DE GRUYTER Clin Chem Lab Med 2024; 62(7): 1327–1338

Tobias Schierscher, Linda Salzmann, Neeraj Singh, Vanessa Fischer, Anja Kobel, Friederike Bauland, Andrea Geistanger, Lorenz Risch, Christian Geletneky, Christoph Seger and Judith Taibon*

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

https://doi.org/10.1515/cclm-2023-1032
Received September 15, 2023; accepted March 12, 2024;

Abstract

Objectives: Primidone is an anticonvulsive drug used in the treatment of epilepsy and essential tremor. It offers beneficial effects in controlling seizures, but its usage is also associated with possible side effects. To ensure optimal therapy, it is crucial to measure its concentration through accurate quantification methods. Therefore, our main goal was to develop and validate a new reference measurement procedure (RMP) for accurately measuring primidone levels in human serum and plasma.

Methods: In our study, we focused on the separation of primidone from both known and unknown interferences using a C18 column. To achieve accurate sample preparation, we developed a protocol involving protein precipitation followed by a high dilution step. The validation of the assay and determination of measurement uncertainty were carried out following guidelines from organizations such as the Clinical and Laboratory Standards Institute, the International Conference on Harmonization, and the Guide to the Expression of Uncertainty in Measurement. These rigorous validation processes ensure the reliability and accuracy of our method for quantifying primidone levels in human serum and plasma samples.

Results: The RMP was shown to be highly selective and specific, with no evidence of matrix interference. It can be used to quantify primidone in the range of 0.150–30.0 µg/mL. Intermediate precision was less than 4.0 %, and repeatability CV ranged from 1.0 to 3.3 % across all concentration levels. The relative mean bias ranged from 0.1 to 3.9 % for native serum levels, and from −2.6 to 2.8 % for lithium-heparin plasma levels. The measurement uncertainties for single measurements and target value assignment were 1.5–4.1 % and 0.9–1.0 %, respectively.

Conclusions: In this study, we introduce an innovative LC-MS/MS-based candidate RMP specifically designed for primidone in human serum and plasma. Our RMP offers a traceable platform, facilitating the standardization of routine assays and enabling the evaluation of clinically relevant samples. With this novel approach, we aim to enhance the accuracy and reliability of primidone measurements, ultimately benefiting the field of clinical research and patient care.

Keywords: primidone; isotope dilution-liquid chromatography-tandem mass spectrometry; qNMR; reference measurement procedure; traceability; standardization

Introduction

Pharmacological background

Primidone ($C_{12}H_{14}N_2O_2$, molecular weight=218.25 Da, conversion factor from µg/mL to molar unti [µmol/L]=4.6) is a synthetic analogue of the antiepileptic drug phenobarbital, first synthesized by Bogue and Carrington in 1949 and associated with fewer sedative and cognitive side effects than phenobarbital [1, 2]. It is metabolized to phenobarbital and phenylethylmalonamide, however the anticonvulsant activity and mechanism are not yet fully understood [3]. Unlike phenobarbital, primidone does not interact directly with GABA-A receptors or ion channels, but it reduces high-

---

***Corresponding author: Judith Taibon**, PhD, Roche Diagnostics GmbH, Nonnenwald 2, Penzberg 82377, Germany, Phone: +49 8856 6012941, E-mail: judith.taibon@roche.com
Tobias Schierscher, Linda Salzmann, Anja Kobel, Lorenz Risch and Christoph Seger, Dr. Risch Ostschweiz AG, Buchs, Switzerland. https://orcid.org/0000-0003-2692-6699 (L. Risch)
Neeraj Singh, Vanessa Fischer, Andrea Geistanger and Christian Geletneky, Roche Diagnostics GmbH, Penzberg, Germany
Friederike Bauland, Chrestos Concept GmbH & Co. KG, Essen, Germany

frequency repetitive firing of neurons and alternates the movement of transmembrane sodium and calcium ions [4]. Moreover, the minor metabolite phenylethylmalonamide has been shown to potentiate the anticonvulsant activity of the major metabolite phenobarbital against tonic-clonic seizures and to decrease the myoclonic jerk threshold and may play an important role in the anticonvulsant activity of primidone [5]. Nevertheless, primidone has been shown to be effective in the treatment of essential tremor and is therefore used primarily for this neurological condition (level A recommendation) rather than for epilepsy [3, 6, 7]. However, it is still used in certain circumstances as it is the only anticonvulsant drug known to shorten QT-interval and correct cardiac arrhythmias [8, 9].

Common adverse effects are confusion, ataxia, and nausea and have been reported in 22–77 % of the patients. These side effects occur even at very low doses [10]. The established therapeutic range of primidone is 5.00–10.0 µg/mL [11]. Primidone is primarily metabolized in the liver mainly to phenobarbital and has a half-life of 7–22 h in adults [12]. The major metabolite, phenobarbital, is metabolized rather slowly and therefore accumulates to high concentrations that may be in the toxic range (>50 µg/mL) [5, 11]. Hence, monitoring of both primidone and phenobarbital is essential.

Analytical background

There are several *in vitro* diagnostic Conformitè Europeenne (CE) certified analytical solutions for therapeutic drug monitoring (TDM) of primidone and phenobarbital by LC-MS/MS. In addition, there are various LC-MS/MS methods published in literature that are used in research and development [13–15]. However, there is currently no reference measurement procedure (RMP) listed in the JCTLM database [16].

An RMP is understood to be free of systematic error, must be traceable to a higher order reference material and in accordance with ISO guidelines (ISO 17511) [17]. The Bureau International des Poids et Mesures (BIPM) and the National Metrology Institutes (NMI) provide metrological control to ensure the accuracy and reliability of RMPs and to provide traceable calibrator and control materials. If materials and/or methods are not provided by BIPM and NMIs, individual traceability approaches must be followed [18]. These systems are essential to improve inter-laboratory measurement accuracy and to reduce inter-laboratory and inter-test variability within routine laboratories.

To establish traceability to SI-units, quantitative nuclear magnetic resonance (qNMR) spectroscopy is used since it is a well-established primary ratio method for determining the mass fraction (absolute content; g/g) of an analyte in a single, non-destructive experiment. This technique offers the potential for complete molecular structure elucidation owing to a plethora of robust 1D and 2D NMR pulse sequences along with a linear response to the amount of analyte and direct traceability to the kilogram via qNMR internal standards [19, 20]. These features provide an unparalleled ability to determine the amount or “counts” of a specific analyte. The highest order qNMR internal standards are directly traceable to NIST benzoic acid 350b (coulometric) and/or NIST PS1 (benzoic acid; first primary qNMR standard) [21]. Furthermore, according to the latest IUPAC Technical Report, qNMR has been identified as a potential primary reference measurement technique that is well suited for the characterization of primary reference materials (PRMs) [22].

The measurement uncertainty of these systems should be no more than one third of the measurement uncertainty of routine methods and/or the biological variability [23]. As described in previous published RMPs [24–27], the target measurement uncertainty (k=1) and the expanded measurement uncertainty (k=2) of the candidate RMP of primidone was determined to be less than 1.5 and 3.0 %, respectively [28].

Herein, we present a novel candidate RMP for primidone that meets the requirements of the ISO 15193 guideline [29]. This manuscript does only cover primidone, an RMP for phenobarbital will be covered in a separate report. The technical implementation of the test procedure, the qNMR-based reference material characterization, and the calculation of the measurement uncertainty are described in three supplemental materials. These supplementary materials provide sufficient detail to enable the RMP to be reproduced in other laboratories.

Materials and methods

A full description of the methods, materials and equipment used is provided in Supplemental Material 1.

Chemicals and reagents

LC-MS grade methanol (CAS 67-56-1) and formic acid (CAS 64-18-6) were purchased from Biosolve (Valkenswaard, The Netherlands). Isopropanol was purchased from Riedel-de Haën (Seelze, Germany). Dimethyl sulfoxide (DMSO) (CAS 67-68-5, ACS reagent, ≥99.99 %), ammonium acetate (CAS 631-61-8, LC/MS grade), DMSO-d6 (CAS 2206-27-1), the analyte primidone (CAS 125-3-7, Art. No. P7295, Lot MKCM2420), and the qNMR internal standard 1,3,5-trimethoxybenzene (TraceCert$^{®}$, Lot Nr. BCBW3670, CAS 621-23-8) were bought from Sigma Aldrich (Taufkirchen, Germany). The deuterated internal standard (ISTD), [$^2H_5$]-primidone (CAS 73738-06-4, Art. No. C629, Lot JF-ALS-19-080-P3) was purchased from Alsachim (Illkirch Graffenstaden, France). Water was produced in-house 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 with different additives (lithium-heparin (Li-Heparin), dipotassium ethylene diamine tetraacetic acid (K_2-EDTA), and tripotassium dipotassium ethylene diamine tetraacetic acid (K_3-EDTA)) was obtained from leftover anonymized patient samples. Patient pools were prepared in accordance with the Declaration of Helsinki.

General requirements for laboratory equipment

A list of equipment used, and their requirements is given in Supplemental Material 1.

qNMR for determination of the purity of the standard materials

The single-pulse ^{1}HNMR measurements were performed on a JEOL 600 MHz NMR spectrometer equipped with an ultra-cool probe head, which provides 4–5 times sensitivity enhancement compared to normal room-temperature probe heads. For Primidone, single-pulse-1HNMR was utilized for the quantitation (Supplemental Material 2 Figure 1; methylene protons; $CH_3C\underline{H}_2$; δ=1.96 ppm; 2H; 1,3,5-trimethoxybenzene as qNMR internal standard; DMSO-d_6 as solvent) with an inter-scan delay of 70 s (Supplemental Material 2, Figure 1). The identity of these methylene protons was established based on the delta value, coupling constant and 2D-TOCSY spectrum. The differentiation of these protons from similar protons which could originate from the starting materials for the synthesis of Primidone viz., 5-Phenyl-5-ethyl-2-thiobarbituric Acid, 2-Ethyl-2-phenylmalonamide, 5-ethyl-2-methoxy-5-phenyl-dihydro-pyrimidine-4,6-dione and Diethyl 2-ethyl-2-phenylmalonate, was established by an extensive analysis of $^{13}C\{^1H\}$ NMR, 2D-TOCSY, g-HSQCAD and CIGAR-HMBCAD spectra (Supplemental Material 2, Figures 3–5). Additional details about FID processing can be found in the supplementary information.

Preparation of calibrators and quality control samples

Two independent calibrator stock solutions were prepared by weighing 30.0 mg of primidone in tin boats on a microbalance. These tin boats were then transferred to 5 mL volumetric flasks and dissolved in DMSO to reach concentrations of 6.00 mg/mL. The concentrations of the stock solutions were calculated using the reference material's purity of 99.8 ± 0.1 % (as determined by qNMR) and the amount weighed. Two working solutions were then prepared by diluting the stock solutions with DMSO in 5 mL volumetric flasks. These working and stock solutions were used to prepare spike solutions in DMSO. Finally, eight matrix-based calibrators were prepared by a volumetrically 1+99 dilution (v/v) into human serum matrix, uniformly distributed from 0.150 to 30.0 µg/mL (0.687–137 µmol/L) (see Figure 1).

A third independent stock solution was used to produce four levels of matrix-based quality control (QC) levels in the same way as described for the calibrators. The final concentrations were selected at four critical points, including levels above the limit of quantitation, below and within the therapeutic reference range, and at the laboratory alert level. Final concentration levels were 0.200, 2.00, 7.50 and 25.0 µg/mL (see Figure 1).

ISTD solution

To prepare the ISTD stock solution, approximately 1 mg of [$^2H_5$]-primidone was dissolved in the appropriate volume of DMSO in the manufacturer's container to obtain a 1000 µg/mL stock solution. The ISTD stock solution was stored at −20 °C for a maximum of 8 months. On each day of sample preparation, a fresh ISTD working solution was prepared by diluting the ISTD stock solution twofold. Specifically, 5 µL of ISTD stock solution was mixed with 95 µL of DMSO, followed by the addition of 3900 µL of Milli-Q water. The final concentration of the ISTD working solution was 1.25 µg/mL.

Sample preparation

Native human serum, TDM free serum (surrogate matrix) and plasma (Li-heparin, K_2-EDTA and K_3-EDTA) were used as sample matrix. 100 µL of the ISTD working solution was transferred into a 2 mL tube (Eppendorf Safe-Lock Tubes; Eppendorf, Hamburg, Germany) and 50 µL of the sample specimen (native sample/calibrator/QC) was added. 1000 µL of precipitation solution (75 % methanol [v/v]) was then added for protein precipitation. Finally, 20 µL of the supernatant was diluted 1+49 (v/v) with mobile phase A.

Liquid chromatography mass spectrometry

An Agilent 1290 Infinity II LC system (Santa Clara, California, USA) with a binary pump, a vacuum degasser, an autosampler at 7 °C, and a temperature-controlled column compartment at 40 °C was used for chromatographic separation. Analyte detection was performed using an AB Sciex Triple Quad 6500+ and Q-Trap 6500+ mass spectrometer (Framingham, Massachusetts, USA) equipped with a Turbo V ion source. Primidone was separated on an Agilent Zorbax Eclipse XDB-C18 column (100 × 3 mm, 3.5 µm, Santa Clara, California, USA). The mobile phases consisted of 2 mM ammonium acetate in Milli-Q water with 0.1 % formic acid (A) and methanol/2 mM ammonium acetate in Milli-Q-water 95+5 (v/v) with 0.1 % formic acid (B). Measurements were performed at a flowrate of 0.6 mL/min with a gradient over 8.0 min. The injection volume was set at 12 µL and a diverter valve was used to divert the eluent flow to the waste until 0.8 min and from 5.0 min to reduce contamination of the MS.

Primidone was detected in multiple reaction monitoring (MRM) mode and positive electrospray ionization mode (ESI+mode). The mass spectrometer parameters were optimized as follows: ion spray voltage of 5000 V; temperature of 400 °C; declustering potential of 50 V; dwell time of 50 ms. Nitrogen gas was used as curtain gas, collision gas, ion gas source 1, and ion gas source 2 and was set to 45, 11, 50, and 60 psi, respectively.

The quantifier mass transition is used as the basis of the quantitative method. Additionally, a qualifier transition was measured to check for interferences by monitoring the quantifier/qualifier ratios of clean system suitability test (SST) samples and clinical samples. The clinical sample and neat sample should not differ from each other

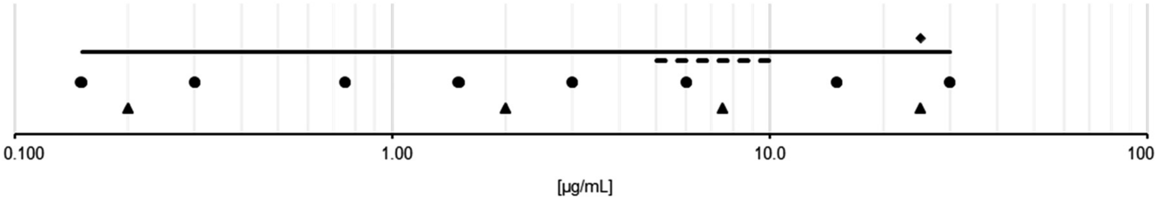

Figure 1: Schematic overview of set calibrator and control levels, which were chosen to allow an optimal coverage of measurement and therapeutic reference ranges. Black circles indicate calibrator spike solution, black triangles are QC spike solutions 1–4, the solid black line is the measurement range, the dashed line is the therapeutic reference range, and the black diamond is the alert level. Cal, calibrator; QC, quality control. Conversion factor µg/mL to µmol/L: 4.6.

Table 1: MS/MS parameters of primidone and [$^2H_5$]-primidone.

Analyte		Precursor ion, *m/z*	Product ion, *m/z*	EP, V	CE, V	CXP, V
Primidone	Quantifier	219.1	162.1	10	20	15
	Qualifier		119.1	10	24	15
[$^2H_5$]-primidone	Quantifier	224.1	167.1	10	20	15
	Qualifier		124.1	10	24	15

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

by more than 20 %. Table 1 summarizes the quantifier and qualifier transitions and the remaining compound-dependent MS settings.

System suitability test

System sensitivity, chromatographic performance, and potential carry-over effect are tested prior to each analysis in an SST. Therefore, two neat samples, SST1 and SST2 (primidone concentration of the processed calibrator levels 1 and 8, respectively), were freshly prepared for each analysis. The signal-to-noise ratio of the quantifier for SST1 had to be ≥10 and the retention times for both SST samples had to be within 3.8 ± 0.5 min to pass the SST, to verify the sensitivity and chromatographic performance, respectively. Potential carry-over effects were examined by injecting the high-concentration sample SST2, followed by two solvent blanks. The analyte peak area observed in the first blank after the high-concentration sample had to be ≤20 % of the analyte peak area of calibrator 1 to pass the SST. This purity criterion applied also to all further blanks in the measurement campaigns.

Calibration and structure of analytical series

The system was calibrated using eight calibrators which were measured in increasing concentrations at the beginning and at the end of the analytical series. Both calibrations were used to generate the final calibration function by linear regression of the area ratios of the analyte and ISTD (y) against the analyte concentration (x) resulting in the function, $y=x \times A+b$. Data evaluation was performed using the Analyst software (version 1.6.3 or higher) using the Intelli Quant algorithm. Retention times for primidone and its ISTD were 3.8 min and were integrated within a 30 s window. Peaks were processed with a smoothing factor of 3 and then integrated with a peak splitting factor of 2 and a noise percent of 80 % with a base sub window of 0.5 min.

Method validation

Assay validation and determination of measurement uncertainty were carried out based on the existing validation guidelines such as the Clinical & Laboratory Standard Guidelines C62A *Liquid Chromatography-Mass Spectrometry Methods* [30], the International Conference on Harmonisation guidance document *Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2 (R1)* [31] and the *Guide to the expression of uncertainty in measurement* (GUM) [32].

Selectivity: Selectivity was evaluated by spiking primidone, the ISTD [$^2H_5$]-primidone and its known metabolite phenobarbital (*m/z* 231.1 to 42.1) into analyte-free human serum, surrogate serum, and Li-heparin plasma at a concentration of 1.50 µg/mL. Furthermore, analyte-free matrices were evaluated at the expected retention time (3.8 min) to identify possible interfering matrix signals for the analyte quantifier and qualifier transitions. To determine the amount of remaining unlabeled analyte within the isotope labelled ISTD analyte-free matrices were spiked with [$^2H_5$]-primidone whereby the amount of unlabeled analyte should not exceed 20 % of the amount of analyte within the lower limit of the measuring interval (LLMI).

Matrix effects and specificity: A neat solution of primidone (10 ng/mL in mobile phase A) was injected at a constant flow rate of 7 mL/min in a post-column infusion setup to establish a stable analyte background signal. Processed matrix samples (native human serum, surrogate serum, and native plasma [Li-heparin, K_2-EDTA, and K_3-EDTA]) were then injected to assess the variation of the background signal. Any change in the MRM analyte signals at the expected retention time would imply a matrix component-mediated effect on the ionization yield of the primidone transitions.

Additionally, calibrators 1–8 were prepared in the following matrices: neat solution (mobile phase A), native human serum, TDM-free human serum, and Li-Heparin plasma. The mean slopes (n=6, sample preparation) of the calibration curves and the coefficients of determination were evaluated [33]. To exclude a matrix effect (ME), the confidence intervals (CI) of the slopes must overlap and the coefficients of determination must be ≥0.99. In addition, the calibrator neat solution was set as standard and the calibrator samples in surrogate matrix, native serum, and plasma were examined as controls. Recoveries were reported as the percentage of recovery of the measured concentration relative to the nominal concentration.

A comparison of absolute peak areas of analyte and ISTD was performed to further demonstrate the matrix independence of the method [34]. Therefore, analyte and ISTD were spiked after protein precipitation for three levels (2.00, 7.50 and 25.0 µg/mL) in four matrices (neat, native human serum, surrogate serum, and Li-heparin plasma). Ion enhancement (values>100 %) and suppression (values<100 %) were evaluated by comparing the peak areas of analyte and ISTD of the matrix samples to the neat samples, and the percentage variance should not exceed 10 %.

Linearity: The linearity of the assay was evaluated over an extended calibration range of 20 % at the lower and upper end of the range (0.120–36.0 µg/mL). For each calibration curve (n=6, sample preparations), the coefficients of determination and the residuals were determined, which had to be ≥0.999 and randomly distributed, respectively.

In addition, the linearity of the method was proven based on the recovery of serially diluted samples. Sample levels were prepared as follows: calibrator level 1 was sample 1 and calibrator level 8 was sample 11. Using these two samples nine dilutions were prepared as follows: 9+1 (v/v), 8+2 (v/v), 7+3 (v/v), 6+4 (v/v), 5+5 (v/v), 4+6 (v/v), 3+7 (v/v), 2+8 (v/v) and 1+9 (v/v). The results of the measurements must exhibit a linear relationship with a coefficient of determination of ≥0.999. Recovery was expressed as a percentage of the measured concentration recovered relative to the nominal concentration of the sample pools.

Lower limit of the measuring interval and limit of detection: Spiked native human serum samples with analyte concentration at the LLMI were prepared and precision and accuracy evaluated. The LLMI corresponded to the lowest calibrator level (0.150 µg/mL). The sample preparation was replicated five times to assess recovery, bias, and precision, which were required to be within the range of the accuracy and precision experiment.

To estimate the limit of detection (LOD), the approach of Armbruster et al. was used. Therefore, the limit of blank was calculated using 10 independent matrix blank samples as follows: $LOB = mean_{blank} + 1.645(SD_{blank})$. The LOD was then estimated using 10 replicates of calibrator level 1, which was used as the low concentration sample: $LOD = LOB + 1.645(SD_{low\ concentration\ sample})$ [35].

Precision and accuracy: To assess the precision of the established method, a five-day validation experiment was carried out. An ANOVA-based variance component analysis was used to evaluate total method variability, including variability components such as between-injection variability, between-preparation variability, between-calibration variability, and between-day variability. Four spiked serum and Li-heparin plasma levels (0.200, 2.00, 7.50 and 25.0 µg/mL) covering the measurement range and two native patient serum sample pools were prepared in triplicate and injected twice. This scheme was performed by two individuals in parallel, defined as validation part A and B, over 5 days (n=12 measurements per day, n=60 measurements per 5 days). For each part and day, independent calibration curves were generated and used for quantitative analysis. To further vary conditions, two batches of columns and two different sample weightings were used for these measurements. Biowarp, an internal statistic program based on the VCA Roche Open-Source software package in R [36], was used to evaluate the data.

Four spiked human serum and plasma samples with concentrations of 0.200, 2.00, 7.50, and 25.0 µg/mL were used to assess the trueness of the candidate RMP. Dilution integrity was evaluated using two spiked native human serum samples at concentrations of 35.0 and 50.0 µg/mL (dilution 1 and 2). All samples were prepared in triplicates for each part A and part B (n=6, sample preparations) on one 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.

Sample stability: After 10 days, the stability of the processed samples stored at 7 °C on the autosampler was investigated. The stability of matrix-based calibrator and control material stored at −20 °C was evaluated after 33 days. Recoveries were calculated by comparing the measured value with freshly prepared native serum samples. The Total Error (TE) was employed as an acceptance criterion and was estimated using the precision and trueness experiment findings, yielding a maximal TE of 5 %. Stability can be ensured for a measurement interval of 2–28 days (x) for x−1 day, and for a measurement interval of >4 weeks (y) for y−1 week.

Equivalence of results between independent laboratories: A method comparison study was performed to evaluate the agreement of the RMP between two independent laboratories (Site 1: Dr. Risch, Ostschweiz AG, Buchs SG and site 2: Roche Diagnostics GmbH, Penzberg). For this purpose, 82 native samples, including 72 native serum patient samples and 10 native patient pool samples were measured. Additionally, a three-day precision experiment was performed at site 2 using the same samples as previously used by site 1. The RMP was transferred to site 2 and system setup was applied as described in the Supplemental Material 1 with some modifications: an ultra-microbalance XP6U/M (Mettler Toledo) and aluminum weighing boats were used for the preparation of stock solutions. Calibrator levels were independently prepared in both laboratories using the qNMR characterized material.

Uncertainty of measurements: Measurement uncertainty was determined according to the GUM [32] and Taibon et al. [37] and considered following step: purity of the qNMR characterized reference material, weighing of the analyte, preparation of stock, working, spike and calibrator solutions, preparation of the ISTD solution, sample preparation of the calibrators, measurement of the calibrators and generation of the calibration curve, preparation and measurement of 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 whereas all other aspects were evaluated as type A evaluation. The total measurement uncertainty of the whole approach was estimated as a combination of type A and type B uncertainties. For further information see Supplemental Material 3.

Results

Traceability to SI units

Traceability to the SI unit of mass (kilogram), the most important parameter for a reference measurement method, has been established by the utilization of 1,3,5-trimethoxybezene as a qNMR internal standard which is directly traceable to the primary qNMR standard NIST PS1. Furthermore, traceability to the SI unit of amount of substance (mole) is also included owing to referencing to NIST PS1. Since Planck's constant is the main parameter for defining kilogram and mole, qNMR methodology is best suited to fulfill both the traceability chains. Additionally, as per the latest IUPAC Technical Report, qNMR has been stated as a potential primary reference measurement procedure ideally suited for the characterization of primary reference materials [22]. Six individual experiments (Table 1 and Figure 2 of Supplemental Material 2), involving six individual weightings of the analyte and 1,3,5-trimethoxybenzene (qNMR ISTD) yield a final content value of 99.8 ± 0.1 % (k=1). The expanded uncertainty (k=2) can be obtained by multiplying the above-mentioned uncertainty by a factor of 2.

Selectivity

Baseline separation of primidone and its metabolite phenobarbital was achieved using reversed phase chromatography with an Agilent Zorbax Eclipse XDB-C18 column and a gradient elution scheme. In this setting a retention time of 3.8 min was realized for primidone. To evaluate the chromatographic selectivity towards phenobarbital, a polarity switch was used since primidone is detected in positive ESI mode and its metabolite phenobarbital in negative ESI mode. Primidone and phenobarbital were spiked at a concentration of 1.50 µg/mL into native human serum, surrogate serum, and Li-heparin plasma. As shown in Figure 2, the phenobarbital signal is well separated resulting in a chromatographic resolution (R) greater than 5.3 for all matrices tested. Evaluation of the primidone quantifier and qualifier transitions showed no interfering signals in the expected retention time window of primidone (3.8 min), neither in analysis-free matrices nor in native patient samples measured in the method comparison study (see Figure 3). In addition, no residual unlabeled analyte was observed in the ISTD [$^2H_5$]-primidone.

Matrix effect and specificity

The possible occurrence of matrix-dependent effects by salts, proteins and phospholipids was minimized by diluting the sample after protein precipitation. The post-column infusion experiment showed that there was no visible matrix-related effect on the ionization of the primidone quantifier and qualifier transitions when processed matrix samples were injected.

To further demonstrate the absence of MEs, mean slopes and coefficients of determination of calibration series in different matrices (native serum, surrogate serum, and Li-heparin plasma) were compared with calibrations in neat solution. The slopes were found to be 0.538 (95 % CI 0.531 to 0.545) for neat solution, 0.543 (95 % CI 0.539 to 0.547) for native serum, 0.550 (95 % CI 0.545 to 0.554) for surrogate serum and 0.538 (95 % CI 0.534 to 0.542) for native Li-heparin plasma. The coefficient of determination was 1.00 independent of the matrix used for calibration. Thus, based on the similar slopes, the overlapping 95 % CI of the slopes, and the coefficients of determination it can be confirmed that no ME is present. Additionally, calibrator levels 1–8 in native human serum and Li-heparin plasma (n=6, sample preparations) were evaluated by applying the neat calibration as a standard. The mean relative bias across all concentrations ranged from −3.4 to 3.7 %, with a CV of ≤5.3 %.

Additionally, the absolute peak areas of the analyte and ISTD and the area ratio of matrix samples were compared to

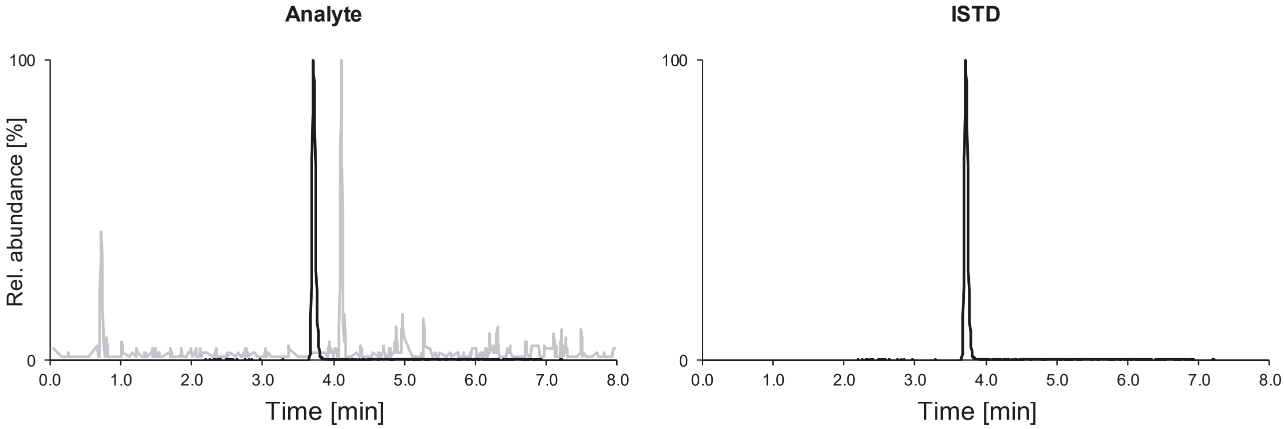

Figure 2: Primidone LC-MS/MS derived analytical readouts. Analyte on the left-hand side, primidone ISTD on the right-hand side. Chromatogram of primidone (black) and its metabolite phenobarbital (grey) both with concentrations of 1.50 µg/mL in native serum. ISTD, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

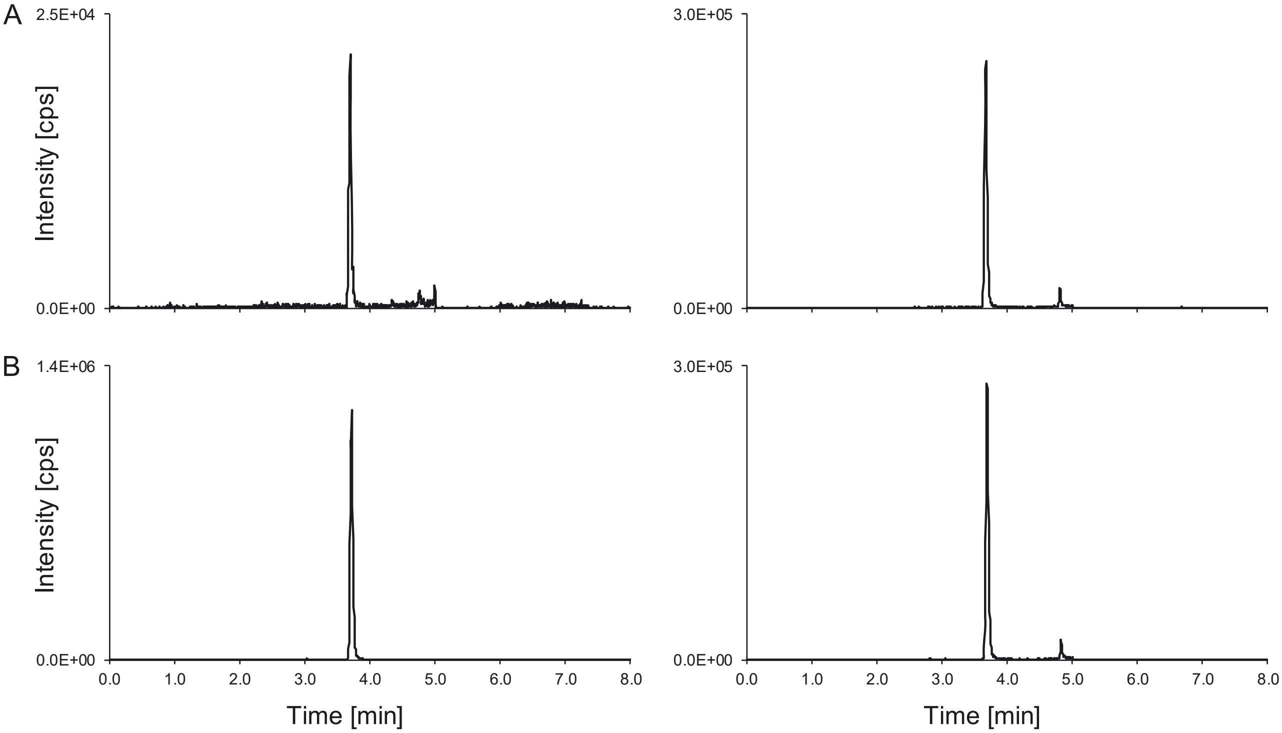

Figure 3: Primidone LC-MS/MS derived analytical readouts. Analyte on the left-hand side, primidone ISTD on the right-hand side. (A) The lowest calibrator level peak (0.150 µg/mL) in native serum; (B) patient pool (n>5, 5.66 µg/mL). ISTD, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

neat samples. There was no significant ME detected and the mean recoveries ranged from 99 to 102 % for the analyte and from 100 to 103 % for the ISTD independent of the matrix. The mean area ratios were between 97 and 102 % (Table 2).

Linearity

Linearity was determined for an extended range of 20 % at the lower and upper end of the range (0.120–36.0 µg/mL). The residuals were randomly distributed in a linear regression model which was therefore selected for assay calibration. The coefficients of determination of all individual calibrators were equal or better than 0.999. The serially diluted samples showed a linear dependence with a coefficient of determination of 0.999 and relative deviations of the samples ranging from −0.3 to 3.6 % with CVs ≤2.8 %.

Lower limit of the measuring interval and limit of detection

The LLMI of primidone is consistent with the lowest calibrator (0.150 µg/mL) and was evaluated by measuring spiked serum matrix samples. The CV (n=4, sample preparations) was found to be 1.5 % and relative bias (n=4, sample preparations) was 0.8 %. One sample was highlighted as an outlier according to Grubbs F [38] and therefore not considered. In addition, the LOD was estimated to be 0.0620 µg/mL.

Accuracy and precision

For the validation of accuracy and precision, spiked human serum and plasma samples at four concentration levels (0.200, 2.00, 7.50 and 25.0 µg/mL) were prepared.

Precision was evaluated with a multi-day validation experiment. Each concentration level was prepared sixfold by two different operators on five different days (n=12 measurements per day and n=60 measurements per 5 days). To estimate the overall variability of the method, various sources including between injections, preparations, calibrations, and days were determined using an ANOVA-based variance component analysis. Intermediate precision was found to be less than 2.5 % for all levels and matrices except for the lowest level in serum which was found to be ≤4.0 %. The repeatability CV was found to be ≤3.3 % for the lowest concentration level in serum and ranged from 1.0 to 1.8 % across all other concentration levels regardless of the matrix type (Table 3).

Table 2: Matrix effect (ME) data of three different matrices compared to neat analyte solutions. Analyte peak areas, ISTD peak areas, and analyte/ISTD area ratios as used in analyte quantification were investigated. Means from five-fold 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.

Primidone level, conc.		**Analyte**		**ISTD**		**Ratio**	
		Mean, %	**95 % CI, %**	**Mean, %**	**95 % CI, %**	**Mean, %**	**95 % CI, %**
Level 1	Natives serum	102	97–107	103	101–105	99	95–103
2.00 µg/mL	Surrogate serum	99	96–102	102	100–104	97	94–101
	Native plasma	101	99–104	103	101–105	99	96–101
Level 2	Natives serum	100	99–100	100	98–101	100	99–102
7.50 µg/mL	Surrogate serum	101	100–102	100	99–101	101	99–103
	Native plasma	102	100–104	103	102–105	99	98–101
Level 3	Natives serum	102	101–104	100	99–102	102	100–103
25.0 µg/mL	Surrogate serum	102	100–104	101	100–102	100	99–102
	Native plasma	102	102–104	102	101–103	101	100–101

CI, confidence interval; ISTD, internal standard; ME, matrix effect.

Table 3: Precision performance parameters for topiramate quantification using the candidate RMP (n=60 measurements).

Variance source	**Serum samples CV, %**					
	Level 1 0.200 µg/mL	**Level 2 2.00 µg/mL**	**Level 3 7.50 µg/mL**	**Level 4 25.0 µg/mL**	**Patient sample 1 8.06 µg/mL**	**Patient sample 2 5.66 µg/mL**
Intermediate precision	**4.0**	**1.4**	**1.4**	**2.0**	**1.2**	**1.9**
Between-day	0.0	0.2	0.4	0.9	0.6	0.9
Between-calibration	2.3	0.7	0.3	0.5	0.0	0.9
Repeatability	**3.3**	**1.2**	**1.3**	**1.7**	**1.1**	**1.4**
Between-preparation	3.0	0.0	0.9	1.3	0.6	1.1

Variance source	**Plasma samples CV, %**			
	Level 1 0.200 µg/mL	**Level 2 2.00 µg/mL**	**Level 3 7.50 µg/mL**	**Level 4 25.0 µg/mL**
Intermediate precision	**2.5**	**1.8**	**1.3**	**1.4**
Between-day	0.0	0.0	0.8	0.8
Between-calibration	1.8	0.9	0.2	0.6
Repeatability	**1.8**	**1.6**	**1.0**	**1.0**
Between-preparation	0.0	1.4	0.4	0.4
Between-injection	1.8	0.7	1.0	0.9

CV, coefficient of variation; RMP, reference measurement procedure. Conversion factor µg/mL to µmol/L: 4.6. The coefficients of variation for repeatability and intermediate precision, which were determined from the individual variances, are printed in bold.

To evaluate the trueness of the method, spiked native serum and plasma samples were evaluated. The mean bias (n=6, sample preparations) within the measurement range (0.200, 2.00, 7.50 and 25.0 µg/mL) was between 0.1 and 3.9 % for serum levels and between −2.6 and 2.8 % for plasma levels. The bias of two high-concentration levels (35.0 and 50.0 µg/mL), which were diluted with native human serum before sample preparation, ranged from −1.8 to 1.9 % (Table 4).

Stability

The stability of processed samples was assessed by re-analyzing stored samples with freshly prepared serum samples. Autosampler stability at 7 °C was demonstrated for 9 days, with calculated recoveries ranging from 98 to 104 %. Moreover, the stability of spiked frozen serum calibrators and QC levels stored at −20 °C was examined after 33 days by comparing them to freshly prepared serum calibrators. The samples showed a recovery between 97 and 103 %.

Equivalence of results between independent laboratories

To demonstrate the equivalence between two independent laboratories, 82 samples (native serum patient samples,

Table 4: Bias and 95 % CI of native human serum and native Li-heparin plasma samples (n=6). The mean bias and corresponding confidence intervals were calculated using the individual sample biases of n=6 preparations.

Concentration, µg/mL	Native serum		Native plasma	
	Mean bias, %	95 % CI, %	Mean bias, %	95 % CI, %
Level 1 0.200 µg/mL	3.9	2.6 to 5.1$_1$	2.8	1.2 to 4.4
Level 2 2.00 µg/mL	0.1	−0.3 to 0.4	−2.4	−3.0 to −1.8
Level 3 7.50 µg/mL	0.5	−1.6 to 2.6	−1.8	−2.5 to −1.2
Level 4 25.0 µg/mL	0.7	−1.7 to 3.1	−2.6	−3.2 to −2.0
Dilution 1 35.0 µg/mL	−1.8	−2.5 to −1.1		
Dilution 2 50.0 µg/mL	1.9	−1.0 to 4.8		

CI, confidence interval. Conversion factor µg/mL to µmol/L: 4.6.

patient pools and samples) were analyzed at site 1 and 2. Two samples were excluded from the analysis as they were below the calibration range. The Passing-Bablok showed a regression equation with a slope of 1.01 (95 % CI 1.00 to 1.01), an intercept of −0.02 (95 % CI −0.07 to 0.01) and a correlation coefficient of 1.00, indicating an excellent agreement between the two laboratories (Figure 4A). The Bland-Altman analysis showed a mean deviation of 0.6 % (95 % CI interval from 0.2 to 1.0 %) and a data scatter which was independent from the primidone concentration. The 2S agreement was 3.6 % (lower limit CI interval from −3.7 to −2.3 %, upper limit CI interval from 3.5 to 4.9 %) (Figure 4B). The precision experiment at site 2 yielded good CVs for repeatability and intermediate precision (n=36) of ≤1.5 and ≤3.0 %, respectively, regardless of concentration level and matrix. These results show that the method is transferable and meets the requirements independent of the laboratory equipment or personnel.

Uncertainty of results

The total measurement uncertainty for single measurements of serum samples was found to be less than 2.1 %, except for the lowest level where it was 4.0 %. It was further reduced for the target value assignment by repeated measurements (n=6, three measurements on two days) where it ranged from 0.9 to 1.0 % regardless of the concentration level and sample type (Tables 5 and 6). The derived total uncertainty is multiplied by a coverage factor of k=2 to obtain an expanded uncertainty.

Discussion

The presented RMP candidate based on ID-LC-MS/MS allows the measurement of primidone levels in human serum and plasma. The method design based on routine measurements

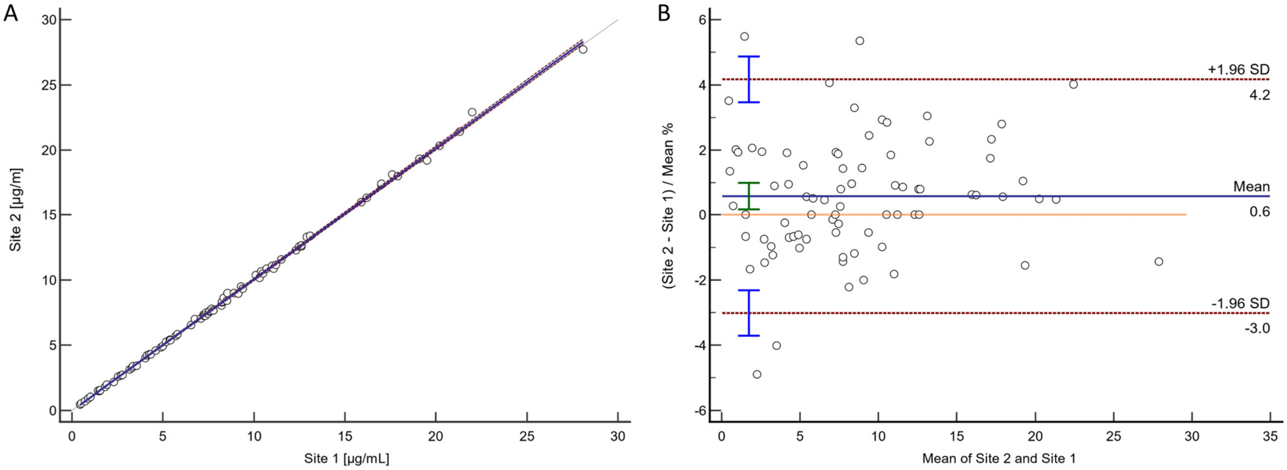

Figure 4: Results from the patient sample-based primidone method comparison study performed between two independent laboratories. (A) Passing-Bablok regression plot including the Pearson regression analysis for the method comparison study of the RMP (n=110 patients) between the independent laboratories (site 1: Risch; site 2: Roche). Passing-Bablok regression analysis resulted in a regression equation with a slope of 1.01 (95 % CI 1.00–1.01) and an intercept of −0.02 (95 % CI −0.07 to 0.01). The Pearson correlation value was ≥999. (B) Bland-Altman plot for the method comparison study of the RMP (n=80, patient samples and patient pool samples) between two independent laboratories (Laboratory 1: Risch site, and Laboratory 2: Roche site). The inter-laboratory measurement bias was 0.6 % (95 % CI interval from 0.2 to 1.0 %) and the 2S interval of the relative difference was 3.6 % (lower limit CI interval from −3.7 to −2.3 %, upper limit CI interval from 3.5 to 4.9 %). CI, confidence interval; RMP, reference measurement procedure; SD, standard deviation.

Table 5: Overview of measurement uncertainty for primidone quantification with the candidate RMP in serum samples for single measurements. For a better readability and interpretability, the determined uncertainties are expressed as CVs.

	Level					
	Level 1 0.200 µg/mL	**Level 2 2.00 µg/mL**	**Level 3 7.50 µg/mL**	**Level 4 25.0 µg/mL**	**Patient sample 1 8.06 µg/mL**	**Patient sample 2 5.66 µg/mL**
Type B uncertainty: calibrator preparation, CV, %	**0.82**	**0.85**	**0.76**	**0.76**	**0.76**	**0.76**
Characterization of reference material	0.10	0.10	0.10	0.10	0.10	0.10
Preparation of						
Stock solution	0.25	0.25	0.25	0.25	0.25	0.25
Working solution	0.45	0.45				
Spike solution	0.54	0.59	0.45	0.45	0.45	0.45
Matrix-based calibrator	0.82	0.85	0.76	0.76	0.76	0.76
Type A uncertainty: intermediate precision, CV, %	**4.0**	**1.4**	**1.4**	**2.0**	**1.2**	**1.9**
Measurement uncertainty (k=1), CV, %	**4.1**	**1.6**	**1.6**	**2.1**	**1.5**	**2.1**
Expanded measurement uncertainty (k=2), CV, %	**8.1**	**3.2**	**3.2**	**4.2**	**2.9**	**4.2**

CV, coefficient of variation; RMP, reference measurement procedure. Conversion factor µg/mL to µmol/L: 4.6. The 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.

Table 6: Overview of measurement uncertainty for primidone target value assignment (n=6) with the candidate RMP in serum samples. For a better readability and interpretability, the determined uncertainties are expressed as CVs.

	Level					
	Level 1 0.200 µg/mL	**Level 2 2.00 µg/mL**	**Level 3 7.50 µg/mL**	**Level 4 25.0 µg/mL**	**Patient sample 1 8.06 µg/mL**	**Patient sample 2 5.66 µg/mL**
Type B uncertainty: calibrator preparation, CV, %	**0.82**	**0.85**	**0.76**	**0.76**	**0.76**	**0.76**
Characterization of reference material	0.10	0.10	0.10	0.10	0.10	0.10
Preparation of						
Stock solution	0.25	0.25	0.25	0.25	0.25	0.25
Working solution	0.45	0.45				
Spike solution	0.54	0.59	0.45	0.45	0.45	0.45
Matrix-based calibrator	0.82	0.85	0.76	0.76	0.76	0.76
Type A uncertainty: intermediate precision, CV, %	**0.6**	**0.6**	**0.5**	**0.5**	**0.5**	**0.4**
Measurement uncertainty (k=1), CV, %	**1.0**	**1.0**	**0.9**	**0.9**	**0.9**	**0.9**
Expanded measurement uncertainty (k=2), CV, %	**2.0**	**2.1**	**1.9**	**1.8**	**1.8**	**1.7**

CV, coefficient of variation; RMP, reference measurement procedure. Conversion factor µg/mL to µmol/L: 4.6. The 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.

and targeted therapeutic analyte concentrations in human serum and plasma specimens. To prepare samples for the selected analytical method, tandem MS with ESI and reversed-phase chromatography, protein precipitation followed by dilution was found to be more effective than liquid-liquid extraction or solid-phase extraction.

Since no primary reference material is available, material characterized by qNMR were utilized to calibrate the assay. qNMR characterization allowed an unbroken traceability chain from the RMP to SI units (kg and mole) to be established. Concentrations of calibrator and control solutions were carefully optimized to adequately cover the measurement range sufficiently. The pipetting scheme including the choice of pipet volumes and number of pipetting steps was optimized to minimize the uncertainty contributions. Summarizing, the preparation of calibrator solutions was presented with transparency, including associated uncertainty terms.

Uncertainty contribution of the measurement was reduced by designing a single analyte RMP. Hence, it was ensured that LC-MS/MS instrument settings depending on physiochemical target analyte properties as the MS source parameters and the LC mobile phase compositions were tailored to the specific needs of primidone including mass transition dwell time optimization to improve the number of data points across the recorded chromatography peaks. Optimization of the presented sample preparation included fluid handling, selection of optimal pipettes; protein precipitation with equilibration times and dilution into the linear range of the MS detector – a point to remember when the procedure is transferred to an instrument with different absolute ion yield.

The validation study confirmed that the developed analytical method met the sensitivity, selectivity, and reproducibility requirements for an RMP. In addition, the absence of MEs was evaluated using a calibration slope comparison and an ion yield attenuation experiment. The measurement uncertainty for multiple measurements (n=6) was found to be within the desired target measurement uncertainty of 3.0 % (k=2).

The successful transfer of the method to a second independent laboratory demonstrated that such a transfer can be performed without significant inter-laboratory bias. This indicates that the calibration solution preparation and sample preparation protocols are robust. In addition, the method comparison study showed that the method can process a high volume of patient samples in a relatively short time, making it suitable for taking a leading role in the traceability chain, conducting method comparison studies and resolving problematic routine samples.

Conclusions

We presented a LC-MS/MS-based candidate RMP for the detection of primidone in human serum and plasma. It provides a traceable and reliable platform for the standardization of primidone routine assays and evaluation of clinically relevant samples.

Acknowledgments: We would like to thank Aline Hoffmeister, Monika Kriner, Alexandra Herbik, Marion Deuster and Michael Dedio for their support in selecting and providing samples.

Research ethics: All procedures were in accordance with the Helsinki Declaration. All samples used were exclusively anonymized leftover samples.

Informed consent: Not applicable.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Tobias Schierscher and Lorenz Risch are employees of Dr. Rich Ostschweiz AG. Linda Salzmann, Anja Kobel, Christoph Seger were all employees of Dr. Risch Ostschweiz AG at the time the study was conducted. Judith Taibon, Neeraj Singh, Andrea Geistanger, and Christian Geletneky are all employees of Roche Diagnostics GmbH. Vanessa Fischer was employed at Roche Diagnostics GmbH at the time the study was conducted. 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 (Genussscheine): Judith Taibon, Christian Geletneky, Andrea Geistanger

Research funding: Not declared.

Data availability: The raw data can be obtained on request from the corresponding author.

References

1. Bogue JY, Carrington HC. The evaluation of “mysoline”, a new anticonvulsant drug. Br J Pharmacol Chemother 1953;8:230–6.
2. Handley R, Stewart AS. Mysoline; a new drug in the treatment of epilepsy. Lancet 1952;1:742–4.
3. Pal PK. Guidelines for management of essential tremor. Ann Indian Acad Neurol 2011;14:S25–8.
4. Guan XM, Peroutka SJ. Basic mechanisms of action of drugs used in the treatment of essential tremor. Clin Neuropharmacol 1990;13:210–23.
5. Baumel IP, Gallagher BB, Mattson RH. Phenylethylmalonamide (PEMA). An important metabolite of primidone. Arch Neurol 1972;27:34–41.
6. Koller WC, Vetere-Overfield B. Acute and chronic effects of propranolol and primidone in essential tremor. Neurology 1989;39:1587–8.
7. Bentué-Ferrer D, Verdier MC, Tribut O. Suivi thérapeutique pharmacologique de la primidone et du phénobarbital [Therapeutic drug monitoring of primidone and phenobarbital]. Therapie 2012;67:381–90.
8. DeSilvey DL, Moss AJ. Primidone in the treatment of the long QT syndrome: QT shortening and ventricular arrhythmia suppression. Ann Intern Med 1980;93:53–4.
9. Loukeris K, Mauri D, Pazarlis P. QT length and heart function in primidone hypocalcaemia. Acta Cardiol 2002;57:367–9.
10. O’Suilleabhain P, Dewey RB Jr. Randomized trial comparing primidone initiation schedules for treating essential tremor. Mov Disord 2002;17:382–6.
11. Hiemke C, Bergemann N, Clement HW, Conca A, Deckert J, Domschke K, et al. Consensus guidelines for therapeutic drug monitoring in neuropsychopharmacology: update 2017. Pharmacopsychiatry 2018;51:e1.
12. Patsalos PN, Spencer EP, Berry DJ. Therapeutic drug monitoring of antiepileptic drugs in epilepsy: a 2018 update. Ther Drug Monit 2018;40:526–48.

13. Kuhn J, Knabbe C. Fully validated method for rapid and simultaneous measurement of six antiepileptic drugs in serum and plasma using ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry. Talanta 2013;110:71–80.
14. D'Urso A, Cangemi G, Barco S, Striano P, D'Avolio A, de Grazia U. LC-MS/MS-Based quantification of 9 antiepileptic drugs from a dried sample spot device. Ther Drug Monit 2019;41:331–9.
15. Serralheiro A, Alves G, Fortuna A, Rocha M, Falcão A. First HPLC-UV method for rapid and simultaneous quantification of phenobarbital, primidone, phenytoin, carbamazepine, carbamazepine-10,11-epoxide, 10,11-trans-dihydroxy-10,11-dihydrocarbamazepine, lamotrigine, oxcarbazepine and licarbazepine in human plasma. J Chromatogr, B: Anal Technol Biomed Life Sci 2013;925:1–9.
16. Bureau International des Poids et Mesures. JCTLM database: laboratory medicine and in vitro diagnostics. Available from https://www.jctlmdb.org/#/app/home [Accessed 20 Jul 2023].
17. International Organization for Standardization (ISO). ISO 17511:2020. In vitro diagnostic medical devices-Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples. Geneva, Switzerland: ISO; 2020.
18. Seger C, Kessler A, Taibon J. Establishing metrological traceability for small molecule measurands in laboratory medicine. Clin Chem Lab Med 2023;61:1890–901.
19. Singh N, Taibon J, Pongratz S, Geletneky C. Absolute content determination by quantitative NMR (qNMR) spectroscopy: a curious case of aldosterone. RSC Adv 2021;11:23627–30.
20. Singh N, Taibon J, Pongratz S, Geletneky C. Quantitative NMR (qNMR) spectroscopy based investigation of the absolute content, stability and isomerization of 25-hydroxyvitamin D2/D3 and 24(R),25-dihydroxyvitamin D2 in solution phase. Sci Rep 2022;12:3014.
21. Nelson MA, Waters JF, Toman B, Lang BE, Rück A, Breitruck K, et al. A new realization of SI for organic chemical measurement: NIST PS1 primary standard for quantitative NMR (benzoic acid). Anal Chem 2018;90:10510–17.
22. Westwood S, Lippa K, Shimuzu Y, Lalerle B, Saito T, Duewer D, et al. Methods for the SI-traceable value assignment of the purity of organic compounds (IUPAC Technical Report). Pure Appl Chem 2023;95:1–77.
23. Braga F, Panteghini M. The utility of measurement uncertainty in medical laboratories. Clin Chem Lab Med 2020;58:1407–13.
24. Salzmann L, Spescha T, Singh N, Schierscher T, Bachmann M, Bauland F, et al. An isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS)-based candidate reference measurement procedure (RMP) for the quantification of lamotrigine in human serum and plasma. Clin Chem Lab Med 2023;61:1930–41.
25. Salzmann L, Spescha T, Singh N, Kobel A, Fischer V, Schierscher T, et al. 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. Clin Chem Lab Med 2023;61:1942–54.
26. Kobel A, Schierscher T, Singh N, Salzmann L, Liesch F, Bauland F, et al. An isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS)-based candidate reference measurement procedure for the quantification of levetiracetam in human serum and plasma. Clin Chem Lab Med 2023;61:1967–77.
27. Salzmann L, Wild J, Singh N, Schierscher T, Liesch F, Bauland F, et al. An isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS)-based candidate reference measurement procedure (RMP) for the quantification of gabapentin in human serum and plasma. Clin Chem Lab Med 2023;61:1955–66.
28. Steele BW, Wang E, Palomaki GE, Klee GG, Elin RJ, Soldin SJ, et al. An evaluation of analytic goals for assays of drugs: a college of American pathologists therapeutic drug monitoring survey study. Arch Pathol Lab Med 2001;125:729–35.
29. International Organization for Standardization. ISO 15193:2009. In vitro diagnostic medical devices – measurement of quantities in samples of biological origin – requirements for content and presentation of reference measurement procedures. Geneva, Switzerland: ISO; 2009.
30. Clinical and Laboratory Standards Institute (CLSI). C62-A. Liquid chromatography-mass spectrometry methods; approved guideline. Malvern, PA: CSLI; 2014.
31. European Medicines Agency (EMA). ICH harmonised tripartite guideline. In: Validation of analytical procedures: text and methodology Q2 (R1). Amsterdam, Netherlands: EMA; 1995.
32. Working Group 1 of the Joint Committee for Guides in Metrology (JCGM/WG1). Evaluation of measurement data – Guide to the expression of uncertainty in measurement (GUM), (2008; GUM 1995 with minor corrections). Available from https://www.bipm.org/documents/20126/2071204/JCGM_100_2008_E.pdf/cb0ef43f-baa5-11cf-3f85-4dcd86f77bd6 [Accessed 01 May 2023].
33. Matuszewski BK. Standard line slopes as a measure of a relative matrix effect in quantitative HPLC-MS bioanalysis. J Chromatogr, B: Anal Technol Biomed Life Sci 2006;830:293–300. https://10.1016/j.jchromb.2005.11.009.
34. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 2003;75:3019–30.
35. Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev. 2008;29:S49–52.
36. Schuetzenmeister A, VCA DF. Variance component analysis. Available from https://cran.r-project.org/web/packages/VCA/index.html [Accessed 01 May 2023].
37. Taibon J, Santner T, Singh N, Ibrahim SC, Babitzki G, Köppl D, et al. An isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS)-based candidate reference measurement procedure (RMP) for the quantification of aldosterone in human serum and plasma. Clin Chem Lab Med 2023;61:1902–16.
38. Grubbs F. Sample criteria for testing outlying observations. Ann Math Stat 1950;21:27–58.

Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/cclm-2023-1032).