/mL for topiramate. The quality control samples (QCs) were prepared in a similar manner at four concentration level, viz. 2700 ng/mL (high, HQC), 225 ng/mL (middle, MQC), 45 ng/mL (low, LQC) and 15 ng/mL (lower limit of Quantification, LLOQ QC) for topiramate; A working IS solution was prepared in same way to get final concentration of 500 ng/mL. All samples were stored at 2 – 8°C until analysis.

2.4. Plasma sample preparation
To 200 µL of spiked plasma, add 50 µL of internal standard and vortex for 30 sec in 2.5 mL polypropylene vial. A 50 µL of extraction buffer (0.1N HCl) was added and vortex for 1 min. Subsequently, 1.3 mL of liquid extraction solvent (tert-butyl methyl ether) was added to above solution and vortex for 3 mins. The resultant mixture was then centrifuged at 4000 rpm at 10°C for 10 mins, the organic phase was transferred to a vial and evaporated to dryness at 40°C under a gentle stream of nitrogen. The dried samples were reconstituted with 100 µL of mobile phase as a reconstitution solvent and a 10µL sample were injected into the chromatographic system.

2.5. Bioanalytical method validation
The method was validated based on recommendation of the United States Food and Drug Administration (USFDA) guidelines.[32]

2.5.1. Specificity
The specificity of the method was evaluated by screening ten different batches of blank human plasma, (seven were K3EDTA and one each of lipidemic, haemolysed and heparinized plasma) to investigate the interferences at the signal of topiramate and IS.[33] Aliquots of
plasma samples were used to prepare LLOQ and blank samples. The baseline noise should be < 20% of analyte response at this concentration level.

2.5.2. Linearity, accuracy and precision
The linearity of the method was determined by analysis of an eight-point calibration curve over the concentration range of 15-3000 ng/mL of topiramate. Each calibration curve (n = 3) was analyzed individually by least square weighted linear regression method (1/x^2) of peak area ratios of topiramate to IS (y) versus actual concentration (x). Slope (m) and intercept (c), were calculated for each standard curve. The regression equation (y = mx + c) for the calibration curve was used to back-calculate the measured concentrations at each standard as well as QC level. A correlation coefficient (r^2) value of greater than 0.990 was desirable for all calibration curves.

The intra and inter assay precision and accuracy were determined by analyzing a set of QC samples (n = 6) at LLOQ QC, LQC, MQC and HQC level (15, 45, 225 and 2700 ng/mL, respectively) on the same day and on three consecutive days respectively. The percentage relative standard deviation (%RSD) and percent deviation from the nominal concentration (% Bias) serves as a measure of precision and accuracy, respectively. The criteria for acceptability of the data included precision and accuracy were within ±15% of nominal value, except for LLOQ, where it should not exceed ± 20% of precision and accuracy as well.

2.5.3. Recovery and matrix effect
The extraction recovery and matrix effect for topiramate were performed in six replicates of LQC, MQC and HQC samples (45, 225 and 2700 ng/mL). The recovery of the topiramate after liquid-liquid extraction was evaluated by comparing peak area obtained from pre (A) and post (B) spiked plasma extracted samples at equivalent concentrations. The percentage recovery was measured by (A/B) × 100. The matrix effect due to plasma matrix was assessed by comparing peak area of the post spiked samples (B) to those of pure standard solutions in mobile phase at the same concentrations (C). The matrix effect (%) was calculated by (B/C) × 100. The extraction recovery and matrix effect of IS were evaluated at the working concentration (500 ng/mL) in the same manner. The %RSD of recovery and matrix effect at each concentration should be less than 15%.
2.5.4. **Dilution integrity**

Dilution integrity was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration in case if study samples go beyond the validated calibration range of method. To demonstrate the dilution integrity of topiramate, pre-determined aliquots were diluted with human plasma (1:2 and 1:10) to form diluted quality control samples (DQCs) having a concentration of DQC (1/2) 1200ng/mL and DQC (1/10) 2700 ng/mL. The back-calculated standard concentrations had to comply both precision of ≤15% and accuracy of 100 ± 15% similar to other QC samples.

2.5.5. **Stability**

The stabilities in human plasma were tested by analyzing six replicates of low and high matrix matched QC samples (45, 2700 ng/mL) in different conditions. The bench top stability (6 h at ambient temperature), freeze-thaw stability (-20 ± 5°C), Autosampler (wet extract) stability (at 4°C for 24 h), dry extract stability (2 - 8°C for 24 h.), and long term stability (-20 ± 5°C for 28 days) were determined. The concentration of stability samples and freshly prepared samples were calculated and stability was shown as the percentage mean stability in calculated concentration. If the % RSD of mean stability was within 15%, the assay was considered stable.

2.6 **Application of the method**

The main objective of this clinical study was to prove the applicability of the analytical technique. Prior to the start, the study protocol and statement of informed consent were approved by human ethics committee of Department of Pharmaceutical Sciences affiliated to Saurashtra University (Rajkot, Gujarat, India). Meanwhile, the clinical study was performed according to the ethical values of the Declaration of Helsinki. An experienced specialist neuro-physicians of Wellcare hospital (Rajkot, Gujarat, India) carried out the diagnosis of the disease and prescribed appropriate drug therapy for its patients. In this manner, total four patients under topiramate therapy were enrolled for the experimental monitoring. The routine dose of 50 mg of topiramate was set for examination in a day. Approximately 3ml of blood sample were drawn into an EDTA tube at 0, 0.5, 1, 2, 3, 4, 12 and 24 h after administration of 50 mg topiramate tablet. The plasma samples were prepared by centrifuging the blood samples at 3000 rpm, for 10 min at Wellcare Pathology Laboratory (Rajkot, Gujarat, India). The plasma samples were stored at -20 °C prior to sample processing.
3. RESULTS

3.1 Optimization of mass spectrometric condition

The topiramate and IS were analyzed firstly by direct infusion of the individual standard solution. Both positive and negative ionization modes were inspected for the detection of topiramate and IS, and negative ionization mode gave more efficiently ionized and therefore employed. In this manner, the molecular ion [M-H]⁻ of topiramate and IS was detected at 338.2 m/z, and 325.20 m/z respectively. The collision-induced (30 eV for topiramate and IS) dissociation at 78.2 m/z due to the stabilized product ion of H₂NO₂S from topiramate, whereas 171.20 m/z due to stabilized product ion of C₆H₄ClN₂O₂⁻. The corresponding mass spectrum as shown in Figure 1.

3.2 Optimization of sample preparation

One of the key central steps in the development of the bioanalytical method is sample preparation. Sample preparation procedure should be rapid, easy and should require the least amount of reagents with maximum recovery of analytes. In this regard, we used LLE method in such a way that the sample preparation took shorten processing time and to acquire desired recoveries of the topiramate and IS. Different organic solvents such as dichloromethane, diethyl ether, n-hexane, ethyl acetate, tert-butyl methyl ether and several buffers such as 0.1 N HCl, 0.1 N NaOH, etc were used in trials. Among them, tert-butyl methyl ether and 0.1 N HCl showed maximum and reproducible recovery. There was no interference from any endogenous or exogenous plasma matrix and IS did not alter topiramate recovery, sensitivity and/or ion suppression as well.

3.3 Optimization of chromatographic condition

The chromatographic conditions were optimized to look for better sensitivity, peak shape and chromatographic run time. The selection of mobile phase was performed taking into account the symmetric peak shape with a shorter run time that further leads to low consumption of organic solvent altogether making the method cost-effective. In this study, we tried Gemini-C18, Kromasil-C18 with various organic solvent and buffer solutions for mobile phases, such as methanol, acetonitrile, 0.1% formic acid, 2mM ammonium acetate and ammonium formate. The satisfactory results were obtained in Gemini C18 (150 x 4.6 mm, 5µm) analytical column with the several combinations showed that acetonitrile: 2mM ammonium acetate buffer (85:15, v/v) serves the desired purpose with utmost effectiveness.
3.4 Method validation

3.4.1 Selectivity

Figure 2 shows the representative mass chromatograms of blank plasma for topiramate and IS, standard zero (IS) and LLOQ sample were presented. No significant interference in the blank plasma traces was observed from endogenous substances in drug free human plasma at the retention time of topiramate and/or IS. The retention times were 3.64 and 4.94 min for topiramate and IS respectively.

3.4.2 Linearity, accuracy and precision

The chromatographic responses were found linear over the concentration range of 15 – 3000 ng/mL in human plasma. The correlation coefficient, $r^2$ was consistently equal or greater than 0.995 during the course of validation. The calibration curve samples were within ± 15% of the theoretical value. The intra and inter-assay precision and accuracy were assessed by analyzing six replicate at each QC level. Concentrations were back-calculated from the calibration curve. The method was reliable and reproducible since the intra and inter-assay precision (% RSD) was less than 9.75% and 8.82%, respectively. On the other hand, intra and inter-assay accuracy (% Bias) was within 6.19% and 7.66%, respectively. Table 1 showed that all values of accuracy and precision fell within the limits as acceptable.

3.4.3 Recovery and matrix effect

The topiramate and IS were recovered by LLE from human plasma. The % recoveries of topiramate were ranged from 89.12 - 93.09%. The recovery of IS was achieved to be 92.81 ± 5.42%. Table 2 showed that the recovery of topiramate and IS were high and consistently precise and reproducible. The mean value of matrix effect of topiramate was varied from 91.59% - 96.16% with acceptable %RSD value. The data represented in Table 2 indicates that no endogenous substance significantly influenced ion suppression of topiramate and IS as well in this analytical method and allowed us to conclude that our method is able to quantify topiramate in human plasma.

3.4.4 Dilution integrity

Precision (%RSD) values for reliability for 1/2nd and 1/10th dilutions were 9.61 and 6.36%, while the accuracy was 102.82 and 97.09% for topiramate, respectively. Results were within the acceptance limit of 15% for precision and 85 – 115% for accuracy as shown in Table 3. This parameter was especially important to prove that the quantification of topiramate was also possible for the out of range sample. In this study, we faced the same for one patient.
Thummar et al. World Journal of Pharmacy and Pharmaceutical Sciences

(Patient A) who’s the plasma concentration of topiramate was significantly higher, in that case, the sample was diluted 2 times and thereafter processed and analyzed.

3.4.5 Stability
Table 4 summarizes the results of the bench-top stability, freeze-thaw stability, dry extract stability, autosampler stability and long term stability in human plasma as the percentage mean stability of the calculated vs. theoretical concentration. No significant deviations were observed compared to theoretical concentration, indicating that topiramate was stable under all tested conditions. Therefore, the method has been proved to be applicable for routine analysis.

3.5 Application of the method
The validated method was applied to monitoring the plasma concentration of topiramate from the four epileptic patients. The calibrations, QCs and real samples were run and analyzed successfully on the same batch. The mean plasma concentration versus time profile is presented in Figure 3. The pharmacokinetic parameters such as peak plasma concentration ($C_{\text{max}}$), time required to achieve maximum plasma concentration ($T_{\text{max}}$), half-life ($t_{1/2}$), Area under curve (AUC), mean residence time (MRT), Elimination rate ($L_z$) and clearance (CL) of topiramate were calculated by pksolver tool in excel sheet and summarized in Table 5.

![Mass spectrum of (a) topiramate and (b) IS, precursor to product ion with its possible fragmentation pattern.](image-url)

Figure 1: Mass spectrum of (a) topiramate and (b) IS, precursor to product ion with its possible fragmentation pattern.
Figure 2: Mass chromatogram of (a) blank plasma (b) Zero standard (IS) (c) LLOQ sample of topiramate (15 ng/mL) (d) ULOQ sample of topiramate (3000 ng/mL).

Figure 3: Plasma concentration of topiramate versus time profile of four epileptic patients.
Table 1: Accuracy and precision values of topiramate in human plasma.

<table>
<thead>
<tr>
<th>Nominal conc. (ng/mL)</th>
<th>Found (mean ± S.D.) (ng/mL)</th>
<th>% RSD</th>
<th>% Bias</th>
<th>Intra-assay (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>14.62 ± 1.42</td>
<td>9.75</td>
<td>2.56</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>42.22 ± 2.41</td>
<td>5.71</td>
<td>6.19</td>
<td></td>
</tr>
<tr>
<td>225</td>
<td>217.50 ± 12.94</td>
<td>5.95</td>
<td>3.33</td>
<td></td>
</tr>
<tr>
<td>2700</td>
<td>2546.15 ± 131.08</td>
<td>5.15</td>
<td>5.70</td>
<td></td>
</tr>
<tr>
<td>Inter-assay (n=18)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>14.87 ± 1.31</td>
<td>8.82</td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>44.07 ± 3.65</td>
<td>8.28</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>225</td>
<td>211.72 ± 11.89</td>
<td>5.62</td>
<td>5.90</td>
<td></td>
</tr>
<tr>
<td>2700</td>
<td>2493.25 ± 126.80</td>
<td>5.09</td>
<td>7.66</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Extraction recovery and matrix effect of topiramate in human plasma.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Spiked conc. (ng/mL)</th>
<th>Recovery (% mean ± SD)</th>
<th>% RSD</th>
<th>Matrix effect (% mean ± SD)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topiramate</td>
<td>45</td>
<td>89.12 ± 4.55</td>
<td>5.10</td>
<td>91.59 ± 3.53</td>
<td>3.86</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>92.24 ± 3.38</td>
<td>3.66</td>
<td>96.16 ± 2.28</td>
<td>2.37</td>
</tr>
<tr>
<td></td>
<td>2700</td>
<td>93.09 ± 4.07</td>
<td>4.37</td>
<td>92.19 ± 2.10</td>
<td>2.28</td>
</tr>
<tr>
<td>IS</td>
<td>500</td>
<td>92.81 ± 5.42</td>
<td>5.83</td>
<td>84.81 ± 4.28</td>
<td>2.08</td>
</tr>
</tbody>
</table>

Table 3: Dilution integrity of topiramate in human plasma.

<table>
<thead>
<tr>
<th>Dilution concentration (ng/mL)</th>
<th>DQC (1/2)</th>
<th>DQC (1/10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SD (n=6) (ng/mL)</td>
<td>1233.83 ± 118.58</td>
<td>2621.50 ± 166.76</td>
</tr>
<tr>
<td>% Mean Accuracy</td>
<td>102.82</td>
<td>97.09</td>
</tr>
<tr>
<td>% RSD</td>
<td>9.61</td>
<td>6.36</td>
</tr>
</tbody>
</table>

Table 4: Stability data of topiramate under different storage conditions.

<table>
<thead>
<tr>
<th>Stability conditions</th>
<th>LQC (45 ng/mL)</th>
<th></th>
<th></th>
<th>HQC (2700 ng/mL)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SD</td>
<td>% RSD</td>
<td>% Mean stability</td>
<td>Mean ±SD</td>
<td>% RSD</td>
<td>% Mean stability</td>
</tr>
<tr>
<td>Bench top stability for 6 h</td>
<td>45.42 ± 3.33</td>
<td>7.34</td>
<td>100.93</td>
<td>2609.48 ± 69.65</td>
<td>2.67</td>
<td>96.65</td>
</tr>
<tr>
<td>(at ambient temperature)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze and Thaw stability at -20 °C (5 cycle)</td>
<td>43.12 ± 3.05</td>
<td>7.08</td>
<td>95.83</td>
<td>2588.33 ± 91.38</td>
<td>3.53</td>
<td>95.86</td>
</tr>
<tr>
<td>Dry extract stability at -20 °C for 24 h</td>
<td>46.03 ± 2.50</td>
<td>5.43</td>
<td>102.30</td>
<td>2623.33 ± 86.58</td>
<td>3.30</td>
<td>97.16</td>
</tr>
<tr>
<td>Auto sampler stability 4 °C for 24 h</td>
<td>44.27 ± 1.94</td>
<td>4.39</td>
<td>98.37</td>
<td>2604.93 ± 34.52</td>
<td>1.33</td>
<td>96.48</td>
</tr>
<tr>
<td>Long term stability at -20 °C for 28 days</td>
<td>42.71 ± 3.28</td>
<td>3.28</td>
<td>94.90</td>
<td>2535.00 ± 83.07</td>
<td>3.28</td>
<td>93.89</td>
</tr>
</tbody>
</table>
Table 5: Pharmacokinetic parameter of topiramate after oral administration 50 mg to 4 epileptic patients.

<table>
<thead>
<tr>
<th>Subject</th>
<th>T_max (h)</th>
<th>C_max (µg/mL)</th>
<th>t_1/2 (h)</th>
<th>AUC_0→24 (µg*h/mL)</th>
<th>MRT_0→24 (h)</th>
<th>Lz (h^1)</th>
<th>CL (L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient A</td>
<td>2</td>
<td>5.98</td>
<td>102.03</td>
<td>78.64</td>
<td>11.21</td>
<td>0.68</td>
<td>0.11</td>
</tr>
<tr>
<td>Patient B</td>
<td>3</td>
<td>1.08</td>
<td>49.97</td>
<td>19.91</td>
<td>11.54</td>
<td>1.39</td>
<td>0.68</td>
</tr>
<tr>
<td>Patient C</td>
<td>0.5</td>
<td>1.92</td>
<td>23.39</td>
<td>25.09</td>
<td>10.10</td>
<td>2.96</td>
<td>0.97</td>
</tr>
<tr>
<td>Patient D</td>
<td>1</td>
<td>2.03</td>
<td>67.77</td>
<td>40.94</td>
<td>11.40</td>
<td>1.02</td>
<td>0.26</td>
</tr>
</tbody>
</table>

A – female, 40 year old, 65 kg., B – female, 29 year old, 75 kg., C – male, 17 year old, 60 kg., D – female, 50 year old, 66kg.

4. DISCUSSION

The topiramate and internal standard were separated on analytical column C18 (150 x 4.6 mm, 5µm) with acetonitrile: 2mM ammonium acetate (85:15, v/v) as a mobile phase. The ionization efficiency of ESI is higher in case of acetonitrile and ammonium acetate because of the properties like low viscosity and volatile salts, respectively. The mass spectrometric conditions were optimized to increase sensitivity and specificity of the method. The MS/MS conditions were optimized to obtain the best possible sensitivity. Multiple reaction monitoring mode was used to monitor precursor to product ion, (338.2 → 78.2 m/z for topiramate and 325.20 → 171.20 m/z for IS), which could reduce interference and enhance selectivity. The method was very sensitive to quantitate 15 ng/mL at LLOQ level which was enough for topiramate estimation. In this study, we follow liquid-liquid extraction for the high throughput recovery of topiramate from human plasma which has advantages to get cleaner sample than protein precipitation technique and also cost-effective than solid phase extraction.

Further, the developed method was accurate and precise, the values of this fell within the limits as acceptable. The high efficient and reproducible liquid-liquid extraction with no matrix effect altogether allowed us to conclude that our method is able to quantify topiramate in human plasma. It is important to have dilution integrity in a method to represent the wide applicability of the method towards the real samples. The topiramate was stable over a variety of stated conditions and it was said to be a rugged method.

This chromatographic method was also checked for its applicability to real samples. The plasma concentration of topiramate with respect to time was helped to calculate the pharmacokinetic parameters. The inter-variability in the pharmacokinetic parameters shows
the need for individual monitoring of topiramate in epileptic patients and dose-related adverse effects may be obviated in most patients by dose optimization.

5. CONCLUSIONS
A novel LC-MS/MS method for the estimation of topiramate from human plasma and its pharmacokinetic application was developed and validated according to the USFDA guidelines. This is significant because the estimation of topiramate is quite difficult because it does not have UV-Vis absorption or emit fluorescence, which is unfavorable for determination by readily accessible techniques. Therefore, the LC-MS/MS method was developed for topiramate in human plasma, prove to have good specificity, improved sensitivity and reproducible linearity. Moreover, this method offers significant advantages over those previously reported methods in the biologic matrix, in terms of highly efficient liquid-liquid extraction method. In addition, the absence of matrix effect and stable under routine handling and processing conditions leads to a reproducible and rugged method for quantification of topiramate in human plasma. The method was successfully applied to the real samples. The plasma concentration profile and its pharmacokinetic parameters of individuals were greatly useful for dosage optimization. Thus, the developed LC-MS/MS method was found to be reliable for an intended application.

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Compliance with ethical standards
The study has been approved by Institutional Ethics Committee, Department of Pharmaceutical Sciences, Saurashtra University, India. And it has been performed in accordance with the ethical standards of the guideline. (Approved protocol no. SUDPS/ETHICLIN/03/2014/14).

Conflict of interest
The authors declare no conflict of interest.
Informed Consent
A written consent was obtained from all the individuals involved in the study.

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