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Rapid assay of topiramate in dried blood spots by a new liquid chromatography-tandem mass spectrometric method

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ABSTRACT

Topiramate (TPM) is a new antiepiletic drug with efficacy in several types of seizures. Therapeutic drug monitoring of TPM is essential for effective patient management. The aim of this study was to evaluate the use of dried blood spot (DBS) specimens to determinate the TPM levels during the treatment. Advantages of DBS include short collection time, low invasiveness, ease and low cost of sample collection, transport and storage. Performance comparison between this method and the commercially available fluorescence-polarization immunoassay (FPIA) was made.

The analysis was performed in selected reaction monitoring (SRM) mode. The calibration curve in matrix using D_{12}-topiramate was linear in the concentration range of 0.0166–1.66 mg/L (0.5–50 mg/L in DBS) of topiramate with correlation coefficient value of 0.9985. In the concentration range of 0.5–50 mg/L, the coefficients of variation in DBS were in the range 2.13–11.85% and the accuracy ranged from 93.93% to 110.67%.

There was no significant differences between the concentrations (ranging 0.5–50 mg/L) measured both FPIA on venous samples and LC-MS/MS assay on simultaneous DBS samples.

The sensitivity and specificity of tandem mass spectrometry allow now high throughput topiramate analysis (the improvement was three times in comparison with FPIA). This new assay has favourable characteristics being highly precise and accurate. FPIA also proved to be precise and accurate, but is not always suitable for the sample collection in neonates in whom obtaining larger blood samples is not convenient or possible.

1. Introduction

Topiramate (TPM) is approved as monotherapy or adjunctive treatment of partial and generalised seizures [1] and of the Lennox–Gastaut syndrome [2] in adults and children.

The anticonvulsant properties of TPM are thought to arise from its inhibitory activity against glutamate receptors, including *N*-methyl-D-aspartate (NMDA), kainate (KA) and α-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA) receptors [3]. TPM can also influence the activity of voltage-activated Na^+ [4] and Ca^+ [5] channels by reducing some carbonic anhydrase isozymes [6] or the mitochondrial permeability transition pore [7].

The clinical effect of this drug correlates better with blood level than with doses, which may provide the rationale for therapeutic TPM monitoring. Indeed, its determination in blood and urine is becoming a routine assay in clinical analysis. In clinical trials, TPM in blood have generally ranged from 2 to 25 mg/L.

Up to now topiramate has been mostly assayed by liquid chromatography (HPLC) with UV detection [8] or mass spectrometry (MS) [9–11]; by gas chromatography (GC) combined with mass

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spectrometry [12,13] or by using flame ionization detection [14]; by capillary electrophoresis with indirect UV detection [15] or by fluorescence polarization immunoassay (FPIA) [16]. However, these approaches are hampered by several drawbacks. The chromatographic run is time demanding and blood sample volumes required for the above methods are in the range between 0.5 and 2 mL, which may represent a limitation in very young babies. In addition, signals from a control specimen are quite difficult to quantify since they are close to baseline (lack of adequate sensitivity) and sometimes they are not sufficiently resolved from other endogenous components, owing to the unspecific wavelength selected for detection. The analysis performed on DBS allows a great advantage in terms of costs, affordability, and ease of sampling, especially in infants. The use of DBS for the collection and analysis of human blood dates back to the early 1960s when Robert Guthrie used the specimens to measure phenylalanine in newborns for the detection of phenylketonuria [17]. This novel application for collecting blood led to the population screening of newborns for the detections of some inborn errors of metabolism. To date, many other clinical applications for measurement of biological markers on DBS have been reported [18].

We evaluated the power of tandem mass-spectrometry (MS/MS) as a rapid method for TPM assay using DBS. We demonstrated that minimal sample preparation, with no derivatization steps, high sensitivity/specificity, high throughput and minimal instrument maintenance make this method a good candidate for a large-scale routine task.

2. Experimental

2.1. Materials

Chemical standard of topiramate (2,3:4,5-bis-*o*-(-1-methyl)-beta]-D-fructopyranose sulfamate) (purity >98%) was purchased from Sigma–Aldrich (Steinheim, Germany); the internal standard, topiramate (2,3:4,5-bis-*o*-(-1-methyl)-beta]-D-fructopyranose sulfamate)-D_{12} (99.8% D purity) was from CDN Isotopes (Quebec, Canada). Stock solutions of both were made in HPLC grade water (corresponding of 1700 mg/L).

Successive dilutions were made using HPLC grade water. All chemicals and solvents were of the highest purity available from commercial sources and used without any further purification.

2.2. Sample preparation

DBS samples were punched and two 3.2 mm blood spots (containing about 3.3–3.4 μL of blood per each) were extracted together with 200 μL 30–70 of water/acetonitrile +0.05% of formic acid solution containing 0.352 mg/L of D_{12}-TPM. Samples were put in an orbital shaker and kept at 37 °C for 20 min.

For the setting-up of this study, a pooled mixture of blood samples was spiked with TPM and 20 μL were put on filter paper (903®, Whatman GmbH, Dassel Germany).

We tested 20 dried blood spots from healthy controls and 21 spots from patients with confirmed epilepsy whose TPM levels had also been monitored with the FPIA method.

The procedure has been approved by the review board of our institution. Blood for DBS controls and patients was collected between 72 h of life and 73 years (controls: *m* 24 years, S.D. 22 years; patients: *m* 29 years, S.D. 21 years) and was spotted on filter paper, dried and sent to the laboratory by courier. Blood spot samples were stored at room temperature until analysis. The analyses were performed on the same day of their arrival in laboratory. The stability of 1 and 5 mg/L of TPM on DBS were measured in triplicates during 1-week period at room temperature. No significant differences were noted (Table 1). The median storage time between the drawing of the blood sample and the analysis was 20 h (minimum 1 h; maximum 48 h).

Table 1
Stability of TMP in dried blood spot at room temperature.

Expected concentration (mg/L)	Mean, analyses in triplicate for 7 days (mg/L)	DS	CV%	Accuracy
1	1.02	0.05	4.94	102.14
5	4.94	0.12	2.53	98.74

3. Methods

3.1. Fluorescence polarization immunoassay (FPIA)

Heparinized plasma samples (20 healthy controls and 21 patients) were analyzed by FPIA by using the commercially available Innofluor® Topiramate Assay System (Oxis, International Inc., OR) on an Abbott TDx analyzer (Abbott Laboratories Inc., Abbott Park, IL). Company-provided heparinized plasma calibrators at concentrations 0.0, 2.0, 4.0, 8.0, 16.0, and 32.0 mg/L were used to establish the calibration curve (Innofluor® 41055) (Oxis, International Inc., OR). The analytic procedure was conducted according to the manufacturer's instructions [19]. Briefly, samples of heparinized plasma (80 μL) were transferred to TDx sample cups and analyzed with low (3.0 mg/L), medium, (10.0 mg/L), and high (24.5 mg/L) control plasma samples (Innofluor® 41056) (Oxis, International Inc., OR). Precision and accuracy in heparinized plasma were determined using the same company-provided heparinized plasma control samples. The intra-run variation was explored analyzing 10 heparinized plasma samples on each of the concentration levels, and the inter-run variation was explored analyzing one heparinized plasma sample on each of the heparinized plasma levels on 10 different days.

3.2. Mass spectrometry

An Applied Biosystems-Sciex (Toronto, Canada) API 4000 bench-top Triple-Quad Mass Spectrometer equipped with the TurboIonSpray source was used for this study. The TurboIonSpray source operated under positive ion mode at a voltage of 5500 V and with a "turbo" gas flow of 10 L/min of air heated at 350 °C (nominal heating-gun temperature).

Mass calibration and resolution adjustments on the resolving quadrupoles were performed automatically by using a PPG 10^{-7} mol/L solution introduced via the built-in Infusion Pump. The resolution was set on both resolving quadrupoles at 0.7 amu (measured at 1/2 height) for all MS and MS/MS experiments.

Collision activated dissociation (CAD) MS/MS was performed through the LINAC Q2 collision cell, operating with 10 mT or pressure of nitrogen as collision gas.

Declustering potential (DP), collision exit potential (CXP) and collision energy (CE) were automatically optimized for TPM and D_{12}-TPM by the "quantitation optimization" option. The resulting DP was +50 V. Optimal CE and CXP were found at 15 and 13 eV, respectively.

MS and MS/MS spectra were collected in continuous flow mode by connecting the Infusion pump directly to the TurboIonSpray source. Standard solutions of 1 ng/mL of TPM and D_{12}-TPM in an aqueous solution of 70% acetonitrile containing 0.05% of formic acid were infused at 10 μL/min.

The quantitation experiments were undertaken by using a Series 1100 Agilent Technologies (Waldbronn, Germany) CapPump coupled to an Agilent Micro ALS autosampler, both being fully

controlled from the API 4000 data system. Liquid chromatography was performed using a Phenomenex Synergi 4u POLAR-RP 80A 4 μm, 2 × 150 mm HPLC column (Phenomenex Italia, Anzola Emilia, Italy). Column flow was 0.2 mL/min using an aqueous solution of 70% Acetonitrile containing 0.05% formic acid. The eluent from the column was directed to the TurboIonSpray probe without split ratio.

Five μL of the extracted sample were injected for the LC-MS/MS experiments.

The data were processed using the Analyst 1.4.1 proprietary software including the "Explore" option (for chromatographic and spectral interpretation) and the "Quantitate" option (for quantitative information generation).

Topiramate concentrations were measured by, and compared to, fluorescence polarization immunoassay (FPIA), performed on a TDxFLx analyser (Abbott Diagnostics, IL, USA) [17].

3.3. Bland–Altman plot

The Bland and Altman plot [20] is a statistical method to compare two measurements techniques in clinical chemistry.

It represents a comparison of a new measurement technique with an established one to establish if they agree sufficiently and therefore, if the new can replace the old.

The plot represents a graphical method in which the differences (or alternatively the ratios) between the two techniques are plotted against the average of the difference of the two techniques.

If the differences between measurements using the two assay methods lie within the limits of agreement of the Bland–Altman test 95% of the time, this indicates that the two methods are not producing different results.

4. Results and discussion

Results are summarized in Figs. 1–3 and in Tables 1 and 2. Fig. 1 shows the MS/MS spectrum obtained by fragmenting the precursor ion (340.1 Th) of TPM and D_{12}-TPM (352.1 Th) under the above-described conditions. From these experiments, the resulting ion-pair transition for the quantitative experiment (SRM) is 340.1 > 264.1 for TPM and 352.1 > 270.1 for D_{12}-TPM. Fig. 2 shows an extract ion chromatogram from DBS spiked with 10 mg/L of TPM (a) versus a TPM standard solution in water at the same concentration (IS concentration 3.5 mg/L). No interferences were revealed but a quenching of the intensity of the signal indicates that the assay performances are not completely independent on the sample matrix. Moreover, the combined effect of all the components of the sample other than the analyte on the measurement of the quantity does not seems to be significant due to the sample dilution rate (30 times) and the isotopic dilution strategy.

The chromatographic conditions selected were set in order to speed-up the running time (3 min) since specificity is provided by the MS/MS measurement. Sample dilution rate and injection volume (5 μL) were selected in order to avoid overloading the chromatographic column even after a high number of sample injections (0.166 μL of original blood per run). The injection sample volume was selected after several trials with different injection volumes. The outcome is that the column shows robust performances regardless of the salt or any other interfering component concentration in the specimen.

The non-weighted regression equation for our LC-MS/MS method was $y = 1.0007x$; $R^2 = 0.9985$. A correlation coefficient of >0.995 is generally considered as the evidence of an acceptable fit

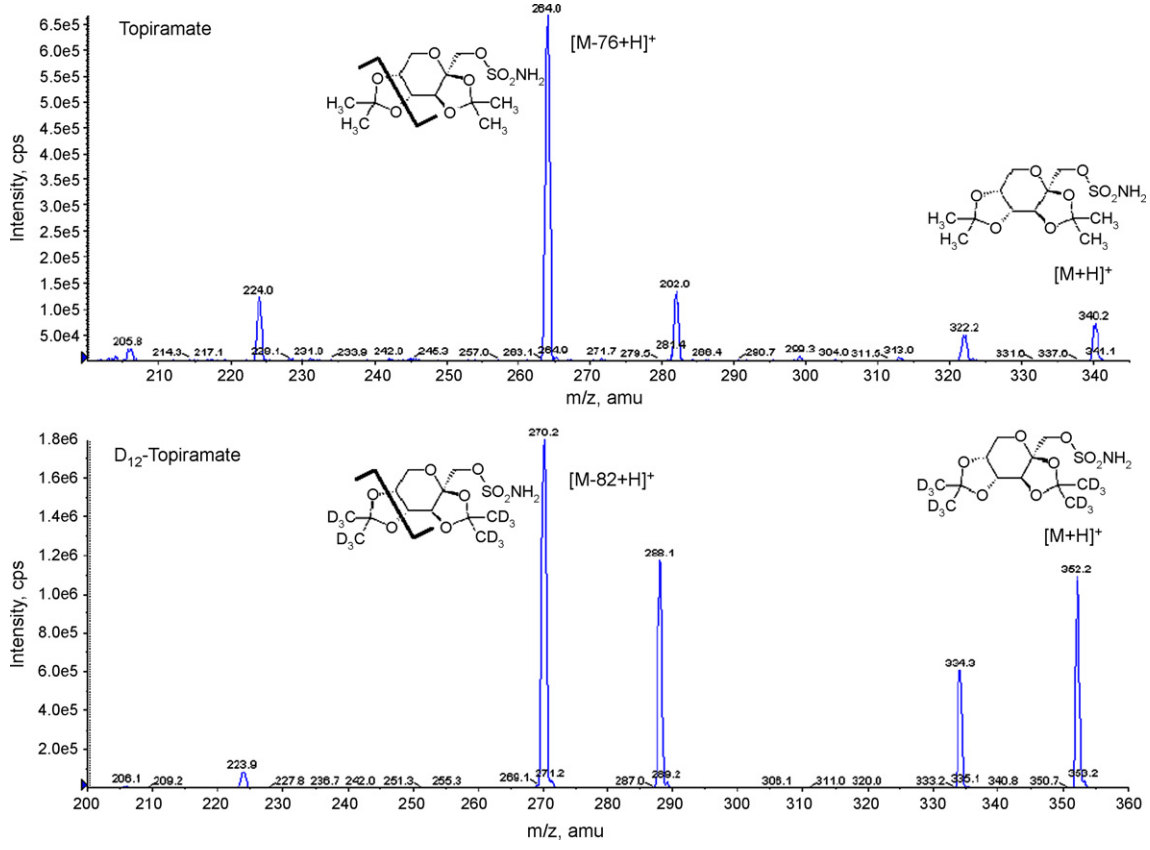

Fig. 1. Product ion scans of topiramate and D_{12}-topiramate.

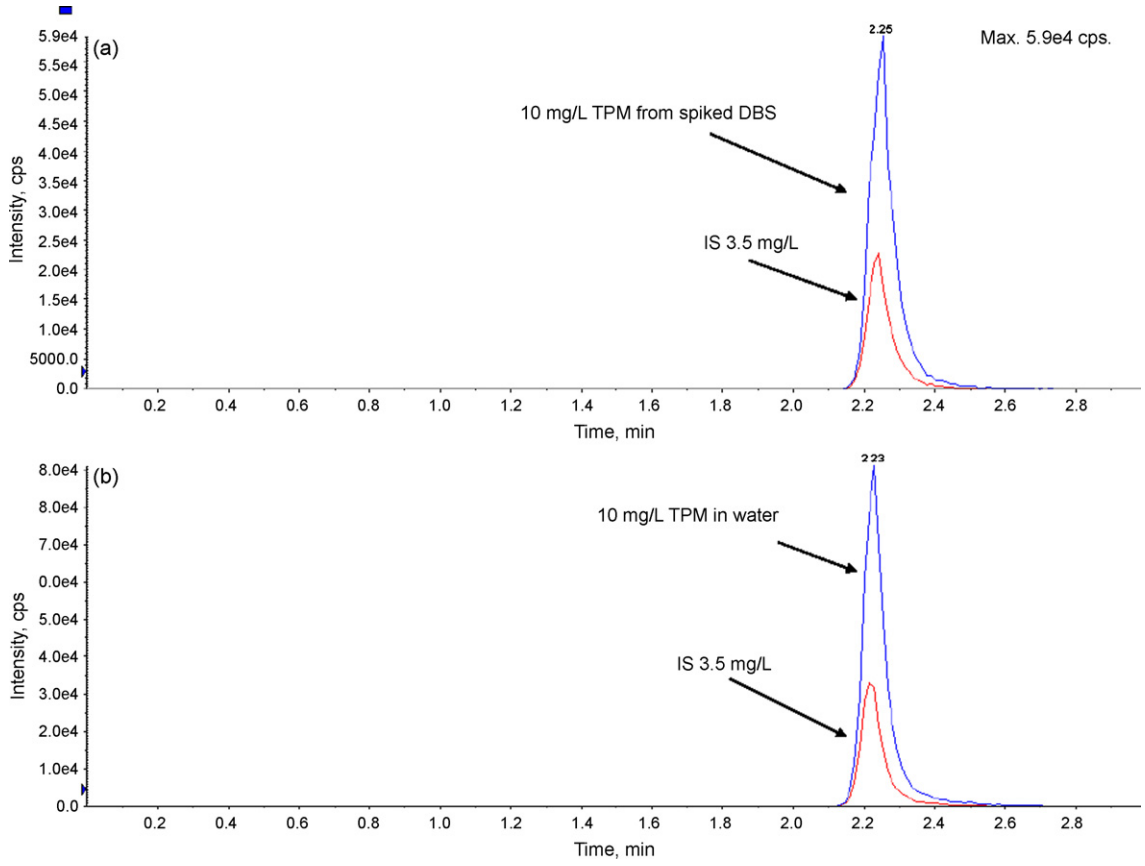

Fig. 2. Extract ion chromatogram from DBS spiked with 10 mg/L of TPM (a) versus a TPM standard solution in water at the same concentration.

of the data to the regression line. The *y*-intercept is less than 3.5% of the response obtained for the analyte at the target level (considered the lower level of the normal range: 2 mg/L).

The calibrators, containing the internal standard at 0.35 mg/L, were at concentrations of 0, 0.5, 1, 2, 5, 10, 20 and 50 mg/L. For spiking studies, we evaluated linearity by analyzing supplemented 3.2 mm dried blood spots prepared at 0, 0.5, 1, 2, 5, 10, 20 and 50 mg/L.

Considering all the examined samples, discrepancies between expected values and measured values were within 12%. In order to assess the robustness of the method, a pooled DBS sample was processed 10 times, resulting an intra-day repeatability below 3.3% for all values (Table 2). Values better than 10.7% were obtained in an interday repeatability test (Table 2).

With the proposed parameters, the estimated limit of detection (Signal to noise ratio >3) in DBS was 3.32 μg/L (0.1 mg/L, 30 times diluted), the limit of quantitation (signal to noise ratio >10) was 16.6 μg/L (0.5 mg/L, 30 times diluted). No deterioration in column efficiency was observed after the analysis of 200 DBS samples.

We noted a significant inverse correlation between TPM plasma and TPM levels in whole blood for the influence of hematocrit on plasma/whole-blood distribution of TPM, as reported in literature

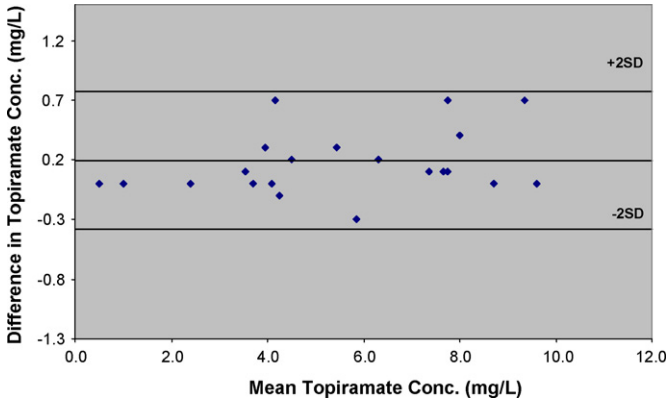

Fig. 3. Bland–Altman plot. The average discrepancy between the two methods is clinically acceptable in spite of a proportional error (2.7%). All differences lie between the mean difference ±2 S.D.

Table 2
Intra- and interday imprecision.

Expected concentration ($n = 10$)	Mean	S.D.	%CV	Accuracy
Intraday				
0	0	0		
0.5	0.52	0.05	10.12	103.24
1	0.95	0.11	11.85	95.06
2	2.00	0.22	10.99	99.80
5	5.12	0.56	10.96	102.45
10	9.93	1.25	12.57	99.33
20	20.16	0.43	2.13	100.79
50	49.99	2.58	5.16	99.98
Interday (10 days)				
0	0	0		
0.5	0.55	0.05	9.66	110.67
1	1.10	0.11	10.19	109.73
2	2.04	0.13	6.5	102.16
5	4.77	0.42	8.69	95.42
10	9.39	0.93	9.89	93.93
20	19.93	0.85	4.20	99.77
50	50.13	2.88	5.74	100.27

[21]. Therefore, assuming an hematocrit range between 50% and 60% (mean 55%) in the case of newborns and 36–52% (mean 44%) in the case of adults [22] we converted the TPM measurements from DBS to plasma concentration multiplying by 2.22 and by 1.79 for newborns (up to 1 month of life) and adults, respectively.

We performed Bland–Altman analysis to compare the proposed method with the FPIA one [20]. We plotted the difference between the methods against the averages of values from the two techniques.

The statistical analysis showed a good agreement between TPM concentration obtained by LC-MS/MS on DBS and FPIA on heparinized plasma. For TPM concentrations, the mean difference (FPIA minus LC-MS/MS) was +0.19 mg/L (error 2.7%) with 95% limits of agreement of Bland and Altman −0.38 mg/L +0.77 mg/L. All differences lie between the mean difference ±2 S.D. (Fig. 3).

TPM is used to treat a wide range of epileptic disorders in adults and children of all ages including newborns [1,2,23,24,25]. It is also used as a first line treatment for the prophylaxis of migraine [26]. As these areas and more additional ones (including alcohol [27] and smoking dependency [28], and obesity [29]), monitoring the drug from a simple blood spot (i.e. about 6 μL), that can be withdrawn even at home, is greatly advantageous compared with classical venous blood sampling (i.e. about 2 mL) required for traditional assay methods. Moreover, the use of this method appears a more reasonable approach in paediatrics area where obtaining larger blood samples is not always convenient or possible.

The new LC-MS/MS assay has favorable characteristics, being highly precise and accurate. FPIA on heparinized plasma also proved precise and accurate, and there was a high agreement with the LC-MS/MS assay in dried blood spot. Either method displayed sufficient precision and accuracy and may thus be implemented in daily routine. The differences between measurements using the two assay methods lie within the limits of agreement of the Bland–Altman test 95% of the time. This indicates that the two methods are not producing different results.

The proposed method requires a fast and easy sample preparation. Instrumental analytical time is less than 3 min. For FPIA assay, the instrument requires 16 min to test a full carousel of calibrators and 8 min to test one sample. The improvement acheived in terms of throughput is at least three times if new LC-MS/MS method is used. Quick chromatography combined with the specificity offered by tandem mass-spectrometry allows a fast, robust and specific procedure for TPM assay.

Several factors have made possible the application of tandem mass spectrometry to topiramate measurement in DBS: the optimal instrument sensitivity in positive-ion mode, with the presence of formic acid (0.05%) in the chromatographic eluent; the MS/MS specificity guaranteed even at such low masses by the resolution of both quadrupoles (typically 0.7 amu, unchanged compared with the higher mass range); the interface robustness, which makes affordable the crude sample injection or the injection of a crude dilution of it. The latter is possible provided the chromatographic conditions are correctly set (i.e. chromatographic retention factor *K*-factor is big enough with respect to the column void volume).

The detection limit of 3.32 μg/L of TPM in DBS should not be intended as the ultimate figure since an increase of sensitivity could be expected by downsizing the LC-column diameter. The value, however, appears to fulfill the sensitivity requirements of the present application so we decided to use the 2.0 mm-column since it proves to be robust for several hundreds of injections. The switching to a smaller column like a 1-mm-one could increase the sensitivity.

This method is well suited for paediatric applications and potentially for domiciliary therapeutic drug monitoring.

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