Scientific paper

Determination of Topiramate in Human Plasma using Liquid Chromatography Tandem Mass Spectrometry

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Abstract

The LC-MS/MS method for determination of the anti-epileptic drug topiramate (TPM) in human plasma was developed and validated for pharmacokinetic and bioequivalence study purposes. For quantitative determination of TPM values the method with deuterated internal standard (topiramate-d_{12}) and liquid chromatography with tandem mass spectrometry was used. TPM was extracted from the human plasma using the solid-phase extraction procedure on a Strata X extraction column. Negative ions were monitored in the selected reaction monitoring mode (SRM) and transitions *m/z* 338.2 > 78.2 and *m/z* 350.3 > 78.2 were used for the quantitative evaluation of TPM and the internal standard, respectively. The results obtained from validation were statistically evaluated according to the requirements of European Medicines Agency (EMA) and Food and Drug Administration (FDA) regulatory guidelines. The linearity of the method was checked within a concentration range from 10 to 2000 ng/mL. Successful validation confirmed that this method is precise, accurate, sensitive and therefore suitable for determination of topiramate plasma levels in pharmacokinetic and bioequivalence studies.

Keywords: Topiramate, liquid chromatography tandem mass spectrometry, human plasma, bioequivalence study

1. Introduction

Topiramate (TPM, 2,3:4,5-bis-*O*-(1-methylethylidene)-β-D-fructopyranose sulfamate) is an anti-epileptic drug that is used for treating various forms of epilepsy in adults and children and preventing frequently recurring migraines in adults.[1]

The measurement of drug concentrations in biological matrices (such as serum, plasma, blood, urine, and saliva) is an important aspect of medicinal product development. Such data may be required to support applications for new active substances and generics, as well as variations to authorised drug products. The results of bioequivalence studies are used to make critical decisions supporting the safety and efficacy of a medicinal drug substance or product. It is therefore paramount that the applied bioanalytical methods used are well characterised, fully validated and documented to a satisfactory standard, in order to yield reliable results.[2]

Several previous reports have described the determination of topiramate in plasma and serum using analytical methods including HPLC with UV detection,[3] HPLC with fluorescence detection,[4] gas chromatography (GC),[5] fluorescence polarization immunoassay (FPIA),[6] liquid chromatographic-mass spectrometric technique (LC-MS)[7] and liquid chromatography-tandem mass spectrometry (LC-MS/MS).[8–13] These analytical methods have usually adopted liquid-liquid extraction procedures, tedious drying and reconstitution processes. The reported methods[3–8,11,12,13] have been developed for therapeutic drug monitoring purposes, therefore the reported sensitivities of the assay are insufficient for human bioequivalence studies. In two reported methods[9,10] the sensitivity is sufficient for pharmacokinetic and bioequivalence studies after the oral administration of a 100 mg TPM tablet. The presented method, which was developed for pharmacokinetic and bioequivalence study purposes for determina-

tion of TPM in human plasma using a 25 mg TPM dosage, includes an automated solid-phase extraction (SPE) as a sample preparation. The automated SPE is suitable for application during routine studies with a large number of samples. Therefore, this method developed for the measurement of TPM plasma concentrations, presented in this article, is faster and simpler regarding the sample preparation step compared to previously reported methods. It has 100% recovery and is the first one that is fully validated according to the new European Medicines Agency (EMA) guide "Guideline on bioanalytical method validation".

This article proposes the use of isotopically labeled internal standard topiramate-d_{12} for the simple, reliable, and accurate measurement of topiramate in human plasma. The usage of isotopically labeled internal standards has been proven to be the ultimate technique that provides results with unchallenged precision and accuracy.[10–15]

2. Experimental

2. 1. Chemicals

Topiramate and its structurally analogous internal standard were supplied as certified reference compounds. Topiramate was obtained from U.S. Pharmacopeia (USA) and the internal standard (IS), topiramate-d_{12} was purchased from TLC PharmaChem (Canada).

All the chemicals and reagents were of analytical grade, and the solvents of HPLC grade. The methanol and water were obtained from Merck (Germany), the acetonitrile from J.T.Baker (Netherlands), and the ammonia solution from Fluka (Germany).

Blank human plasma with K_2-EDTA was supplied by Trina Bioreactives AG (Switzerland).

2. 2. Instrumental and Operating Conditions

2. 2. 1. Instrumentation

The solid phase extraction unit: RapidTrace SPE Workstation (Zymark, USA) was used for automated SPE procedures.

Tandem triple quadrupole mass spectrometer Quattro Premier Micromass (Waters Corporation, Milford, USA), equipped with liquid chromatographic system Waters 2795 Alliance HT system (Waters Corporation, Milford USA) was used for LC-MS/MS analysis. The peak area ratios between the analyte versus internal standard were generated by the MassLynx software version 4.0.

2. 2. 2. HPLC-MS/MS

In order to achieve quantitative determination of TPM in plasma, the electrospray ionization interface parameters were optimized for maximum abundance of the molecular ions of the compounds. Acquisition parameters were determined by the direct infusion of a 200 ng/mL solution of TPM and IS into the mass spectrometer at a flow rate of 50 µL/min. The variable mass spectrometric conditions had been optimized previously. The main working parameters were as follows: desolvation temperature 220 °C, source temperature 120 °C, capillary voltage 2.95 kV, cone voltage 43.00 V, desolvation gas flow 750 L/h, cone gas flow 96 L/h and collision energy for TPM was 28 eV, for IS was 29 eV, respectively.

An aliquot of human K_2-EDTA plasma containing topiramate and internal standard was extracted using the solid-phase extraction procedure. The extracted samples were injected into a liquid chromatograph equipped with tandem mass spectrometric detector. Negative ions were monitored in the selected reaction monitoring (SRM) mode. The SRM transitions at *m/z* 338.2 > 78.2 and *m/z* 350.3 > 78.2 were selected for the quantification of TPM and IS, respectively. The mass spectra of TPM and IS are presented in Figure 1. Quantitation was performed, using weighted linear regression analysis (1/*c*), of the peak area ratios between the analyte and internal standard.

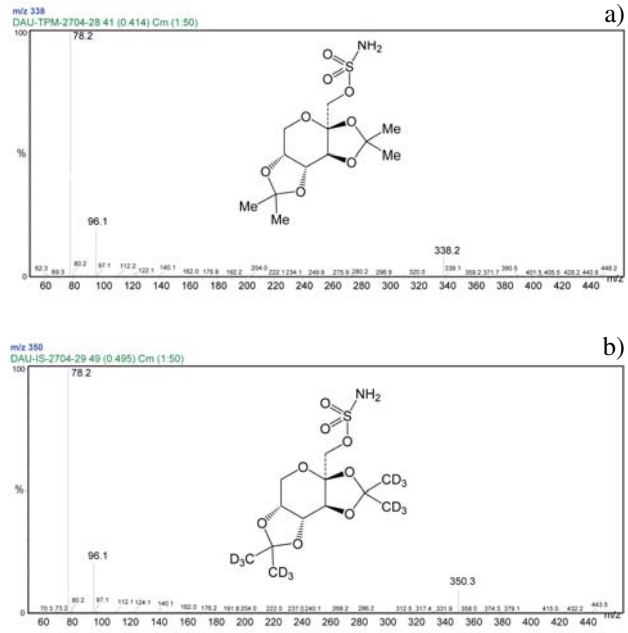

Figure 1: Product ion mass spectra and chemical structure of (a) topiramate and (b) topiramate-d_{12}.

2. 2. 3. Chromatographic Conditions

The autosampler temperature was kept at 5 °C. A Gemini C18 110 Å column from Phenomenex (50 mm × 2 mm internal diameter (ID), 5 µm) preceded by a guard column Zorbax Eclipse XDB C8 from Agilent Technologies (12.5 mm × 2.1 mm ID, 5 µm) were used for separation. The columns were operated at ambient temperature.

The mobile phase consisted of acetonitrile and 0.1% ammonia solution at a ratio of 75:25 (v/v). The

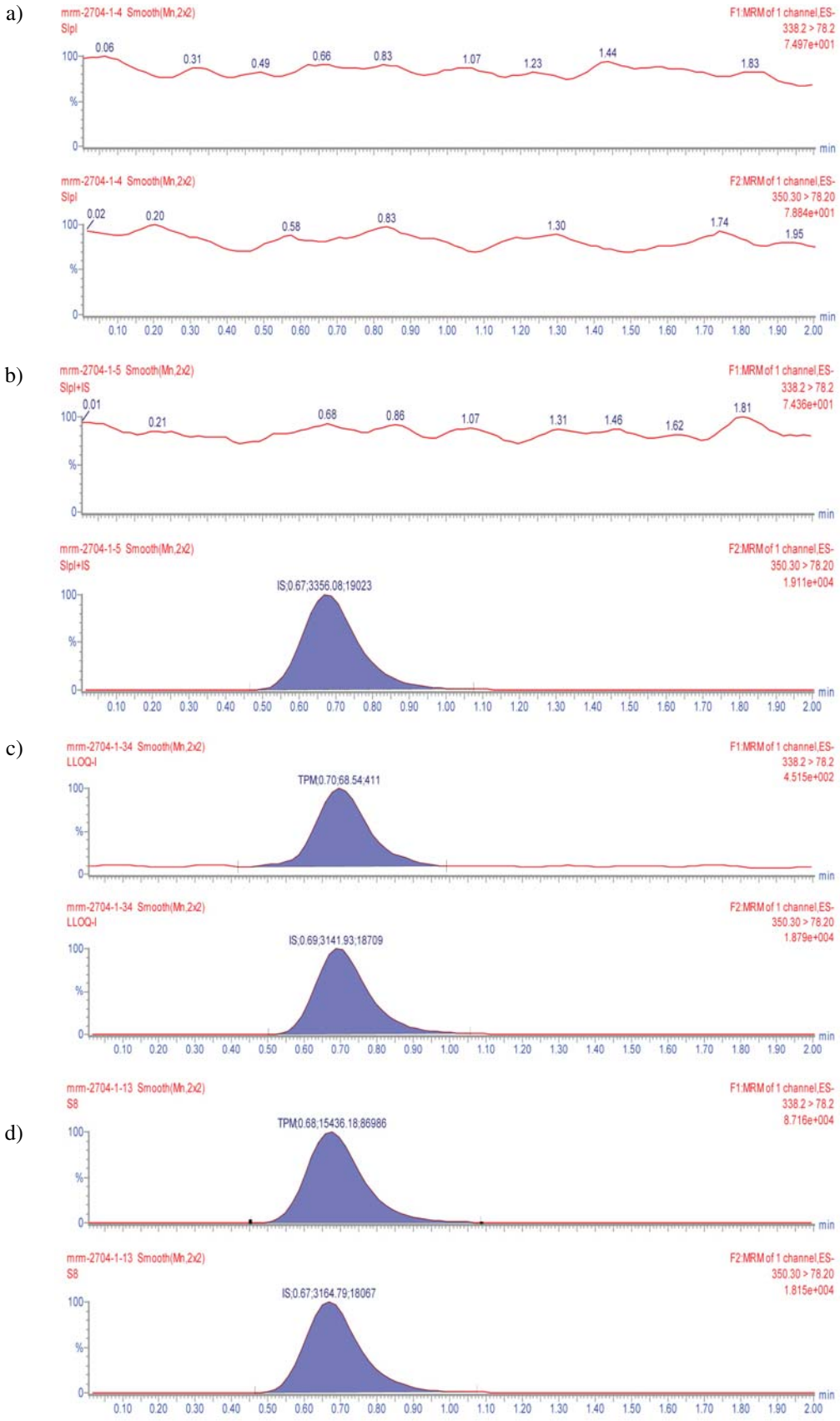

Figure 2: Representative LC-MS/MS chromatograms for topiramate and internal standard in human plasma. (a) Blank human plasma; (b) Blank human plasma with IS; (c) LLOQ plasma sample at 10 ng/mL of TPM; (d) ULOQ plasma sample at 2000 ng/mL of TPM.

flow rate was set at 0.4 mL/min. The retention time was 0.67 min for analyte and IS, which yielded a total run time of 2 min. Figure 2 shows representative LC-MS/MS chromatograms for TPM and IS in the plasma samples.

2. 2. 4. Sample Preparation

A 200 μL aliquot of human plasma sample was mixed with 50 μL of internal standard (2000 ng/mL) and TPM working solutions. 350 μL of water was added and mixed well. The sample mixture was loaded onto a Strata X (60 mg/3 mL) extraction cartridge that had been first pre-conditioned with 2.0 mL of acetonitrile and then with 3.0 mL water. The extraction cartridge was washed twice with 2.0 mL water. Topiramate and internal standard were eluted with 1.0 mL of acetonitrile; and 50 μL of the extract was injected into the LC-MS/MS system. The drying time applied to each step of the solid phase extraction was 0.2 min. All the plasma samples including the blanks, calibration standards, quality control samples and the validation samples were treated using the same sample preparation.

2. 2. 5. Standard Solutions, Calibration Curve (CC) and Quality Control (QC) Samples

Stock solutions of TPM and IS (topiramate-d_{12}) were prepared by dissolving an appropriate amount of analyte in methanol. Working standard solutions for TPM were prepared from a stock solution using acetonitrile – water (80:20, v/v) as diluents. All the solutions were stored at +5 °C.

The drug-free human plasma was stored at –18 °C until used. After having been thawed at room temperature, 200 μL of the drug-free human plasma was spiked daily with 50 μL of TPM working solutions and 50 μL of IS for calibration samples, to provide eight TPM concentrations (10, 20, 50, 100, 500, 1000, 1500, 2000 ng/mL) across the range from 10 to 2000 ng/mL. Quality control samples were prepared at three TPM concentrations at low, medium, and high calibration levels (30, 250, 1750 ng/mL). At least two replicates of each QC sample were analyzed together with the CC and unknown samples within one sequence.

3. Results and Discussion

Method validation was performed according to the guidelines for bioanalytical assays in biomatrices.[2,16,17] The validated parameters: selectivity,[2,16] accuracy and precision,[2,16,17] lower limit of quantification,[2,16] linearity,[2,16,17] matrix effect and matrix factor,[2,17] extraction recovery,[16] dilution integrity,[2] stability,[2,16,17] carry-over.[2,17]

3. 1. Specificity

The specificity was studied by checking the chromatograms obtained from eight different sources of human plasma including one haemolytic and one lipemic plasma. By comparing the chromatograms of those plasma samples spiked with TPM and IS with the chromatograms of the blank plasma samples, no peak was found at the retention time of TPM and IS in eight of the blank plasma samples.

3. 2. Accuracy and Precision

Intra-day and inter-day precision and accuracy of the method were obtained by the analysis of the plasma QC samples' replicates at three concentration levels. The precision of the method was expressed as the coefficient of variation (CV) of the repeatable assays, and accuracy was calculated as the ratio between the mean found and nominal TPM concentrations. Table 1 shows a summary of the individual QC data obtained during the seven runs of validation. As can be seen, the assay for TPM was both accurate and precise between days and within day for each level. The inter-day precision (CV) of the assay was no more than 4.4% at the three concentration levels of QC samples, and the CV for the intra-day precision was no more than 4.5%. The inter- and intra-day accuracy of assay ranged from 92.9 to 96.4% and from 95.9 to 97.0%, respectively.

3. 3. Lower Limit of Quantification

The sensitivity (lower limit of quantification; LLOQ) was determined by plasma spiked with TPM at the lowest calibration level (10.0 ng/mL) and analyzed independently from the calibration curve. Satisfactory intra-day and inter-day precision and accuracy was found at this concentration. The signal to noise ratio at LLOQ was above 10. The detailed results are presented in Table 1.

Table 1. Inter- and intra-day accuracy and precision of TPM determination in human plasma

Nominal conc.		Intra-day (N = 8)		Inter-day (N = 21)	
	(ng/mL)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
LLOQ	10	4.5	107.4	7.1	111.4
QC1	30	4.5	97.0	4.4	95.7
QC2	250	1.6	96.2	3.5	96.4
QC3	1750	2.3	95.9	3.2	92.9

3. 4. Linearity

For the validation of linearity, the blank human plasma was spiked with TPM at eight concentration lev-

Table 2. Precision and accuracy of calibration samples and calibration curve parameters of TPM in human plasma

Nominal conc. (ng/mL)	10.0	20.0	50.0	100	500	1000	1500	2000	Slope	Intercept	Correlation
	Calculated concentration of TPM (ng/mL)										r
run 1	10.52	20.30	49.51	95.81	469.73	1037.81	1558.68	1937.65	0.00265	–0.00471	0.9992
run 2	10.81	19.69	48.68	97.88	468.47	1038.29	1555.04	1941.15	0.00256	–0.00348	0.9992
run 3	10.68	20.44	48.23	95.00	471.62	1045.10	1574.97	1913.95	0.00255	–0.00579	0.9988
run 4	10.03	20.14	51.26	97.68	473.54	1037.41	1549.87	1940.07	0.00243	–0.00344	0.9993
run 5	11.15	19.47	47.83	95.62	471.78	1053.20	1569.65	1911.30	0.00253	–0.00239	0.9988
run 6	11.11	19.29	48.28	95.31	483.02	1030.29	1582.46	1910.26	0.00241	–0.00512	0.9989
run 7	11.37	19.41	47.25	95.10	460.94	1086.17	1552.84	1906.92	0.00241	–0.00481	0.9982
Mean	10.81	19.82	48.72	96.06	471.30	1046.90	1563.36	1923.04			
SD	0.453	0.466	1.321	1.211	6.589	18.743	12.403	15.680			
CV (%)	4.2	2.4	2.7	1.3	1.4	1.8	0.8	0.8			
Accuracy (%)	108.1	99.1	97.4	96.1	94.3	104.7	104.2	96.2			
N	7	7	7	7	7	7	7	7			

els ranging from 10 to 2000 ng/mL, and with one concentration level of IS (500 ng/mL), over different days. The calibration curves were constructed from the linear regression analysis of the peak-area ratio vs. the concentration of TPM respectively, weighted with 1/concentration. A weighted linear regression curve was determined to best represent the concentration/detector response relationship for TPM in human plasma. The correlation coefficient (r) was 0.9982 or higher. The precision and accuracy of the calibration samples' and calibration curves' parameters of TPM in human plasma are presented in Table 2. The average curve of the seven calibration curves data is shown in Figure 3. The CV at each level was less or equal to 4.2%, with accuracy ranging from 94.3 to 108.1%.

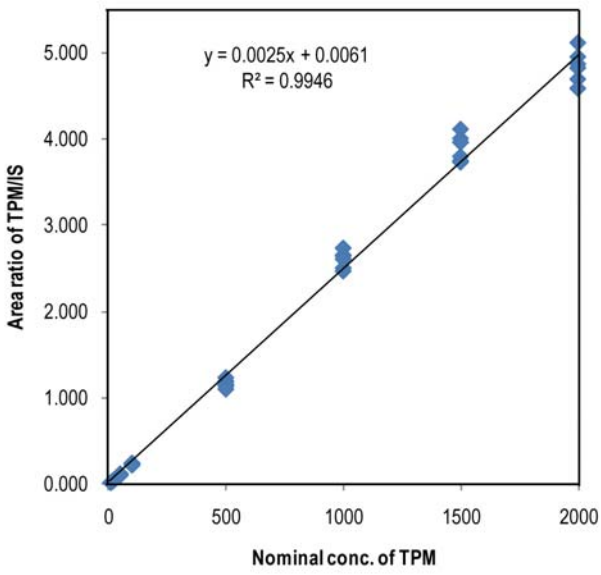

Figure 3: The response of the instrument with regard to the concentrations of TPM as an average of the seven calibration curves.

3. 5. Matrix Effect and Matrix Factor

Samples for determination of matrix effect (ME) were prepared at a low QC level (30 ng/mL) in human plasma samples from eight different sources including one haemolytic and one lipemic plasma. Three replicates of the low QC sample for each matrix were analyzed in a run. The accuracy of TPM determination in each matrix was calculated. The matrix effect was found in 1 of the 8 tested plasma samples. The mean accuracy for 24 replicates of low QC samples was 91.5%, and the CV was 5%.

For the matrix factor (MF), the responses for TPM in the extracted blank human plasma samples from eight different sources, including one haemolytic and one lipemic plasma (spiked with low and high QC standards and IS), were compared with the responses for TPM in the standard solution samples (low and high QC standard solutions and IS solution). The mean MF of TPM was 0.669 and the mean MF for IS was 0.680. The IS-normalised MF was also calculated by dividing the MF of the analyte by the MF of the IS. The mean IS-normalised MF was 0.988 and CV of the IS-normalised MF was 4.1%.

3. 6. Extraction Recovery

The recovery of TPM was evaluated by comparing the extracted plasma samples (mean analyte responses of eight processed samples at 30, 250, 1750 ng/mL) with the reference samples (mean analyte responses of eight replicates of QC solutions at three concentrations that were added to the blank plasma extracts). The sample preparation recovery of the internal standard at concentration 500 ng/mL was also calculated. Kuchekar et al.[10] reported that the percent of mean recovery of solid phase extraction at one QC concentration was 78.2%.[10] However, the mean recoveries of TPM at 30, 250 and 1750 ng/mL obtained by our method were between 103.0 and 105.5%, with CV less than 4.6%.

3. 7. Dilution Integrity

The dilution integrity test was not presented in any of the reported methods. However, the dilution of plasma samples was checked at the two fold value – at the high concentration level of the QC samples (3500 ng/mL). A pool of sample was prepared by spiking drug-free plasma with TPM. Eight replicates with dilution factors of 2 were analyzed. No significant deviation in the accuracy of TPM concentration by sample dilution was observed. The dilution accuracy was 98.9%.

3. 8. Stability

The stability of TPM under different storage conditions in the plasma samples and the extracted samples prepared for LC-MS/MS analysis was tested at two different concentration levels (30 and 1750 ng/mL). The mean concentration at each level was compared to the mean of the freshly prepared concentrations. The stability of TPM in the human plasma was investigated after one and three freeze-thaw cycles (–18 °C to room temperature), after 4 hours at room temperature, after freezing at –18 °C for 91 days and after 96 hours of the extracted samples being in the autosampler at +5 °C. The results are listed in Table 3 and indicate that the analyte remained considerably stable under each of the above mentioned conditions.

Table 3. Stability of TPM in human plasma

Reference conc. (ng/mL) (N = 8)	Calculated conc. (ng/mL) (N = 8)	CV (%)	Accuracy (%)
Autosampler stability after 96 h			
29.13	31.53	4.5	108.2
1622.36	1821.67	1.3	112.3
Freeze-thaw stability (one cycle) and 4 h at 23 °C			
29.13	31.13	3.5	106.9
1622.36	1726.35	2.3	106.4
Freeze-thaw stability (three cycles)			
31.27	34.40	2.0	110.0
1768.03	1915.35	1.2	108.3
Long-term stability after 91 days at –18 °C			
31.27	34.99	3.1	111.9
1768.03	1956.08	1.8	110.6

3. 9. Carry-Over

Carry-over should be addressed in validation and minimized, and an objective determination should be made in the evaluation of analytical runs.[17] Nevertheless, the carry-over during the SPE and during the analysis, developed by Kuchekar et al.,[10] was not tested.

In order to determine the carry-over in the sample preparation step (SPE unit: RapidTrace SPE Workstation, Zymark), a sample of blank human plasma was extracted immediately after the extraction of the sample at the highest concentration level of the calibration curve (2000 ng/mL) of the eight replicates. For all the carry-over blank samples, the peak area at the retention time of TPM was <20% relative to the peak area of the lowest calibration sample at 10 ng/mL TPM. In order to verify that there was no carry-over in the LC-MS/MS instrument, the solution of the mobile phase was injected immediately after the plasma sample extracts of the highest concentration of the calibration curve were injected with eight consecutive injections. There were no peaks observed at the retention times of the TPM and IS.

4. Conclusions

A simple, specific, rapid, and sensitive liquid chromatographic tandem mass spectrometric method was developed and presented for the quantification of topiramate in human plasma samples. Isotope topiramate-d_{12} was used as the internal standard. Solid phase extraction was adopted for sample preparation, and a 2 min assay per sample was achieved.

Sample stability was demonstrated under different conditions. No significant degradation products of topiramate or other interferences due to a possible contribution of matrix constituents were detected in the stability samples. A small but irrelevant matrix effect and matrix factor of topiramate was observed in the human plasma. However, the internal standard compensated for this effect – a matrix factor in the same order of magnitude was present for the internal standard.

Formal validation according to the recommendations of the European Medicines Agency (CHMP guidelines) and Food and Drug Administration showed that the topiramate plasma assay is appropriate for use in pharmacokinetic and bioequivalence studies of topiramate.

5. References

1. R. Shank, J. Gardocki, A. Streeter, B., *Epilepsia* **2000,** *41*, S3–9.
2. Guideline on Bioanalytical Method Validation, EMEA/CHMP/EWP/1992217/2009, 21 July **2011.**
3. G. Bahrami, S. Mirzaeei, B. Mohammadi, A. Kiani, *J. Chromatog. B* **2005**, *822*, 322–325.
4. T. Vovk, M. B. Jakovljević, M.K. Kos, S. M. Janković, A. Mrhar, I. Grabnar, *Biol. Pharm. Bull.* **2010**, *33*, 1176–1182.
5. C. E. Wolf, R. Crooks, A. Poklis, *J. Anal. Toxicol.* **2000**, *24*, 661–663.
6. J. Christensen, C. S. Hojskov, J. H. Poulsen, *Ther. Drug Monit.* **2002**, *24(5)*, 658–664.
7. S. Chen, P. M. Carvey, *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1980–1984.
8. S. Chen, P. M. Carvey, *Rapid Commun. Mass Spectrom.* **2001,** *15*, 159–163.

9. J. H. Park, Y. S. Park, M. H. Lee, S. Y. Rhim, J. C. Song, S. J. Lee, J. M. Kim, L. M. Shaw, J. S. Kang, *Biomed. Chromatogr.* **2008**, *22*, 822–829.
10. S. R. Kuchekar, M. L. Kundlik, B. H. Zaware, *J. Bioanal. Biomed.* **2011**, *2*, 107–112.
11. K. M. Matar, *Clin. Chim. Acta.* **2010**, *411*, 729–734.
12. S. S. C. Tai, C. Y. Yeh, K. W. Phinney, *Anal. Bioanal. Chem.* **2011**, *401*, 1915–1922.
13. K. B. Kim, K. A. Seo, S. E. Kim, S. K. Bae, D. H. Kim, J. G. Shin, *J. Pharm. Biomed. Anal.* **2011**, *56*, 771–777.
14. M. Juris, M. Zoltan, *Anal. Chim. Acta* **2008**, *607*, 115–125.
15. E. Stokvis, H. Rosing, J. H. Beijnen, *Rapid Commun. Mass Spectrom.* **2005**, *19*, 401–407.
16. Guidance for Industry: Bioanalytical Method Validation, U. S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Rockville, USA, **2001.**
17. C. T. Viswanathan, S. Bansal, B. Booth et al, *AAPS Journal* **2007**, *9*, E30–E42.

Povzetek

Razvili in validirali smo LC-MS/MS metodo za določevanje antiepileptičnega zdravila topiramat (TPM) v humani plazmi za namene študij farmakokinetike in bioekvivalence. Za kvantitativno določitev smo uporabili metodo devteriranega internega standarda (topiramat-d_{12}) in tekočinsko kromatografijo s tandemsko masno spektrometrijo. Učinkovino TPM smo ekstrahirali iz humane plazme z ekstrakcijo na trdnem nosilcu na Strata X ekstrakcijskih kolonah. Spojine smo ionizirali z elektrorazprševanjem in merjenjem negativnih ionov, spremljali smo SRM prehoda 338,2 > 78,2 za TPM in 350,3 > 78,2 za interni standard.
Rezultate vsebnosti topiramata smo statistično ovrednotili v skladu z zahtevami EMA regulative in FDA smernicami. Linearnost metode smo preverili v koncentracijskem območju od 10 do 2000 ng/ml. Uspešni rezultati testov so pokazali, da je razvita metoda natančna, točna in občutljiva ter primerna za določanje topiramata v humani plazmi v študijah farmakokinetike in bioekvivalence.