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An isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS)-based candidate reference measurement procedure for the quantification of topiramate in human serum and plasma

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Abstract

Objectives: Topiramate is an antiepileptic drug (AED) used for the monotherapy or adjunctive treatment of epilepsy and for the prophylaxis of migraine. It has several pharmacodynamic properties that contribute to both its clinically useful properties and observed adverse effects. Accurate measurement of its concentration is therefore essential for dose adjustment/optimisation of AED therapy. Our aim was to develop and validate a novel reference measurement procedure (RMP) for the quantification of topiramate in human serum and plasma.

Methods: An isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) method in combination with a protein-precipitation-based sample preparation allows for quantification of topiramate in human serum and plasma. To assure traceability to SI units, quantitative nuclear magnetic resonance (qNMR) was applied to characterize the reference material used as primary calibrator for this RMP. Matrix effects were determined by performing a post-column infusion experiment and comparing standard line slopes. Accuracy and precision was evaluated performing an extensive five day precision experiment and measurement uncertainty was

evaluated according Guide to the Expression of Uncertainty in Measurement (GUM).

Results: The method enabled topiramate quantification within the range of 1.20–36.0 µg/mL without interference from structurally related compounds and no evidence of a matrix effect. Intermediate precision was ≤3.2 % and repeatability was 1.4–2.5 % across all concentration levels. The relative mean bias was –0.3 to 3.5 %. Expanded measurement uncertainties for target value assignment (n=6) were found to be ≤2.9 % (k=2) independent of the concentration level and the nature of the sample.

Conclusions: In human serum and plasma, the RMP demonstrated high analytical performance for topiramate quantification and fulfilled the requirements on measurement uncertainty. Traceability to SI units was established by qNMR content determination of the topiramate, which was used for direct calibration of the RMP. This RMP is, therefore, fit for purpose for routine assay standardization and clinical sample evaluation.

Keywords: isotope dilution-liquid chromatography-tandem mass spectrometry; qNMR; reference measurement procedure; SI units; topiramate; traceability.

Introduction

Establishing the traceability chain is considered a key activity in laboratory medicine to ensure global comparability of results. The aim is to avoid systematic error contributions (bias) between different *in-vitro* diagnostic (IVD) manufacturers. A reference measurement procedure (RMP) in the sense of ISO 17511 must be interference-free, highly accurate and precise to optimally support the IVD industry and method developers. Metrological oversight is guaranteed by National Metrology Institutes (NMIs) and reference/calibration laboratories. IVD industry relies on

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reference materials, reference measurement procedures and reference measurement procedures performed in reference or calibration laboratories for material value assignments to provide routine laboratories with traceable calibration and control materials. If materials and/or methods are not provided by the NMIs or reference/calibration laboratories, individual traceability concepts must be followed. Currently in most cases, only one or no calibration/reference laboratory is available for routine calibration value assignment [1, 2].

Topiramate ($C_{12}H_{21}NO_8S$, molecular weight=339.4 Da, conversion factor from $\mu\text{g/mL}$ to molar unit [$\mu\text{mol/L}$]=2.9) is a sulfamate-substituted monosaccharide derivative used as a broad-spectrum antiepileptic drug (AED). It is indicated for monotherapy or adjunctive treatment of epilepsy in both adults and children, as well as for the prophylaxis of migraine. Topiramate possesses several pharmacodynamic properties such as the blockade of ion channels and interaction with neurotransmitter systems [3]. These effects contribute both to its clinically useful attributes and to its observed adverse effects [3–7]. Adverse effects include lack of appetite, weight loss, fatigue, drowsiness, sensory disturbances, visual disturbances, headache, and dizziness [3–7]. Topiramate is quickly absorbed after oral administration. It has a half-life of 19–23 h; steady-state concentration is reached in approximately four days in patients with normal renal function [8]. Most of the drug (81 %) is excreted in the urine, metabolism via liver monooxygenases is not described [4].

Therapeutic drug monitoring (TDM) of topiramate is indicated owing to its use in combination with other anti-convulsant drugs (e.g., phenytoin, carbamazepine, and phenobarbital), which may lead to drug–drug interactions [4, 9]. In addition, topiramate TDM can be useful in dose adjustment/optimization and thus is required for patients with concomitant AED therapy [10, 11]. Various IVD CE (Conformité Européenne)-certified analytical solutions are available for topiramate TDM. Aside from immunoassays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) solutions are available from at least two commercial vendors [12]. A multitude of LC-MS/MS-based assays have been reported in research settings [13–16]. One of these was designed as a reference method by the National Institute of Standards and Technology (NIST) and subsequently listed by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) [17]. An expanded uncertainty of 2.4 % was reported by the authors, however, intermediate imprecision data and data to demonstrate the transferability of the method to a second laboratory were not. The reference material “Antiepilepsy Drugs in Frozen Human Serum, SRM 900a” issued by NIST was based on this method but has since been

discontinued [18]. Hence, currently no method traceability to highest metrological order was found possible. Since the activity status of the NIST RMP was unclear, and we were not aware of reference laboratories that have instituted the NIST RMP for antiepilepsy drugs, it was decided to go for a *de novo* design and validation of a candidate RMP in accordance with the ISO 15193 guideline requirements [19, 20].

The RMP methodology proposed includes all technical information, calculation of the measurement uncertainty (based on the *Guide to the Expression of Uncertainty in Measurement* (GUM) [21]) and transferability of methods to a second laboratory. The RMP must also fulfil imprecision acceptance criteria based on the approach of Braga and Panteghini, who stated that a reference method must remain below one-third of the measurement uncertainty of the routine method [22]. The desired topiramate routine uncertainty can be derived from different approaches as outlined by Steele and colleagues for several AEDs in 2001 [23]. Topiramate routine TDM should show a measurement uncertainty ($k=1$) of better than 5.0 % if the pharmacokinetic model by Fraser [23] is applied. If the model by Glick [23] is followed, a measurement uncertainty ($k=1$) of less than 8.0 % is considered sufficient. A recent investigation dealing with the inter-laboratory measurement uncertainty in CAP (College of American Pathologist) proficiency testing showed that, independent of the method chosen, topiramate TDM routine services performed within a method independent measurement uncertainty ($k=1$) ranging from of 5–13 % [24].

With an analysis of variance (ANOVA) approach the intra-laboratory variance component can be estimated from PT results if repeated challenges are utilizing identical samples. Steele and coworkers did show for TDM CAP data in 1996 that the relative intra-laboratory variance component was about 80 % of the total variance found in CAP-PT [25]. Applied to the topiramate CAP-PT data compiled by Krasowski et al. [24], the intra-laboratory uncertainty can be estimated to be in the range of 4–11 %. These data allow to assume that currently in clinical routine, topiramate TDM assays do not always or not for all analyte concentrations present, provide the analytical quality that modelling approaches based on either the therapeutic range (according to Glick <8 %) or pharmacokinetic model considerations (according to Fraser <5 %) assume to be necessary for clinical meaningful TDM [23].

Combining the analytical quality demands from pharmacokinetic model considerations (Fraser <5 %) with the Braga and Panteghini argument, that a candidate reference method should be performed with a measurement uncertainty not exceeding one third of routine [22], an intra-laboratory measurement uncertainty not exceeding 1.7 %, translating to an expanded measurement uncertainty of

3.4 % should be achieved. It is, of course, a paradigmatic imperative that the measurement bias of an RMP is as low as possible, if not zero [26].

A prerequisite to achieve this goal is to characterize reference materials thoroughly, e.g., by the mass balance approach or by quantitative nuclear magnetic resonance (qNMR) spectroscopy [27, 28]. The latter option is an effective method of determining absolute quantities of analytes with a single destruction-free analysis [28, 29]. Direct traceability to SI units is facilitated via NIST traceable primary benzoic acid standards as standard reference material (SRM) 350b or PS1 [30]. Recently, owing to its operational ease and non-destructive nature, qNMR has gained acceptance by national metrological institutes for measuring absolute amounts in primary reference standards.

Herein, we describe a novel candidate RMP for topiramate that meets the requirements of the ISO 15193 guideline. To facilitate the reproduction of the RMP candidate method by other laboratories, the approach and method are described in full in the Supplementary Materials focusing on the technical implementation of the procedure (Supplementary Material 1), the qNMR-based reference material characterization (Supplementary Material 2), and the calculation of measurement uncertainty (Supplementary Material 3).

Materials and methods

A full description of method, materials, equipment used, and instructions for the application of the method is given in Supplementary Material 1.

Materials

Chemicals and reagents: LC-MS grade methanol was purchased from Biosolve (Valkenswaard, The Netherlands). Dimethyl sulfoxide (ACS reagent, ≥99.99 %), acetic acid (LC-MS grade), and isopropanol were purchased from Sigma Aldrich (Taufkirchen, Germany). Water was purified using a Millipore Milli-Q 3 UV system from Merck (Darmstadt, Germany). Native human serum (Art. No. S1-LITER) was obtained from Merck (Darmstadt, Germany). TDM-free human serum (multi-individual pooled; defined as surrogate matrix, ID No. 12095432001) was obtained from Roche Diagnostics GmbH (Mannheim, Germany), native plasma matrix (Li-heparin, K₂-EDTA and K₃-EDTA) was obtained from anonymized patient samples. The patient samples were anonymized residual samples, and pools prepared from them, in accordance with the Declaration of Helsinki. Topiramate (Art. No. PHR1600, Lot Nr. LRAC2801) was purchased from Sigma Aldrich (Taufkirchen, Germany), its deuterated internal standard, [²H₁₂]-topiramate (Art. No. TRCT540252), was purchased from Toronto Research Centre (Toronto, Canada). NMR solvent CD₃OD and qNMR internal

standard duroquinone (Art. No. 06856, Lot Nr. BCBRS528V) were obtained from Sigma Aldrich (Taufkirchen, Germany). 5 mm × 10 in. NMR tubes were purchased from Sigma Aldrich (Taufkirchen, Germany) and/or Euroisotop GmbH (Hadfield, United Kingdom).

General requirements for laboratory equipment: A list of equipment used, and their requirements is given in Supplementary Material 1.

qNMR for determination of the purity of the standard materials: qNMR measurements were performed on a JEOL 600 MHz NMR spectrometer (Jeol Ltd, Tokyo, Japan) equipped with a He-cooled cryoprobe. Single-Pulse ¹H/¹³C NMR (Supplementary Material 2, Figures 1 and 2) was utilized for the quantitation (CH₂OSO₂NH₂) with an inter-scan delay of 70 s. These diastereotopic methylene protons (AB_{quartet} spin-system as a single resonance) were chosen as the quantifiable resonance since the impurities (Supplementary Material 2, Figure 3) found in topiramate have functionalization at the –NH₂ of the sulfamic acid functional group and any such substitution/oxidation/reduction there, would be directly reflected on the chemical shift value of these protons. Moreover, the AB_{quartet} spin-system is extremely sensitive to changes in the stereochemistry (inversion, etc.) of the asymmetric centres in the molecule as well as the solvolysis of the two 1,3-dioxolanes in topiramate. Additional details about NMR acquisition and FID processing parameters can be found in the Supplementary Material 2.

Preparation of calibrators and quality control (QC) samples: The preparation of calibrators, QC levels, and the estimation of calibrator level uncertainties (type B uncertainty) were performed based on Taibon et al. [31] and are described in more detail in Supplementary Materials 1 and 3. For the calibrator stock solutions (1 and 2), 50 mg of topiramate was weighed on a micro balance XPR2 (Mettler Toledo, Columbus, Ohio, USA) and dissolved in 5 mL dimethyl sulfoxide (DMSO) using a volumetric flask to reach concentrations of 10 mg/mL. Concentrations of the stock solutions were calculated based on the purity of the reference material (99.2 ± 0.1 %, k=1, determined by qNMR) and the exact amount weighed.

These stock solutions were used to prepare working and spike solutions, which were further used to prepare eight final matrix-based calibrator levels. Calibrators were prepared volumetrically by a 1 + 99 dilution (v/v) into human serum matrix and were uniformly distributed from 1.20–36.0 µg/mL (3.54–106 µmol/L) (see Figure 1). Four levels of matrix-based QC were prepared in the same way as the calibrator levels using a third independent stock solution and resulting working and spike solutions.

The concentrations for the QC levels were defined at four critical control points: above the limit of quantification (LOQ), below and within the therapeutic reference range, and at the laboratory alert level (see Figure 1). Final concentration levels were 1.60, 4.00, 8.00 and 16.0 µg/mL.

Internal standard solution: To prepare the internal standard (ISTD) stock solution with a concentration of 1,000 µg/mL, 1 mL DMSO was pipetted directly into the manufacturer's container, which contained approximately 1 mg of [²H₁₂]-topiramate. The solution was stored at –20 °C. The ISTD working solution was prepared freshly by a twofold dilution of the ISTD stock solution: 150 µL DMSO was mixed with 50 µL of ISTD stock solution, followed by the addition of 7,800 µL Milli-Q water to obtain a final concentration of 6.25 µg/mL.

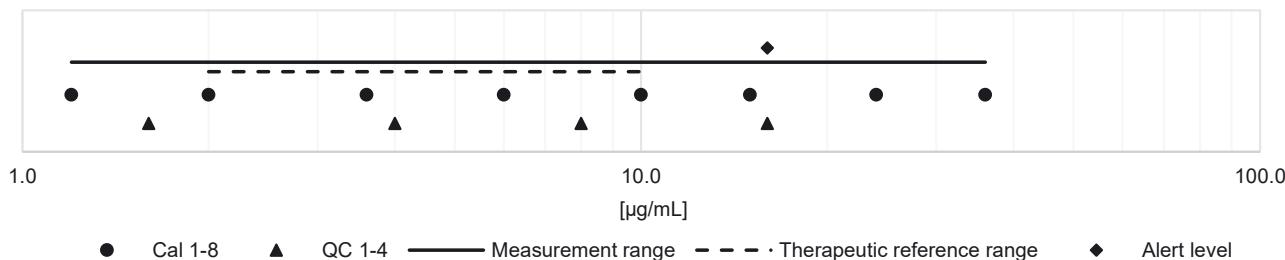


Figure 1: Schematic overview of set calibrator and control levels chosen to allow optimal coverage of measurement and therapeutic reference range. Black circles indicate calibrator spike solution, black triangles are QC spike solutions 1–4, the solid black line is the measurement range, the broken black line is therapeutic reference range, and the black diamond is the alert level. Cal, calibrator; QC, quality control. Conversion factor $\mu\text{g/mL}$ to $\mu\text{mol/L}$: 2.9.

Sample preparation: Native human serum, TDM-free human serum (surrogate matrix) and plasma (Li-heparin, K₂-EDTA and K₃-EDTA) was used as sample matrix. ISTD working solution (100 μL) was transferred into a 2 mL tube (Eppendorf Safe-Lock Tubes) to which 50 μL of the sample specimen (native sample/calibrator/QC) was added. For protein precipitation, 1,000 μL 75 % methanol in Milli-Q water (v/v) was added. A 10 μL aliquot of the supernatant was further diluted 1 + 99 (v/v) using mobile phase A, followed by a second dilution (1 + 9, v/v) with mobile phase A.

Liquid chromatography-mass spectrometry: An Agilent 1290 Infinity II LC system (Santa Clara, California, USA) equipped with a binary pump, a vacuum degasser, an autosampler and a column temperature control device, was used for the chromatographic separation. The detection of the analyte was performed by an AB Sciex Triple Quad 6500+ mass spectrometer with an electrospray ionization source (ESI) (Framingham, Massachusetts, USA).

Chromatographic separation of topiramate was achieved using an Agilent Zorbax Eclipse XDB-C8 column (100 \times 3 mm, 3.5 μm , Santa Clara, California, USA), which was kept in the column compartment at 40 °C. The mobile phases consisted of 10 % methanol in Milli-Q water (v/v) containing 0.1 % acetic acid (A) or 95 % methanol in Milli-Q water (v/v) (B).

All measurements were performed at a flowrate of 0.6 mL/min using the following gradient: t=0.0 min, 100 % A; t=1.0 min, 100 % A; t=3.0 min, 65 % A; t=3.2 min, 50 % A; t=4.2 min, 40 % A; t=4.3 min, 20 % A; t=5.2, 0 % A; t=8.0 min, 0 % A; t=8.1 min, 10 % A; t=10.0 min, 100 % A. The injection volume was set at 5 μL . To reduce contamination of the mass spectrometer the eluent flow was switched to the waste via a divert valve until 0.8 min and from 6.5 min.

Topiramate was detected by multiple reaction monitoring (MRM) operating in the negative ESI mode. An ion spray voltage of $-3,500$ V and a temperature of 600 °C were applied. Curtain gas, collision gas, ion gas source 1 and ion gas source 2 were, respectively, set at 35, 10, 70 and 60 psi. A declustering potential of -70 V and a dwell time of 50 ms were applied for all mass transitions. The quantifier transition sets the basis for the quantitative method and is linked to a corresponding transition of the ISTD. An additional specific mass transfer (qualifier) was monitored to exclude interfering substances in native matrix samples by comparing the quantifier/qualifier ratios of clean SST and native matrix samples, which should not differ by more than 20 %. Table 1 shows an overview of the SRM transitions as well as the remaining compound-dependent MS settings.

Table 1: MS/MS parameters of topiramate and its ISTD.

Analyte	Precursor ion, m/z	Product ion, m/z	EP, V	CE, V	CXP, V
Topiramate	Quantifier	338.2	78.0	-10	-50
	Qualifier		95.9	-10	-29
² H ₁₂ -topiramate	Quantifier	350.2	78.0	-10	-50
	Qualifier		95.9	-10	-29

EP, entrance potential; CE, collision energy; CXP, collision exit potential; ISTD, internal standard; MS/MS, tandem mass spectrometry.

System suitability test: To check the sensitivity of the system, chromatographic performance and potential carry-over effects, a system suitability test (SST) was performed before each analysis. Concentration levels of SST1 and SST2 corresponded to the analyte concentration within the processed calibrator levels 1 and 8, respectively. To pass the SST the signal to noise (S/N) ratio of the quantifier transition had to be ≥ 200 for SST1 and the retention time for SST1 and SST2 had to be within 4.6 ± 0.5 min. To analyze carry-over effects, the highly concentrated sample SST2 was injected, followed by the injection of two solvent blanks. The analyte peak area observed in the first blank after the injection of the SST2 sample had to be ≤ 20 % of the analyte peak area of SST1 to pass the SST.

Calibration, structure of analytical series and data processing: Calibrator levels were measured in increasing concentration at the beginning and at the end of the analytical series. Both calibration functions are used to generate the final calibration function by linear regression of the area ratios of the analyte and internal standard (y) against the analyte concentration (x) resulting in the function, $y=x \times A + b$. Data evaluation was performed using Analyst software (version 1.6.3) and the Intelli Quant algorithm. Topiramate and its ISTD showed a retention time of 4.6 min and were integrated within a 30 s window. To integrate peaks, a smoothing factor of 3 and a peak-splitting factor of 2 were used. The calibration curve was generated from eight calibrator levels using area ratios of the analyte and internal standard (y), based on a linear model with intercept and $1/x^2$ weighting. The reportable result is calculated in $\mu\text{g/mL}$ to three significant figures. Measurement uncertainty standard deviation (SD, $\mu\text{g/mL}$) is reported to three significant figures, and the bias and coefficient of variation (CV, %) to 1 decimal place.

Method validation

Assay validation and determination of measurement uncertainty were performed according to existing validation guidelines such as Clinical & Laboratory Standards Institute's C62A *Liquid Chromatography-Mass Spectrometry Methods* [32], the International Council of Harmonization's (ICH) guidance document *Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2 (R1)* [33] and the GUM [21].

Selectivity: To determine selectivity, topiramate and the ISTD [$^2\text{H}_{12}$]topiramate were spiked in analyte-free human serum, native human serum, and a plasma pool. To analyze possible interfering matrix signals for the analyte quantifier and qualifier transition an analyte-free human serum pool was tested at the expected retention time. Additionally, analyte-free human serum was spiked with the deuterated internal standard to evaluate a possible amount of residual unlabeled analyte within the stable isotope labeled internal standard. The amount of unlabeled analyte in the internal standard must not exceed 20 % of the amount of the lower limit of the measuring interval, corresponding to the concentration level of the lowest calibrator.

Matrix effects and specificity: To determine possible matrix effects, a qualitative post-column infusion experiment was performed. A neat solution of topiramate (75 ng/mL in mobile phase A/mobile phase B 1 + 1 (v/v)) was infused post-column via a T-piece with a flow rate of 7 $\mu\text{L}/\text{min}$ to generate a stable analyte background signal in the MS. Next, processed matrix samples (native human serum, surrogate serum, and plasma (Li-heparin, K₂-EDTA, and K₃-EDTA)) and a blank (mobile phase A) were injected.

In addition, eight calibrator levels were prepared sixfold in neat solution (mobile phase A), native human serum, TDM-free human serum (surrogate serum), and Li-heparin plasma to evaluate the specificity (for sample preparation, see Section [Sample preparation]). The calibration curves of each matrix were compared in terms of the mean slope (n=6) and correlation coefficient (n=6). The confidence intervals of the slopes must overlap to exclude a matrix effect. The correlation coefficient must be ≥ 0.99 . Calibrator samples in native human serum, surrogate serum, and Li-heparin plasma were evaluated as controls by employing the neat calibration as a standard. All samples were prepared in six replicates. Recoveries were reported as the percentage of recovery of the measured concentration in relation to the nominal concentration.

A comparison of absolute peak areas of analyte and internal standard was then performed. Analyte and ISTD solution were spiked in the matrices mentioned above after protein precipitation for three levels spread over the working range (4.00, 8.00, and 16.0 $\mu\text{g}/\text{mL}$). By comparing the peak areas of the analyte and internal standard of matrix samples against neat samples, ion enhancement or suppression was evaluated. All samples were prepared in five replicates. A value $>100\%$ indicates an ionization enhancement, and a value $<100\%$ indicates ion suppression. The percentage deviation should not be greater than $\pm 10\%$.

Linearity: To determine linearity, calibration levels were as described in Section [Preparation of calibrators and QC samples]. To obtain spiked serum samples for an extended calibration range by $\pm 20\%$, with final concentrations of 0.960 and 43.2 $\mu\text{g}/\text{mL}$, two additional spike solutions were prepared. A plot of the peak area ratio of analyte to the

corresponding internal standard against the respective analyte concentration ($\mu\text{g}/\text{mL}$) was made. For each calibration curve (n=6 sample preparations) the correlation coefficient and the residuals were determined, which had to be ≥ 0.99 and randomly distributed.

Additionally, the linearity of the method was demonstrated based on the recovery of serially diluted samples using the selected regression model for calculation. The measurement results have to show a linear dependence with a correlation coefficient of ≥ 0.99 . Sample levels were prepared as described in the following: calibrator level 1 was sample 1 and calibrator level 8 was sample 11. Using these two samples nine mixtures were diluted as follows: 9 + 1, 8 + 2, 7 + 3, 6 + 4, 5 + 5, 4 + 6, 3 + 7, 2 + 8, and 1 + 9 (v/v). Recovery was reported as the percentage recovery of the measured concentration relative to the nominal concentration of the sample pool.

Precision and accuracy: Precision was evaluated over a five-day validation experiment using two individual calibrator preparations for two different measurement sequences (Parts A and B) on each day. An ANOVA-based variance components analysis was performed to estimate total variability of the method including between-injection variability, between-preparation variability, between-calibration variability, and between-day variability. On each day, four spiked native serum and native Li-heparin plasma samples spread over the measuring range (1.60, 4.00, 8.00, and 16.0 $\mu\text{g}/\text{mL}$) as well as two native patient serum samples, which were close to the medical decision point, were prepared in triplicate for parts A and B and injected twice (n=12 measurements per day and n=60 measurements per five days). Independent calibration curves were generated for each part and day and used for quantitative analysis. In addition, as measurements should be performed under varying conditions one operator was responsible for sample preparation for parts A and B, respectively, and two different column batches were used. The data evaluation was carried out with an internal statistical program, based on the VCA Roche Open-Source software package in R [34].

Accuracy was evaluated using four spiked human serum and plasma samples with the following concentrations: 1.60, 4.00, 8.00, and 16.0 $\mu\text{g}/\text{mL}$. Dilution integrity was determined using two spiked serum samples (topiramate free human serum) at concentration levels of 42.0 and 60.0 $\mu\text{g}/\text{mL}$. All samples were prepared in triplicate for part A and for part B (n=6 measurements) on the same day. Accuracy was determined as the percentage recovery of the measured concentration related to the spiked concentration, whereas trueness was reported as the percentage recovery of the mean measured concentration related to the spiked concentration.

Lower limit of measuring interval and limit of detection: Precision and accuracy at the lower limit of the measurement interval (LLMI) were determined by measuring spiked serum matrix samples in the expected concentration range of the quantitation limit (QL). The QL matches the lowest calibrator level (1.20 $\mu\text{g}/\text{mL}$). Sample preparation was replicated fivefold and recovery, bias, and precision were determined and had to be within the range determined within the accuracy and precision experiment. The limit of detection (LOD) was estimated by determining the mean and SD of blank matrix samples (100 data points at the retention time of the analyte, 0.2 min time window, 10 independent samples from the precision experiment) and calculating LOD as the mean +3 SD with the mean peak height of calibrator 1 analysis serving as quantification reference.

Sample stability: The stability of the processed samples on the auto-sampler was investigated at 7 °C after 4 and 8 days using samples from the accuracy and precision experiment. Recoveries were calculated by comparing the measured value with freshly prepared samples. The stability of matrix-based calibrator and control material stored at -20 °C was evaluated after 28 days. Recoveries were calculated by comparing the measured value with freshly prepared samples. The Total Error was used as an acceptance criterion and estimated based on the results from precision and trueness experiment, resulting in a TE of $\pm 5\%$. Stability can be ensured for a measurement interval of 2–28 days for $y = 1$ day, and for a measurement interval of >4 weeks for $y = 1$ week.

Equivalence of results between independent laboratories: To evaluate agreement of the RMP between two independent laboratories (laboratory 1: Labor Risch, Buchs, Switzerland; and laboratory 2: Roche Diagnostics GmbH, Penzberg, Germany) a method comparison study was performed including 84 native samples provided by laboratory 2. The samples consisted of native, anonymized serum and plasma patient samples as well as sample pools. Additionally, a three-day precision experiment was performed at laboratory 2 based on the experimental design described in Section [Precision and accuracy]. Spiked serum and native patient samples were provided by laboratory 1. The RMP was transferred to laboratory 2 and the system setup applied as described in Supplementary Material 1 with some modifications: an ultramicrobalance XP6U/M (Mettler Toledo) and aluminum weighing boats were used for the preparation of stock solutions.

Uncertainty of measurements: Measurement uncertainty was determined according to the GUM [21] and Taibon et al. [31] and considered the following steps: purity of the reference material, weighing of the analyte, preparation of stock, working, spike and calibrator solutions, preparation of the internal standard solution, sample preparation of the calibrators, measurement of the calibrators and generation of the calibration curve, preparation and measurement of the unknown samples as well as evaluation of the sample results. The estimation of the uncertainty in the preparation of the calibrators was performed as a type B evaluation. All other aspects were evaluated as type A evaluation. The measurement uncertainty of the whole approach was estimated as a combination of types A and B uncertainties (Supplementary Material 3).

Results

Traceability to SI units

Traceability to the SI unit kilogram, the deterministic parameter for an RMP, has been established using qNMR ISTDs that are directly traceable to NIST PS1 (primary qNMR standard) or NIST benzoic acid 350b primary standards. Six individual experiments (Supplementary Material 2, Figure 2), involving six individual weightings of the analyte and the internal standard duroquinone, yield a final content value of $99.2 \pm 0.1\%$ ($k=1$).

Selectivity

The developed gradient combined with the reversed phase column (Agilent Zorbax Eclipse XDB-C8) yielded a retention

time of 4.6 min for topiramate (Figure 2). Selectivity was determined by analyzing sample pools of analyte-free human serum, surrogate serum, and human Li-heparin plasma. No signals were observed at the expected retention time. Furthermore, no residual analyte was measured in the internal standard [$^2\text{H}_{12}$]-topiramate and no interferences were observed in the retention time window of topiramate while measuring native patient samples within the method comparison study.

Matrix effect

A sample preparation protocol (with a high dilution after protein precipitation) was used to avoid matrix-dependent effects caused by proteins, phospholipids, and salts. No suppression or enhancement at the expected retention time of topiramate independent from the matrix was shown by the post-column infusion experiment.

Furthermore, a comparison of slopes and correlation coefficient of calibrators was performed in different matrices. Slopes were 0.0763 (95 % CI 0.0747–0.0779) for native serum matrix, 0.0761 (95 % CI 0.0755–0.0767) for neat solution, 0.0766 (95 % CI 0.0758–0.0773) for surrogate serum matrix, and 0.0771 (95 % CI 0.0764–0.0777) for plasma matrix. Correlation coefficients were ≥ 0.998 independent of the matrix used for calibration. Since the CIs of the slopes overlap, it can be assumed that they are not significantly different from each other, supporting the absence of matrix effects.

Additionally, calibrator samples (levels 1–8) in surrogate serum, native serum, and Li-heparin plasma were evaluated as controls ($n=6$ sample preparations) by applying the neat calibration as a standard. Relative bias for all matrices and levels ranged from -1.2 to 2.5 % except for one level in Li-heparin plasma (calibrator level 6) where the bias was 9.1 %. CVs were found to be less than 3.1 %. Although one level within the Li-heparin plasma matrix showed an increased bias, which may be explained by a pipetting problem during the spike process for this level, the method was shown to be matrix independent over the entire measuring range as all remaining confidence intervals of the bias overlapped.

Matrix effects (ME) were determined based on Matuszewski et al. [35]. ME were calculated by comparing the sets above and results reported as percentage recovery. No ME were observed at any level with mean values ranging from 98–103 % for the analyte and the corresponding internal standard. The mean area ratios were between 97 and 102 % (Table 2). Thus, the compensating effect of the labeled ISTD was confirmed.

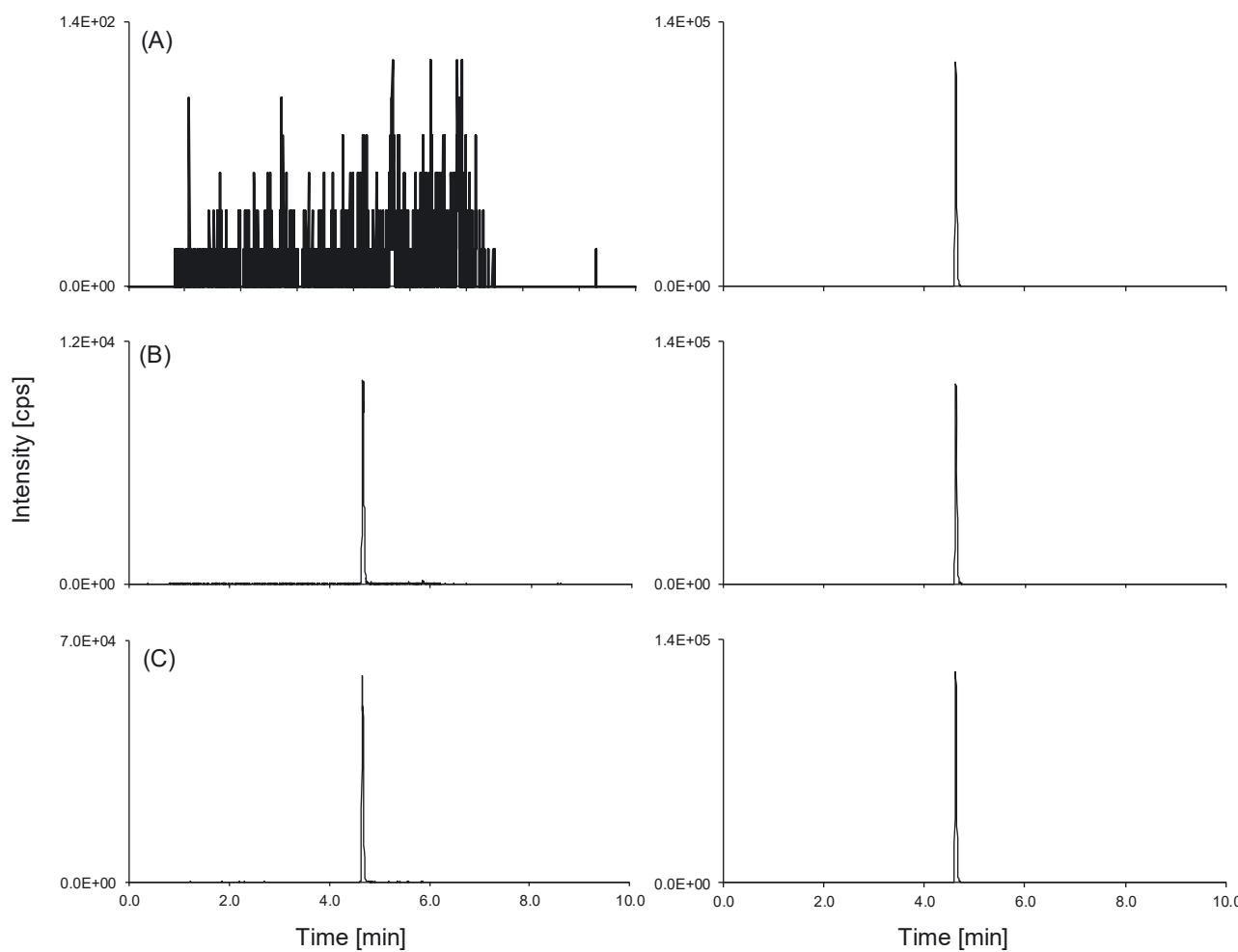


Figure 2: Topiramate LC-MS/MS-derived analytical readouts. Analyte on the left-hand side, topiramate ISTD on the right-hand side. (A) Chromatogram of a blank native serum sample without analyte but with ISTD. (B) The lowest calibrator level peak (1.20 µg/mL). (C) Patient sample pool (n>5, 6.12 µg/mL). ISTD, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

Table 2: Matrix effect data of three different matrices compared with neat analyte solutions. Analyte peak areas, ISTD peak areas, and analyte/ISDT area ratios as used in analyte quantification were investigated. Means from fivefold analysis were used as data input. The relative ME was calculated as ME (%) = set 2/set 1 × 100, where set 2 corresponds to the respective matrix samples and set 1 to the neat samples. No matrix effect is present if ME=100 %.

Topiramate level, conc.		Analyte		ISTD		Area ratio	
		Mean, %	95 % CI, %	Mean, %	95 % CI, %	Mean, %	95 % CI, %
Level 1 4.00 µg/mL	Native serum	100	97–103	101	98–104	99	96–101
	Surrogate serum	100	99–101	100	99–101	100	99–101
	Native plasma	100	99–102	103	102–104	97	95–99
Level 2 8.00 µg/mL	Native serum	100	98–102	100	99–102	99	98–101
	Surrogate serum	101	99–102	101	99–102	100	98–102
	Native plasma	102	100–103	100	98–103	102	99–104
Level 3 16.0 µg/mL	Native serum	98	97–99	99	98–100	99	98–100
	Surrogate serum	99	97–102	100	98–103	99	98–101
	Native plasma	101	99–104	102	101–103	99	98–101

CI, confidence interval; ISTD, internal standard.

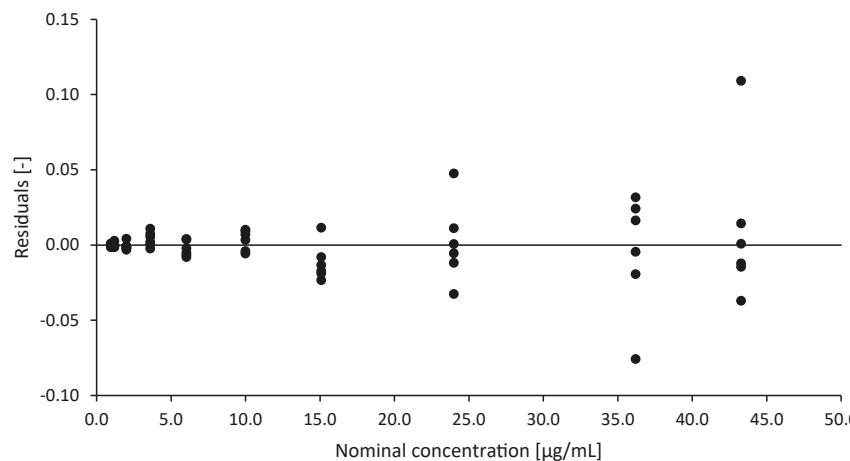


Figure 3: Residuals plot derived from the evaluation of the final linear calibration model. Six individual sample set preparations were performed to evaluate the repeatability of the procedure.

Linearity and LLMI

By analyzing calibration curves extended by $\pm 20\%$ of the assay measuring range ($n=6$, sample preparations) the linearity of the method was proven over the range 0.960 – $43.2\text{ }\mu\text{g}/\text{mL}$. Figure 3 shows that residuals were randomly distributed in a linear regression model, wherefore it was chosen for assay calibration. Correlation coefficients were 1.00 for all individual calibrations. Based on these results, samples 1–11 were evaluated and showed a linear dependence with a correlation coefficient of 0.999. The relative deviation ranged from -0.1 to 3.2% and the CV was $\leq 3.7\%$.

The LLMI was determined using spiked samples at the concentration of the lowest calibrator level ($1.2\text{ }\mu\text{g}/\text{mL}$). Relative bias showed a deviation of 0.7% while the CV was 2.2% . The LOD was estimated as $0.0239\text{ }\mu\text{g}/\text{mL}$ with an intensity corresponding approximately to 150th of the average calibrator 1 peak height.

Accuracy and precision

Precision and accuracy were validated using spiked serum and plasma samples at four concentration levels with a focus on medical decision points. Each level was prepared sixfold and injected twice by two different operators on five different days ($n=60$). To determine the bias for levels in the measurement range (1.60 , 4.00 , 8.00 and $16.0\text{ }\mu\text{g}/\text{mL}$) data from the first day were evaluated. To assess the bias for highly concentrated samples (42.0 and $60.0\text{ }\mu\text{g}/\text{mL}$) samples were diluted with matrix prior to sample preparation. Levels within the calibration range were found with a bias between -0.3 and 1.4% for serum levels and between -0.3 and 1.1% for Li-heparin plasma levels. High-concentration native serum samples showed a bias between -0.4 and 3.5% (Table 3).

Table 3: Bias evaluation results.

Concentration, $\mu\text{g}/\text{mL}$	Serum		Plasma	
	Mean bias, %	95 % CI, %	Mean bias, %	95 % CI, %
Level 1 $1.60\text{ }\mu\text{g}/\text{mL}$	-0.3	-0.8 to 0.2	-0.3	-2.7 to 2.0
Level 2 $4.00\text{ }\mu\text{g}/\text{mL}$	1.4	0.5 to 2.4	1.0	-0.5 to 2.6
Level 3 $8.00\text{ }\mu\text{g}/\text{mL}$	0.1	-1.2 to 1.3	0.4	-1.2 to 1.9
Level 4 $16.0\text{ }\mu\text{g}/\text{mL}$	0.5	-0.3 to 1.2	1.1	0.0 to 2.2
Dilution 1 $42.0\text{ }\mu\text{g}/\text{mL}$	-0.4	-2.2 to 1.5	-	-
Dilution 2 $60.0\text{ }\mu\text{g}/\text{mL}$	3.5	1.7 to 5.3	-	-

Measurements were performed in triplicate within parts A and B ($n=6$ measurements). The bias and corresponding confidence intervals were calculated using the individual sample biases of $n=6$ preparations.

Precision was evaluated in a multi-day validation experiment. To assess the overall variability of the method, variability components such as variability between injections, between preparations, between calibrations, and between days were determined using an ANOVA-based variance component analysis. Intermediate precision including variances such as between-day calibration, preparation, and injection was found to be less than 3.2% independent of the matrix. Repeatability CV ranged between 1.4 and 2.5% over all concentration levels (Table 4).

Stability

Stability of processed samples was determined by re-analyzing the original sample with freshly prepared

Table 4: Precision performance parameters for topiramate quantification using the candidate RMP (n=60 measurements).

Variance source	Serum samples CV, %					
	Level 1 1.60 µg/mL	Level 2 4.00 µg/mL	Level 3 8.00 µg/mL	Level 4 16.0 µg/mL	Patient sample 1 1.60 µg/mL	Patient sample 2 6.12 µg/mL
Intermediate	2.5	2.1	1.7	1.8	2.1	2.3
Between-day	0.0	0.0	0.3	0.0	0.6	0.0
Between-calibration	0.0	0.0	0.0	0.6	0.6	0.9
Repeatability	2.5	2.1	1.7	1.7	1.9	2.0
Between-preparation	0.0	1.0	0.9	0.9	0.0	0.3
Between-injection	2.5	1.9	1.5	1.4	1.9	2.0

Variance source	Plasma samples CV, %			
	Level 1 1.60 µg/mL	Level 2 4.00 µg/mL	Level 3 8.00 µg/mL	Level 4 16.0 µg/mL
Intermediate	3.2	2.1	1.7	1.7
Between-day	0.0	0.0	0.3	0.7
Between-calibration	2.2	0.4	0.0	0.7
Repeatability	2.3	2.0	1.7	1.4
Between-preparation	0.0	0.5	1.2	0.0
Between-injection	2.3	2.0	1.2	1.4

The coefficients of variation for repeatability and intermediate precision, which were determined from the individual variances, are in bold. CV, coefficient of variation; RMP, reference measurement procedure; SD, standard deviation. Conversion factor µg/mL to µmol/L: 2.9.

frozen serum calibrators. Autosampler stability of processed samples (7 °C) was demonstrated for seven days. Recovery was between 98 and 104 %. The stability of spiked calibrator and control samples was 27 days (–20 °C). The recovery was between 95 and 102 % of the original value.

Precision experiments (n=36) performed at laboratory 2 resulted in very comparable CVs for repeatability ($\leq 2.4\%$) and intermediate precision ($\leq 3.0\%$). Thus, the method is transferable and meets the requirements independent of laboratory equipment or personnel.

Equivalence of results between independent laboratories

The method comparison study was performed using 84 native anonymized patient samples (native serum, native plasma, and pooled samples). Twelve of these samples were below the limit of quantitation and, therefore, not considered for further analysis. Passing–Bablok regression analysis (Figure 4A) showed good agreement between the two laboratories and resulted in a regression equation with a slope of 1.01 (95 % CI 1.00–1.03) and an intercept of –0.05 (95 % CI –0.13 to –0.01). The Pearson correlation coefficient was found to be ≥ 0.999 . In addition, the Bland–Altman analysis (Figure 4B) showed a good agreement within the two laboratories. The data scatter shows a mean bias of 0.1 % and is statistically not different from zero (95 % CI interval from –0.7 to 0.8 %). Moreover, the data scatter is independent from the concentration with a 2S agreement of 6.1 % (lower limit CI interval from –7.3 to –4.8 %, upper limit CI interval from 4.9 to 7.4 %).

Uncertainty of measurements

The combined measurement uncertainties for single measurements of serum samples ranged from 1.9 to 2.7 % and the combined measurement uncertainties for target value assignment (n=6, three measurements on two days) ranged from 0.9 to 1.4 % regardless of concentration level and sample type (Tables 5 and 6). For a better readability and interpretability, the determined uncertainties are expressed as coefficients of variation (CVs).

Consequently, the expanded measurement uncertainties are between 3.8 and 5.4 % for single measurements and between 1.8 and 2.8 % for multiple measurements. The derived combined uncertainty is multiplied by a coverage factor of k=2 to obtain an expanded uncertainty.

Discussion and conclusions

The presented ID-LC-MS/MS-based RMP candidate for the quantification of topiramate in human serum and plasma

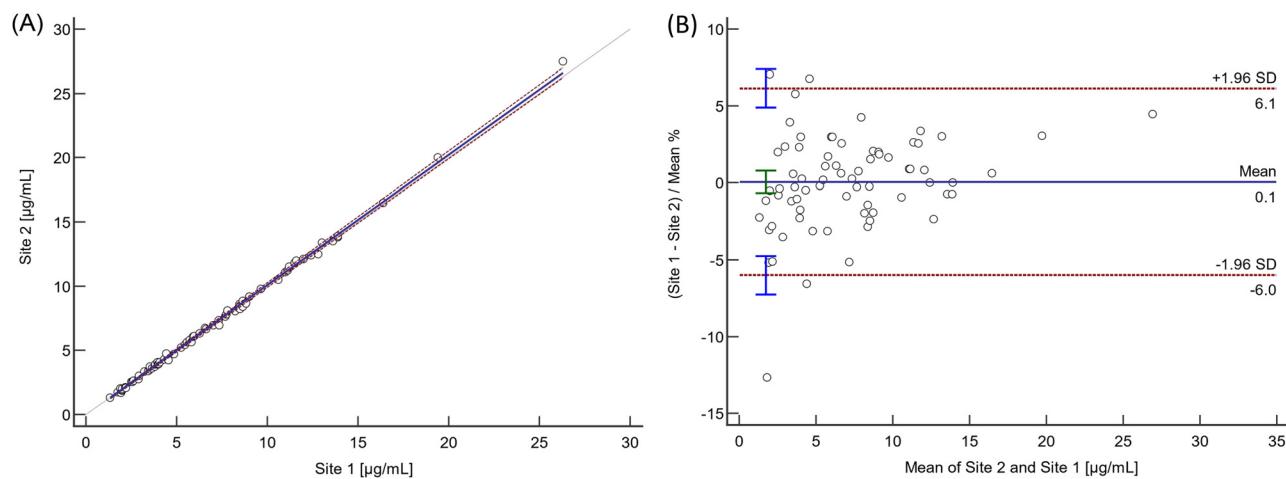


Figure 4: Results from the patient sample-based topiramate method comparison study. (A) Passing-Bablok regression plot including the Pearson regression analysis for the method comparison study of the RMP ($n=72$) between the independent laboratories (Site 1: Risch; Site 2: Roche). Passing-Bablok resulted in a regression equation with a slope of 1.01 (95 % CI 1.00–1.03) and an intercept of –0.05 (95 % CI –0.13 to –0.01). The Pearson correlation value was ≥ 0.999 . (B) Bland-Altman plot for the method comparison study of the RMP ($n=72$ patients) between two independent laboratories (Laboratory 1: Risch site, and Laboratory 2: Roche site). The interlaboratory measurement bias was 0.1 % (95 % CI interval from –0.7 to 0.8 %) and the 2S interval of the relative difference was 6.1 % (lower limit CI interval from –7.3 to –4.8 %, upper limit CI interval from 4.9 to 7.4 %).

Table 5: Overview of measurement uncertainty for topiramate quantification with the candidate RMP in serum samples for single measurements.

	Level					
	Level 1	Level 2	Level 3	Level 4	Patient sample 1	Patient sample 2
	1.60 µg/mL	4.00 µg/mL	8.00 µg/mL	16.0 µg/mL	1.60 µg/mL	6.12 µg/mL
Type B uncertainty	0.91	0.86	0.86	0.77	0.91	0.86
Calibrator preparation, CV %						
Characterization of reference material	0.1	0.1	0.1	0.1	0.1	0.1
Preparation of						
Stock solution	0.25	0.25	0.25	0.25	0.25	0.25
Working solution	0.40	–	–	–	0.40	–
Spike solution	0.68	0.61	0.61	0.46	0.68	0.61
Matrix-based calibrator	0.91	0.86	0.86	0.77	0.91	0.86
Type A uncertainty	2.5	2.1	1.7	1.8	2.1	2.3
Intermediate precision, CV %						
Combined measurement uncertainty ($k=1$), CV %	2.7	2.3	1.9	2.0	2.2	2.4
Expanded measurement uncertainty ($k=2$), CV %	5.4	4.6	3.8	3.9	4.5	4.8

The total measurement uncertainty of the whole approach for a single measurement estimated as a combination of the uncertainty of calibrator preparation (type B uncertainty) and uncertainty of the precision experiment (type A uncertainty) are given in bold. CV, coefficient of variation; RMP, reference measurement procedure. Conversion factor µg/mL to µmol/L: 2.9.

is suitable for its intended use. The design was derived considering routine measurements of topiramate by LC-MS/MS. Therefore, tandem mass spectrometry with ESI and reversed-phase chromatography was chosen for the analytical method. Owing to relatively high concentrations of topiramate in serum and plasma samples, sample preparation of protein precipitation followed by a dilution was applied. Development and optimization of the analytical

method included mobile phase composition and gradient, stationary phase selection, and ion source optimization during the setup of the selected reaction monitoring experiment. All these elements were carefully evaluated prior to method validation. Moreover, co-elution of unknown interferences in patient samples was ruled out beforehand.

Optimization of sample preparation comprised fluid handling, including selection of optimal pipettes; protein

Table 6: Overview of measurement uncertainty for topiramate target value assignment (n=6) with the candidate RMP in serum samples.

	Level					
	Level 1	Level 2	Level 3	Level 4	Patient sample 1	Patient sample 2
	1.60 µg/mL	4.00 µg/mL	8.00 µg/mL	16.0 µg/mL	1.60 µg/mL	6.12 µg/mL
Type B uncertainty	0.91	0.86	0.86	0.77	0.91	0.86
Calibrator preparation, CV %						
Characterization of reference material	0.1	0.1	0.1	0.1	0.1	0.1
Preparation of						
Stock solution	0.25	0.25	0.25	0.25	0.25	0.25
Working solution	0.40	—	—	—	0.40	—
Spike solution	0.68	0.61	0.61	0.46	0.68	0.61
Matrix based calibrator	0.91	0.86	0.86	0.77	0.91	0.86
Type A uncertainty	0.5	0.7	0.8	0.5	1.1	0.4
Mean of measurement results, CV %						
Combined measurement uncertainty (k=1), CV %	1.0	1.1	1.2	0.9	1.4	1.0
Expanded measurement uncertainty (k=2), CV %	2.1	2.2	2.4	1.8	2.9	1.9

The total measurement uncertainty of the whole approach for target value assignment estimated as a combination of the uncertainty of calibrator preparation (type B uncertainty) and uncertainty of the precision experiment (type A uncertainty) are given in bold. CV, coefficient of variation; RMP, reference measurement procedure. Conversion factor µg/mL to µmol/L: 2.9.

precipitation, including equilibration times; dilution into the linear range of the MS detector; establishment of an optimized calibration and control step scheme; and optimized preparation of calibrator and control materials including pipetting. The use of qNMR-characterized reference standards for calibration of the candidate RMP allows direct traceability to SI units.

The validation study confirmed the absence of matrix effects by a calibration slope comparison in addition to an ion yield attenuation experiment. Furthermore, the study showed that the method meets the requirements for an RMP for topiramate in terms of sensitivity, selectivity, and reproducibility. However, single measurements of serum samples did not fulfill the requirements of RMP measurement uncertainty derived from the pharmacokinetic model by Fraser [23], which is described in the introduction. Therefore, multiple measurements (n=6) must be performed for the target value assignments in order to reduce measurement uncertainty and to meet the requirement.

A second independent laboratory showed that the method is transferable without a significant increase in inter-laboratory bias. This confirms that the preparation of the calibrator solutions and the sample preparation protocols are robustly designed. Moreover, the platform comparison study showed that the method is suitable for processing a high volume of patient samples in a relatively short time. This gives the user confidence in this RMP for the evaluation of routine samples with unclear results. Consequently, the method fulfills both the requirements to

take a leading role in the traceability chain and to perform method comparison studies and check problematic routine samples.

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Competing interests: Tobias Schierscher and Lorenz Risch are employees of Dr. Rich Ostschweiz AG. Linda Salzmann, Tino Spescha, Anja Kobel, and Christoph Seger were all employees of Dr. Risch Ostschweiz AG at the time the study was conducted. Judith Taibon, Neeraj Singh, Vanessa Fischer, Andrea Geistanger, and Christian Geletneky are all employees of Roche Diagnostics GmbH. Friederike Bauland is an employee of Chrestos Concept GmbH & Co. KG, (Girardetstr.1-5, 45131 Essen, Germany) and did the work on behalf of Roche Diagnostics GmbH. Roche employees holding Roche non-voting equity securities (Genusscheine): Judith Taibon, Christian Geletneky, and Andrea Geistanger.

Informed consent: Not applicable.

Ethical approval: Not applicable.

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