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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

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Abstract

Objectives: We developed an isotope dilution (ID)-liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based candidate reference measurement procedure (RMP) for lamotrigine in human serum and plasma, using quantitative nuclear magnetic resonance-characterized reference standards to ensure traceability to the International System of Units.
Methods: A sample preparation protocol based on protein precipitation combined with LC-MS/MS analysis using a C18 column for chromatographic separation was established for the quantification of lamotrigine in human serum and plasma. Assay validation was performed according to current guidelines. Spiked serum and plasma samples were used to assess selectivity and specificity; a post-column infusion experiment and comparison of standard line slopes were performed to ascertain possible matrix effects. Precision and accuracy were determined in a 5 days validation experiment. Measurement uncertainty was determined per the Guide to the Expression of Uncertainty in Measurement.
Results: The method allowed the quantification of lamotrigine in serum and plasma in a range of 0.600–24.0 µg/mL without any observable matrix effects. The relative mean bias (n=6) ranged from 1.7 to 3.7%; intermediate precision, including variances in between-day, -calibration, and -injection, was ≤2.4%, independent of the level and matrix. Total measurement uncertainty for a single measurement was ≤2.6%; expanded uncertainty was ≤5.2% (coverage factor k=2).
Conclusions: This candidate RMP based on ID-LC-MS/MS provides a traceable and reliable platform for the standardization of routine assays and the evaluation of clinical samples.

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

Introduction

In clinical chemistry, the establishment of reference methods and traceability chains in accordance with the International Organization for Standardization (ISO) guidance documents ISO 15195 and ISO 17511, respectively, comprises an opportunity to provide consistent standards and best practice without bias contributions between test providers, both intra-individually (e.g., when tracking a medical intervention in a single patient) as well as inter-individually (e.g., across multiple patients in meta-analysis of clinical trials). There is a strong tradition of reference method use in the field of therapeutic drug monitoring (TDM), but this approach is currently limited to only a few analytes. To expand methodological traceability in TDM, the establishment initiative has developed candidate reference methods for several drugs in accordance with ISO 15193 which serves to communicate the reference method candidate for lamotrigine.

Lamotrigine ($C_9H_7Cl_2N_5$, molecular weight = 256.1 Da, conversion factor to molar unit [µmol/L] = 3.9), a chlorinated phenyl-triazine derivative, is an anticonvulsant agent common among antiepileptic drugs. It is used for the treatment of epilepsy and for the prevention of depressive episodes in

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patients with bipolar disorder. Its action is mainly due to the blockade of voltage-gated sodium channels and the associated inhibition of the release of excitatory neurotransmitters such as glutamate and aspartate from the pre-synapse [1–3].

Lamotrigine use is associated with therapeutic trough level ranges. For its application as a mood stabilizer, a commonly used therapeutic range is 1–6 µg/mL, but if used as an anticonvulsant medication, a trough level of 3–15 µg/mL must be targeted [4]. If the effective range is left to higher concentrations, adverse side-effects such as visual disturbances, fatigue, dizziness, headache, gait disturbances, drowsiness, nausea, and diarrhea occur more frequently. In rare cases, severe skin reactions such as Stevens-Johnson syndrome and toxic epidermal necrolysis have been observed. Hence, a laboratory alarm limit of 20 µg/mL is recommended to alert the user of the increased likelihood of adverse reactions occurring [4, 5]. An important challenge for the application of lamotrigine is the significant change in its elimination half-life that occurs with co-medication, with both a slowing (e.g., 45–75 h with co-medication with valproic acid) and acceleration (e.g., 9–14 h with co-medication with carbamazepine) of its metabolism being observed [4]. In addition, dose adaptions must be considered in elderly patients, in patients with renal insufficiency or during pregnancy [4, 6]. Furthermore, dose-related trough concentration deviations have also been observed [7]. Not being able to ascertain drug levels makes dose adjustments challenging. For these reasons, TDM support for the use of lamotrigine in routine clinical practice is considered necessary. Consequently, various assay formats have been developed to date. Besides ligand binding assays, some of the FDA-approved [8–10], chromatographic assays [11–14], and LC-MS/MS [15, 16] are in worldwide clinical routine use.

As mentioned, reference measurement systems improve inter-laboratory measurement accuracy and reduce variability between laboratories and assays [17, 18]. The reference measurement system comprises a reference measurement procedure (RMP) and higher order reference materials. An RMP must be virtually free of systematic errors (i.e., measurement bias). This can be provided by careful analytical operations and using bias-free reference materials. The random error (measurement uncertainty) must be well below the allowable measurement of uncertainty in routine use; as a rule of the thumb, one third of this figure of merit should not be exceeded to allow for a sufficient error propagation reserve [19].

The reference material for a particular analyte must be characterized with respect to its molecular identity and purity, and solutions of this material of defined concentration are used as primary calibrators. These materials are then used for the calibration of a secondary RMP which can be used to assign values to industrial master calibrators, and from there, to routine calibrators used for assessment of analyte concentrations in routine patient samples. According to the ISO guidelines, laboratories wishing to achieve "reference measurement laboratory" accreditation must implement RMPs of a higher order and produce results that are accurate and traceable to national or international reference materials [20]. The non-destructive, primary ratio method of quantitative nuclear magnetic resonance (qNMR) spectroscopy provides a linear response to the amount of analyte and allows direct traceability to the International System of Units (SI) kilogram via qNMR internal standards (ISTDs), delivering an unmatched ability to determine the quantity, or 'count', of a given analyte [21]. The highest order qNMR ISTDs are traceable directly to National Institute of Standards and Technology (NIST) Standard Reference Material for benzoic acid 350b (using acidimetric methods) and/or NIST Primary Standard 1 for benzoic acid (the first primary qNMR standard) [22]. Thus, in addition to its ability to elucidate structures, qNMR can be used to determine the mass fraction (absolute content; g/g) of a calibration standard in a single experiment [23]. Since there is no primary reference material for lamotrigine, we developed an in-house qNMR protocol for target value assignment.

Isotope dilution (ID) liquid chromatograph (LC)-tandem mass spectrometry (MS/MS) is a technique with the potential to accurately measure serum and plasma lamotrigine, as shown by its high capability in small molecule reference method establishment, as a result of its inherent selectivity, sensitivity, and linearity [24]. For lamotrigine, a single analyte ID-LC-MS/MS-based RMP using a combination of off-line solid phase extraction (SPE) and LC-MS/MS utilizing stable isotope internal standards and impurity (mass balance approach) corrected calibrator materials was previously reported by the NIST [25]. That method, listed by the Joint Committee for Traceability in Laboratory Medicine (JCTLM), was established to characterize a reference material (NIST SRM 900a) which is no longer available. For lamotrigine which has been measured without fragmentation, hence without the selectivity of an SRM MS/MS experiment, an expanded uncertainty of 2.3% was reported by the authors. However, neither an inter-laboratory comparison to a second independent laboratory nor intermediate imprecision figures of merit were provided to strengthen that claim.

Consequently, we aimed to develop and validate a novel candidate RMP for lamotrigine that meets the requirements of the ISO 15193 guidelines. We directed our analytical imprecision goal on the therapeutic ranges available for lamotrigine and the desired routine measurement imprecision derived thereof. Following the approaches of Glick, Burnett, or Fraser as outlined by Steele and colleagues for several

drugs in 2001 [26], lamotrigine routine TDM should show a measurement uncertainty better than 8% with the pharmacokinetic model by Fraser leading to significantly lower figures of merit, if the expected half-life time exceeds the dosing interval by several fold. A recent investigation dealing with the inter-laboratory lamotrigine measurement uncertainty in proficiency testing showed that mass spectrometry-based services did not always reach that goal, with an observed uncertainty range of 8–18% [9]. Correcting for the inter-laboratory expansion factor, e.g., by applying the average inter-laboratory/intra-laboratory variance component factor of 0.8, published by Steele [27], an intra-laboratory uncertainty range of approximate 6.5–14.5% can be expected, which is in good agreement with the data derived from approaches based on the therapeutic range or pharmacokinetic consideration.

Thus, if the approach of Braga and Panteghini is to be followed, which grants the reference method only one third of the measurement uncertainty of the routine method [19], the latter must have a measurement uncertainty of greater than ~2.5%, translating to an expanded measurement uncertainty of 5%. It is a paradigmatic imperative that the bias associated with a reference measurement procedure must be nil [28]. Thoroughly and transparently performed reference material characterization ensures that bias terms between RMPs are minimized, and only laboratory comparison studies can allow such estimation.

To facilitate the reproduction of the RMP candidate method by other laboratories, this is described in the supplementary materials including the technical implementation of the test 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 detailed description on the test procedure methodology can be found in the Supplementary 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). Dimethyl sulfoxide (CAS 67-68-5, ACS reagent, ≥99.99%) and ammonium acetate (CAS 631-61-8, LC/MS grade) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Isopropanol (CAS 67-63-0, HPLC grade) was bought from Riedel-de Haën (Seelze, Germany). Water was purified in-house using a Millipore Milli-Q 3 UV system from Merck (Darmstadt, Germany).

Native human serum was obtained from Merck (Cat No. S1-Liter) and TDM-free human serum (surrogate matrix) was obtained from Roche Diagnostics GmbH (ID No. 12095432001, Mannheim, Germany). Native plasma matrix (lithium [Li]-Heparin, K_2-ethylenediaminetetraacetic acid [EDTA], and K_3-EDTA) was obtained from anonymized leftover patient samples. Lamotrigine (CAS 84057-84-1, Cat. No. PHR1392, Lot LRAC3640) was purchased from Sigma-Aldrich; its isotope labelled ISTD [$^{13}C_3D_3$]-lamotrigine (CAS 1246815-13-3, Cat. No. TRCL173253-1 mg, Lot No. 19-SBT-136-3) was purchased from Brunschwig AG (Basel, Switzerland). CD_3OD (CAS 811-98-3, Cat No. 151947), the qNMR internal standard tecnazene (CAS 117-18-0, Cat No. 40384-1G, Lot No. BCBW6288) and NMR tubes (diameter 5 mm, length 8 inches) were purchased from Sigma-Aldrich.

General requirements for laboratory equipment

All equipment used was calibrated and certified by the manufacturer. The minimum sample weight for the microbalance used (XPR2, Mettler Toledo, Columbus, OH, USA) was determined according to the United States Pharmacopeial Convention (USP) guidelines (USP Chapters 41 and 1251). Direct displacement pipettes were used to measure organic solvents and serum. For the preparation of stock and spike solutions, volumetric glassware (Class A volumetric flasks) that fulfilled the requirements of ISO 1042 and USP was used.

qNMR methodology for the determination of absolute content (g/g) of lamotrigine

qNMR measurements were performed on a 600 MHz NMR (Jeol Ltd, Tokyo, Japan) with a He-cooled Cryoprobe. Single pulse-$^1H\{^{13}C\}$ NMR was utilized for the quantitation (proton *ortho* to the chloro-substituent, 1H, Supplementary Figures 1 and 2). The NMR solvent optimal for achieving the required dispersion of the spin systems was methanol-d_4, wherein the chemically and magnetically identical protons were resolved completely allowing a successful quantitation. The identity of the resonances, in addition to the published literature, was confirmed by additional 2D pulse sequences such as TOCSY (Supplementary Figure 3) wherein even long range coupling between the protons can be clearly ascertained. Additional details about NMR acquisition and processing parameters can be found in Supplementary Material 2.

Preparation of calibrators and quality control (QC) samples

The preparation of calibrator and QC levels, as well as the estimation of calibrator level uncertainties (type B uncertainty), was done based on Taibon et al. (manuscript submitted to CCLM, [29]). A detailed description of calibrator preparation, including information on pipettes and volumetric flasks used, and calculation of measurement uncertainty, is given in Supplementary Material 1.

In brief, two individual calibrator stock solutions were prepared and further used for the preparation of spike solutions and the final matrix-based calibrator levels. For each stock solution, 30 mg of lamotrigine was weighed in tin boats on a microbalance (XPR2, Mettler Toledo) and dissolved in 5 mL dimethyl sulfoxide (DMSO) using a volumetric flask to achieve concentrations of 6 mg/mL. The exact concentration of the stock solutions was calculated based on the purity of the reference material (100.0 ± 0.1%, determined by qNMR) and the exact amount weighed.

For the preparation of the eight spike solutions, the produced stock solutions were further diluted by pipetting 500 µL into a 5 mL volumetric flask, which was then filled with DMSO (working solution). Based on

stock and working solutions, the calibrator spike solutions were generated by alternating dilution with DMSO. Final matrix-based calibrators, uniformly distributed from 0.600 to 24.0 µg/mL (see Figure 1), were prepared with a 1 + 99 dilution (v/v) in human serum matrix.

For the preparation of the four levels of matrix-based quality control samples (QC levels), 25 mg of lamotrigine was weighed in tin boats. The levels were then prepared in the same way as described for the calibrator levels, using a third independent stock solution, and resulting spike solutions. The concentrations for the control levels were set at four critical control points: above the limit of quantification, below and within the therapeutic reference range, and at the laboratory alert level [4] (Figure 1). Final concentration levels were 0.900, 2.00, 8.00, and 20.0 µg/mL. Matrix-based calibrators and QC levels were incubated on a thermomixer for 15 min at 37 °C and 500 rpm. All samples were stored at −20 °C for maximum of 28 days.

ISTD solution

For the preparation of the ISTD stock solution, [$^{13}C_3D_3$]-lamotrigine was dissolved in the appropriate amount of DMSO to obtain a 1,000 µg/mL ISTD stock solution and stored at −20 °C until further use. The ISTD working solution was prepared by a twofold dilution of the ISTD stock solution: 95 µL of DMSO was mixed with 5 µL of ISTD stock solution, followed by the addition of 3,900 µL Milli-Q water, to provide a final concentration of 1.25 µg/mL.

Sample preparation

As sample matrix native human serum, TDM-free serum (surrogate matrix) and plasma (Li-Heparin, K_2-EDTA, and K_3-EDTA) can be used. ISTD working solution (100 µL) was pipetted into a 2 mL Safe-Lock Tube (Eppendorf, Hamburg, Germany) and 50 µL of the sample specimen (native sample/calibrator/QC) was added. To ensure an entire ISTD equilibration, the sample was then incubated for 15 min (500 rpm, 37 °C) on a thermomixer (Eppendorf, Hamburg, Germany). Proteins were precipitated by adding 1,000 µL of precipitation solution (75% methanol [v/v]) and shaken on a thermomixer for 10 min (2,000 rpm, 23 °C). The samples were subjected to further centrifugation (4 °C, 20,000×*g*) for 10 min. Subsequently, a two-step dilution was carried out by pipetting 70 µL of the supernatant and 980 µL of mobile phase A (described in the 'Liquid chromatograph-mass spectrometry [LC-MS]' section) into a 1.5 mL Eppendorf tube followed by thorough mixing. This solution was then further diluted 1 + 9 (v/v) using mobile phase A in a HPLC-vial (Wicom, Heppenheim, Germany) to achieve the final sample.

Liquid chromatograph-mass spectrometry (LC-MS)

An Agilent 1290 Infinity II LC system (Santa Clara, CA, USA) equipped with a binary pump, a vacuum degasser, an autosampler, and a tempered column compartment was used for chromatographic separation. Analyte detection was performed using a Triple Quad 6500+ and Q-Trap 6500+ mass spectrometer (AB Sciex, Framingham, MA, USA) with a Turbo V ion source operating in positive electrospray ionization (ESI) mode.

Chromatographic separation of lamotrigine was achieved using an Agilent Zorbax Eclipse XDB-C18 column (100 × 3 mm, 3.5 µm), maintaining 40 °C in the column compartment during measurements. 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).

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 = 1.9 min, 55% A; t = 2.9 min, 55% A; t = 3.8 min, 0% A; t = 6.0 min, 0% A; t = 6.1 min, 100% A; t = 8.0 min, 100% A. A divert valve, switching the eluent flow before 0.8 min and after 5.0 min to the waste, was used to reduce contamination of the mass spectrometer.

Lamotrigine was detected in multiple reaction monitoring (MRM) mode. The ion spray voltage was adjusted to 5,000 V and the temperature was set to 400 °C. Curtain gas, collision gas, ion gas source 1, and ion gas source 2 were set at 45, 11, 50, and 60 psi, respectively. For all mass transitions, a declustering potential of 89 V and a dwell time of 50 ms was applied.

The quantifier transition serves as a basis of the quantitative method and is linked to a correspondent transition of the ISTD. The additional qualifier transition enables the investigation of possible interferences in clinical samples by applying the "branching ratio concept". Table 1 shows an overview of the selected reaction monitoring transitions as well as the remaining compound-dependent MS settings.

System suitability test (SST)

Prior to each analysis, a SST was performed to check the sensitivity, chromatographic performance, and possible carryover effects of the system. SST1 and SST2 concentration levels of lamotrigine corresponded to the final dilutions of calibrator 1 and 8, respectively. The retention time for SST1 and SST2 was required to be within 3.3 min (±0.5 min) to pass the SST. Further, the signal-to-noise ratio of the quantifier transition was required to be ≥10 for SST1. To examine potential carryover effects, the injection of high concentrated sample SST2 was followed by two solvent blanks. The analyte peak area observed in the first blank was required to be ≤20% of the analyte peak area of SST1 (same concentration as calibrator 1) to pass.

Calibration and structure of analytical series

The system was calibrated using the calibrators prepared as described in the 'Preparation of calibrators and quality control (QC) samples' section. Calibration was performed in bracketing mode by measuring the calibrator levels in increasing concentration at the beginning and at the end of the analytical series. A linear regression of the area ratios of lamotrigine and ISTD (y) against the analyte concentration (x) was used to obtain the calibration, resulting in the function, $y = ax + b$. The sequence setup is described in Supplementary Material 1.

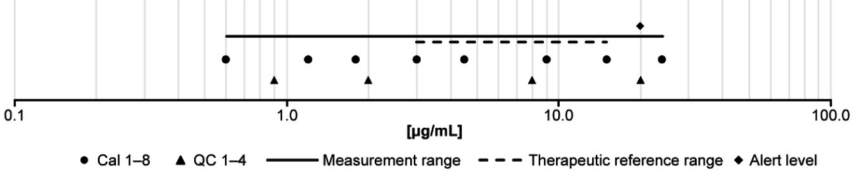

Figure 1: Schematic overview of lamotrigine calibrator and control levels chosen to allow optimal coverage of measurement and therapeutic reference range.

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

Analyte		Precursorion, *m/z*	Production, *m/z*	EP, V	CE, V	CXP, V
Lamotrigine	Quantifier	256.1	211.0	10	35	15
	Qualifier	256.1	145.0	10	55	15
[$^{13}C_3D_3$]-lamotrigine	Quantifier	262.0	217.0	10	35	15
	Qualifier	262.0	148.0	10	55	15

EP, entrance potential; CE, collision energy; CXP, collision exit potential; ISTD, internal standard; MS/MS, tandem mass spectrometry; *m/z*, mass-to-charge ratio; V, volts.

Data processing

Data evaluation was performed using Analyst software, Version 1.6.3, employing the IntelliQuan algorithm (ABSciex). Lamotrigine an d its ISTD showed a retention time of 3.3 min and were integrated within a 30 s window. The noise percentage was set to 90% with a base sub window of 0.5 min. A smoothing factor of 3 and a peak splitting factor of 2 were used for the peak integration.

Method validation

The assay was validated and measurement uncertainty was determined according to the Clinical & Laboratory Standard Institute (CLSI) Guidelines C62A "Liquid Chromatography-Mass Spectrometry Methods", the International Conference on Harmonization guidance document "Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2 (R1)" [30], and the Guide to the expression of uncertainty in measurement (GUM) [31].

Selectivity: Analyte-free matrices (analyte-free human serum [surrogate matrix], native Li-Heparin plasma and in a native human serum pool) were checked at the expected retention time to examine possible interfering matrix signals for lamotrigine and the ISTD [$^{13}C_3D_3$]-lamotrigine quantifier and qualifier transition. In addition, matrices were spiked with the isotope labelled ISTD to evaluate the peak height of residual unlabelled analyte in the stable isotope labelled ISTD. An analyte peak height ≤20% of the peak height of the analyte at the quantitation limit was required.

Specificity and matrix effects: A qualitative post-column infusion experiment, an experiment based on the comparison of standard line slopes [32], and a comparison of absolute areas of analyte and ISTD were performed to determine possible matrix effects.

In the qualitative post-column infusion experiment, a neat solution of the analyte (10 ng/mL lamotrigine in mobile phase A) was infused with a flow rate of 7 µL/min via a T-piece into the HPLC column effluent. The processed matrix sample was injected and the change of the background signal was acquired due to a stable analyte background signal in the MS. To exclude possible matrix effects, native human serum, surrogate serum, and plasma (Li-Heparin, K_2-EDTA, and K_3-EDTA) (all matrix pools assembled from five anonymized individual donors) were analyzed. A matrix component-mediated effect on the ionization yield of the lamotrigine transitions would be indicated by any change (decrease or increase) of the MRM analyte signal at the expected retention time.

A comparison of standard line slopes was performed, comparing calibrator levels 1–8 in the following matrices: neat solution (mobile phase A), native human serum pool, TDM-free human serum, and Li-Heparin plasma. Calibrator levels were prepared as described in the 'Preparation of calibrators and quality control (QC) samples' section. Slopes and coefficients of determination were compared. Therefore, the neat calibration was set as standard, evaluating the calibrator samples in surrogate matrix, native serum, and plasma as controls. Recoveries were reported as the percentage of recovery of the measured concentration relative to the nominal concentration.

A comparison of absolute areas of analyte and ISTD slopes was performed in order to exclude further matrix effects. Analyte and ISTD solution were spiked in the four abovementioned matrices after protein precipitation for three levels spread over the working range (2.00, 8.00, and 20.0 µg/mL). The mean peak areas of analyte and ISTD were compared against the neat samples to evaluate the matrix effect. All samples were prepared in five replicates.

Linearity: The linearity of the method was determined by preparing calibration curves, two preparations on 3 days, as described in the 'Preparation of calibrators and quality control (QC) samples' section. Two additional stock solutions were prepared to obtain serum samples for an extended calibration range of ±20%, with a final concentration of 0.480 and 28.8 µg/mL. The regression model was chosen according to the determined correlation coefficient and residuals for each curve.

The linearity of the method was proven by using the preferred regression model for calculation 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 mixtures were diluted: 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 of the measured concentration relative to the nominal concentration of the sample sets. Measurement results were required to show a linear dependency.

Lower limit of measuring interval (LLMI): Precision and accuracy at the quantitation limit (QL) were determined by measuring spiked serum matrix samples at the expected concentration range of the QL. The QL matches with the lowest calibrator level (0.600 µg/mL). Samples were prepared in five replicates and recovery, bias and precision were determined.

Precision and accuracy: A five-day validation experiment was performed to evaluate precision and accuracy of the developed method, as described previously (manuscript submitted to CCLM, [29]). A variance component analysis (VCA) was used to estimate total method variability and included items such as between-injection variability, between-preparation variability, between-calibration variability, and between-day variability. On each day, four spiked serum and Li-Heparin plasma samples covering the measuring range (0.900, 2.00, 8.00, and 20.0 µg/mL), as well as two

anonymized native patient serum samples, that 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 over 5 days). An independent calibration curve was generated for each part and used for quantitative analysis. Data evaluation was done using Biowarp, an internal statistic program based on the VCA Roche Open Source software package in R [33].

Accuracy was assessed using the same spiked human serum and plasma samples as for the precision experiment. Dilution integrity was performed using two spiked serum samples (lamotrigine-free human serum) at elevated concentration levels of 30.0 µg/mL and 50.0 µg/mL. All samples were prepared in triplicate for part A and part B (n=6 measurements) on one day. Accuracy was reported as the percentage of recovery of the measured concentration related to the spiked concentration.

Sample stability: The samples from the linearity experiment were used to investigate sample stability at 7 °C after 15 days; recoveries were calculated by comparing the measured value with freshly prepared samples. Stability of the matrix-based calibrator and control material stored at −20 °C was evaluated after 29 days; recoveries were calculated by comparing the measured value with freshly prepared samples.

Equivalence of results between independent laboratories: To assess the agreement of the RMP between two independent laboratories (Laboratory 1: Dr. Risch Ostschweiz AG, Buchs SG; and Laboratory 2: Roche Diagnostic GmbH, Penzberg), a method comparison study was performed on 130 native samples provided by Laboratory 2. The samples consisted of native, anonymized serum and plasma patient samples, sample pools, and spiked samples to cover the whole measuring range. All samples were prepared once each and measured over 4 days. A three-day precision experiment was also performed at Laboratory 2 based on the experimental design described in the 'Precision and accuracy' section. All samples needed were provided by Laboratory 1.

The RMP was transferred to Laboratory 2 and system setup was applied as described within the Supplementary Material 1 with the following adaptions: an ultra-microbalance XP6U/M (Mettler Toledo) and aluminium weighing boats were used for the preparation of stock solutions. For the preparation of spike solutions an Eppendorf Multipette® E3/E3x was used.

Uncertainty of measurements: Measurement uncertainty was determined according to the Guide to the Expression of Uncertainty in Measurement (GUM) [34], and evaluated according to the following parameters: (i) uncertainty estimation of qNMR target value assignment of primary reference material; (ii) uncertainty estimation of the preparation of calibrator materials; and (iii) estimation of uncertainty of the LC-MS/MS method (manuscript submitted to CCLM, [29]).

Results and discussion

Traceability to SI units

Since there is no primary reference material available, the characterization and determination of absolute content of lamotrigine was done by qNMR. Therefore, six individual experiments, involving six individual weightings of the analyte and the qNMR internal standard tecnazene were performed. The absolute content of the lamotrigine used within this study was found to be 100.0 ± 0.1% (k=1).

Selectivity

The separation of lamotrigine was reached with the reversed-phase column (Agilent Zorbax Eclipse XDB-C18) combined with the developed gradient (see Figure 2). The chromatogram of the different matrix blanks showed no interfering signals at the respective retention time of the analyte (3.3 min). In addition, no residual unlabelled analyte was observed in the ISTD [$^{13}C_3D_3$]-lamotrigine.

Matrix effects

Possible ion suppression or enhancement effects from different matrices (neat solution, serum, and plasma matrices) were evaluated performing a post column infusion experiment and experiment based on the comparison of standard line slopes. Salts, proteins, and phospholipids can cause matrix-dependent effects, which may have an impact on the analyte signal and the resulting concentration. These effects were reduced by developing a sample preparation protocol employing a high dilution after precipitation. No matrix component-mediated effect on the ionization field of lamotrigine was shown at the expected retention time for serum and plasma matrix (K_2-EDTA plasma, K_3-EDTA plasma, and Li-Heparin plasma).

For further evaluation of matrix effects, slopes, and coefficients of determination of calibrations in different matrices (native serum, surrogate serum, and Li-Heparin plasma) were compared against calibrations in neat solution. Slopes (n=6 sample preparations) were 0.273 (95% CI 0.270–0.276) for the calibration in native serum, 0.278 (95% CI 0.276–0.280) for neat solution, 0.274 (95% CI 0.273–0.276) for surrogate serum, and 0.279 (95% CI 0.278–0.281) for native Li-Heparin plasma. The CIs of the slopes overlap, leading to the assumption that they are not significantly different from each other, supporting the absence of matrix effects. Moreover, mean r^2 values were ≥0.999 independent of the matrix used for calibration. Data for matrix-based calibration samples vs. neat showed a CV (n=6) of ≤3.0% and the mean bias ranged from −5.8 to 2.5% (n=6).

Additionally, matrix effects were evaluated based on previously reported strategies [35]. A comparison between analyte peak areas, ISTD peak areas, and area ratios from spiked matrix samples to spiked neat samples was made (Table 2); matrix effect values of >100% indicate

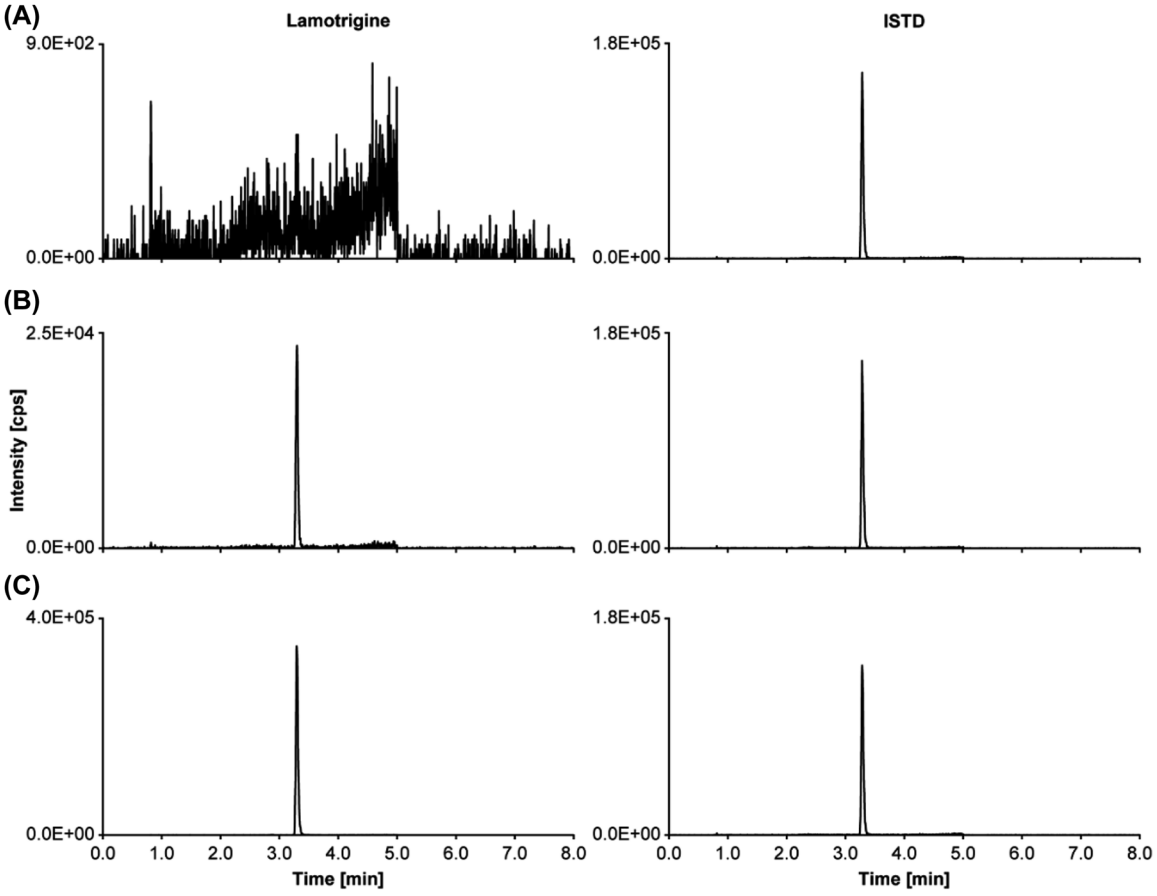

Figure 2: Lamotrigine LC-MS/MS derived analytical readouts. (A) Chromatogram of a matrix blank showing the analyte SRM ion trace (left) and the ISTD SRM ion trace (right); (B) chromatogram of the lowest calibrator level with a concentration of 0.600 µg/mL spiked in native serum; analyte (left) and ISTD (right). (C) Pooled patient sample with a concentration of 9.66 µg/mL; analyte (left) and ISTD (right).

an ionization enhancement and values <100% indicate an ionization suppression. There was no matrix effect observed with values ranging from 90 to 99% for the analyte and from 89 to 101% for the ISTD in all tested matrices. The area ratios were between 99 and 102%. Thus, the compensating effect of the present labelled ISTD is confirmed.

Linearity

Linearity was determined by analyzing six native serum calibration curves which were expanded for ±20% of the measuring range. The residuals were randomly distributed in a linear regression model (see Figure 3), wherefore it was chosen for assay calibration. The correlation coefficients were r≥0.998 for all individual calibration curves.

Furthermore, the linearity of the method was confirmed using serially diluted samples. The measurement results showed a linear dependence with a correlation coefficient of 0.999. The relative deviation of serially diluted samples (n=6 for each level) ranged from –1.1 to 3.5% and CV was determined to be ≤3.6%.

LLMI

The LLMI was determined using spiked samples at the concentration of the lowest calibrator level (0.600 µg/mL) (Figure 2). Relative bias and CV were found to be 1.2 and 1.9%, respectively.

Precision and accuracy

Precision and accuracy were determined using spiked serum and plasma as well as native patient samples. In the absence of certified secondary reference materials, accuracy was assessed using four levels of spiked serum

Table 2: Matrix effect data of three different matrices compared to neat analyte solutions.

Concentration level		Analyte		ISTD		Area ratio	
		Mean, %	95% CI, %	Mean, %	95% CI, %	Mean, %	95% CI, %
2.00 µg/mL	Native serum	90	81–98	89	81–97	100	99–102
	Surrogate serum	90	82–98	89	82–96	101	99–103
	Native plasma	92	84–101	91	83–99	102	99–104
8.00 µg/mL	Native serum	92	84–99	91	83–99	101	100–102
	Surrogate serum	91	84–98	90	83–98	101	100–102
	Native plasma	91	84–98	90	83–97	101	100–102
20.0 µg/mL	Native serum	97	94–99	97	96–99	99	97–101
	Surrogate serum	99	97–102	101	98–103	99	97–100
	Native plasma	99	96–101	100	98–101	99	97–101

Analyte peak areas, ISTD peak areas, and analyte/ISDT area ratios as used in analyte quantification were investigated. Means from five-fold analysis were used as data input. The relative matrix effect ME (%) was calculated as ME (%) = set 2/set 1 × 100, whereby set 2 corresponds to the respective matrix samples and set 1 to the neat samples. No matrix effect is present, if ME (%) = 100.

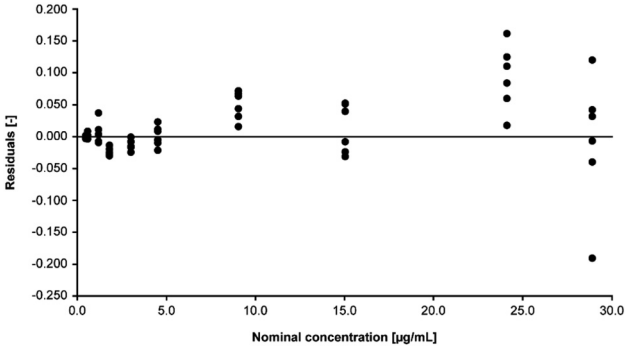

Figure 3: Residuals plot of the calibration model evaluation. A linear model was chosen and six individual sample set preparations were performed to evaluate repeated model performance.

and plasma samples. The relative mean bias (n=6, three preparations of two individual operators) ranged from 1.7 to 3.7% for all levels and matrices showing a slight positive bias (see Table 3). Intermediate precision including variances as between-day, between-calibration, between-preparation, and between-injection was found to be ≤2.4% independent of the matrix. Repeatability CV ranged between 1.2 and 2.3% over all concentration levels (Table 4).

Sample stability

Stability of processed samples (7 °C) was demonstrated for 15 days with recoveries ranging from 95 to 102%. The stability of frozen serum calibrator and control samples (stored at −20 °C) was shown for 28 days. The recovery was found to be within 95 and 102% compared to freshly prepared levels.

Table 3: Bias evaluation results.

Concentration, µg/mL		Bias evaluation results		
		Mean bias, %	SD of bias, %	95% CI of bias, %
Human native serum				
Level 1	0.900	2.5	1.7	1.2–3.8
Level 2	2.00	3.1	2.2	1.3–4.8
Level 3	8.00	1.7	1.1	0.8–2.6
Level 4	20.0	2.0	2.6	−0.1–4.0
Dilution 1	30.0	3.4	1.3	2.4–4.5
Dilution 2	50.0	3.7	2.2	2.0–5.5
Human native Li-heparin plasma				
Level 1	0.900	1.8	1.6	0.5–3.0
Level 2	2.00	2.9	1.3	1.8–3.9
Level 3	8.00	2.4	1.9	0.9–3.9
Level 4	20.0	2.8	0.9	2.1–3.5

Measurements were performed in triplicate within part A and part B (n=6 measurements). Reported are the mean bias, the standard deviation (SD) of the bias, as well as the respective confidence interval of the bias (CI).

Equivalence of results between independent laboratories

The method comparison study was performed on 130 native anonymized patient samples. Fourteen samples were not considered for further analysis: nine were below the limit of quantification, one sample was above the calibration range, three samples were identified as outliers by application of the generalized extreme Studentized deviate test [36], and one sample was excluded due to a pipetting error within one laboratory. Unfortunately, a re-analysis was of this sample not possible as there was no additional volume left. Passing-

Table 4: Detailed precision performance obtained by variance component analysis' (n=60 measurements).

Sample ID	Concentration, µg/mL	Repeatability				Intermediate precision			
		Serum		Plasma		Serum		Plasma	
		SD, µg/mL	CV, %	SD, µg/mL	CV, %	SD, µg/mL	CV, %	SD, µg/mL	CV, %
Spike level 1	0.900	0.0215	2.3	0.0197	2.1	0.0221	2.4	0.0221	2.4
Spike level 2	2.00	0.0295	1.5	0.0311	1.5	0.0336	1.7	0.0353	1.7
Spike level 3	8.00	0.0965	1.2	0.139	1.7	0.111	1.4	0.178	2.2
Spike level 4	20.0	0.278	1.4	0.332	1.6	0.336	1.6	0.366	1.8
Patient sample 1	2.09	0.0384	1.8	–	–	0.0428	2.0	–	–
Patient sample 2	9.66	0.163	1.7	–	–	0.228	2.4	–	–

CV, coefficient of variation; SD, standard deviation; VCA, variance component analysis.

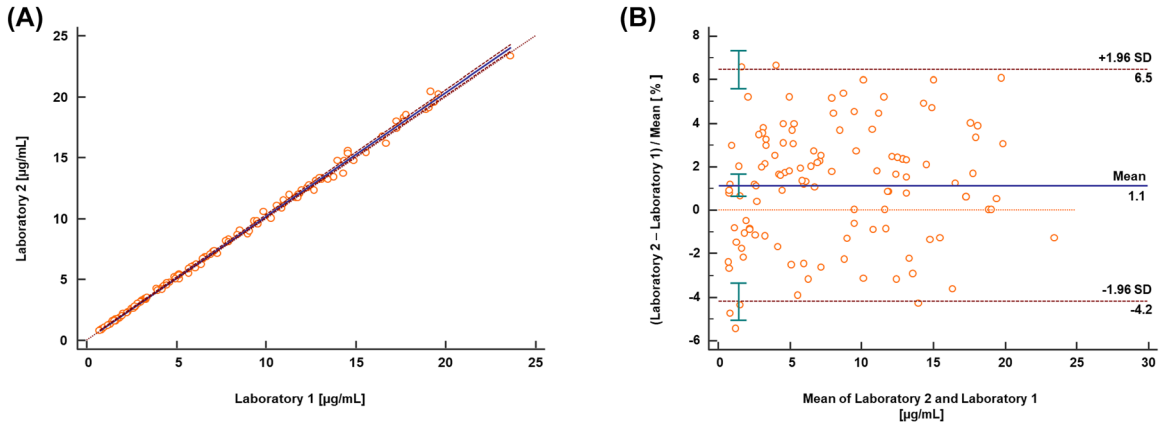

Figure 4: Results from the patient sample-based lamotrigine method comparison study performed between two independent laboratories. (A) Passing–Bablok regression plot including the Pearson regression analysis for the method comparison study of the RMP (n=116 patients) between the independent laboratories (laboratory 1: Risch site, and laboratory 2: Roche site). The regression analysis resulted in a regression equation with a slope of 1.02 (95% CI 1.01–1.03) and an intercept of −0.013 (95% CI −0.043 to 0.023). The Pearson correlation value was ≥0.999. (B) Bland–Altman plot for the method comparison study of the RMP (n=116 patients) between two independent laboratories (laboratory 1: Risch site, and laboratory 2: Roche site). Relative measurement difference, based on the mean of the individual values, are plotted against the mean of the individual measurement values ("relative Bland-Altman plot"). The inter-laboratory measurement bias was +1.1% (95% CI interval from 0.6 to 1.6%) and the 2 S interval of the relative difference was 5.4% (lower limit CI interval from −5.0 to −3.3%, upper limit CI interval from 5.6 to 7.3%).

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

	Level					
	Spike level 1	Spike level 2	Spike level 3	Spike level 4	Patient sample 1	Patient sample 2
Concentration, µg/mL	**0.900**	**2.00**	**8.00**	**20.0**	**2.09**	**9.66**
Type B uncertainty calibrator preparation, CV %	**0.85**	**0.78**	**0.81**	**0.76**	**0.78**	**0.76**
Characterization of reference material	0.1	0.1	0.1	0.1	0.1	0.1
Preparation of						
Stock solution	0.26	0.26	0.26	0.26	0.26	0.26
Working solution	0.46					
Spike solution	0.59	0.48	0.53	0.46	0.48	0.46
Matrix based calibrator	0.85	0.78	0.81	0.76	0.78	0.76
Type an uncertainty intermediate precision, CV %	**2.4**	**1.7**	**1.4**	**1.6**	**2.0**	**2.4**
Total measurement uncertainty, CV %	**2.6**	**1.8**	**1.6**	**1.8**	**2.2**	**2.5**

Bablok regression analysis showed very good agreement between the two laboratories for the remaining samples (n=116) and resulted in a regression equation with a slope of 1.02 (95% CI 1.01–1.03) and an intercept of −0.013 (95% CI −0.043 to 0.023) (Figure 4A). Pearson's correlation coefficient was ≥0.999.

Furthermore, Bland-Altman analysis showed good agreement between the two laboratories (Figure 4B). The data scatter (two standard deviations) between the laboratories was 5.4% (95% CI interval = 1.6%). Since the performance evaluation in the laboratory 2 yielded an intermediate precision comparable to laboratory 1 (better than 2.7%), the found scatter is in good agreement with the error propagation of two independent measurements. The result bias in the patient cohort was +1.1% statistically significantly different from zero (95% CI interval of the bias ranges from 0.6 to 1.6%). It is however in the range to be expected from the uncertainty associated with the production of independent calibrator samples (details see above). Both data scatter and data bias indicate that the proposed lamotrigine RPM is transferable between independent laboratories. Evaluation of precision performance in the laboratory 2 yielded CVs for repeatability and intermediate precision of ≤2.4 and ≤2.7%, respectively. The found data scatter prove the transferability of the method to independent laboratories.

Uncertainty of results

The total measurement uncertainties for lamotrigine for single measurements were evaluated by combining Type A and B uncertainty. It was found to be ≤2.6% independent of the concentration level and the nature of the sample (see Table 5).

Conclusions

The presented ID-LC-MS/MS based candidate RMP allows lamotrigine quantification in human serum and plasma. Its layout is derived from considerations for lamotrigine RMP by LC-MS/MS. Hence, ESI and reverse-phase chromatography were chosen as method of choice. Considering the relatively high concentration of the analyte in serum/plasma, protein precipitation was understood to be advantageous over liquid-liquid extraction or solid-phase extraction processes which introduce additional error terms. All elements of the procedure were carefully evaluated in the design phase of the undertaking, a method prototype was evaluated prior to undergoing method validation. Elements covered by optimization of the LC-MS/MS setup were mobile phase composition and mobile phase gradient, stationary phase selection, and ion source optimization during establishment of the SRM experiment. Elements covered by the optimization of the sample preparation procedure comprised liquid handling procedures including choosing optimal pipettes, protein precipitation regimen including equilibration times, dilution schemes into the linear range of the MS detector, establishment of an optimized calibration and control level scheme, optimized calibrator, and control material production including pipetting.

Since there are currently no primary reference materials for lamotrigine on the market, the use of qNMR to characterize the material used for calibrator preparation is optimal to ensure traceability to SI units. This includes both the identification of possible impurities and the determination of the absolute content of lamotrigine. In addition, the use of a primary reference material or a reference material characterized by qNMR is of utmost importance to keep the measurement bias of an RMP as low as possible.

The validation study presented here showed that the developed procedure meets the reference method requirements for lamotrigine in terms of sensitivity, selectivity, and reproducibility. The method transfer to the second independent laboratory has demonstrated that such a transfer can be achieved without significant increase in inter-laboratory bias. This proves that the production of calibration solutions as well as the sample preparation protocols have been robustly designed. Furthermore, the platform comparison shows that the method is suitable for processing large numbers of patient samples in reasonably short time periods. This gives the users the confidence to use this RMP also for the evaluation of problematic patient samples (e.g., unusual signal positions when using high-performance liquid chromatography-ultraviolet or immunoassay systems). This method thus fulfils both the requirement of taking on a lead role in the traceability chain and the requirement of being able to carry out method comparison studies and review problematic routine samples.

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