Linda Salzmann, Janik Wild, Neeraj Singh, Tobias Schierscher, Franziska Liesch, 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 gabapentin in human serum and plasma

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Abstract

Objectives: To describe and validate a reference measurement procedure (RMP) for gabapentin, employing quantitative nuclear magnetic resonance (qNMR) spectroscopy to determine the absolute content of the standard materials in combination with isotope dilution-liquid chromatograph-tandem mass spectrometry (ID-LC-MS/MS) to accurately measure serum and plasma concentrations.

Methods: A sample preparation protocol based on protein precipitation in combination with LC-MS/MS analysis using a C8 column for chromatographic separation was established for the quantification of gabapentin. Assay validation and determination of measurement uncertainty were performed according to guidance from the Clinical and Laboratory Standards Institute, the International Conference on Harmonization, and the Guide to the expression of uncertainty in measurement. ID-LC-MS/MS parameters evaluated included selectivity, specificity, matrix effects, precision and accuracy, inter-laboratory equivalence, and uncertainty of measurement.

Results: The use of qNMR provided traceability to International System (SI) units. The chromatographic assay was highly selective, allowing baseline separation of gabapentin and the gabapentin-lactam impurity, without observable matrix effects. Variability between injections, preparations, calibrations, and days (intermediate precision) was <2.3%, independent of the matrix, while the coefficient of variation for repeatability was 0.9–2.0% across all concentration levels. The relative mean bias ranged from −0.8–1.0% for serum and plasma samples. Passing-Bablok regression analysis indicated very good inter-laboratory agreement; the slope was 1.00 (95% confidence interval [CI] 0.98 to 1.03) and the intercept was −0.05 (95% CI -0.14 to 0.03). Pearson's correlation coefficient was ≥0.996. Expanded measurement uncertainties for single measurements were found to be ≤5.0% (k=2).

Conclusions: This analytical protocol for gabapentin, utilizing traceable and selective qNMR and ID-LC-MS/MS techniques, allows for the standardization of routine tests and the reliable evaluation of clinical samples.

Keywords: gabapentin; ID-LC-MS/MS; qNMR; reference measurement procedure; standardization; traceability.

Introduction

Establishing reference methods and traceability chains in accordance with ISO guidance documents as ISO 15195 and ISO 17511, respectively, is considered as a possibility to provide both the provider and the user of laboratory results, with confidence to follow and intra- and inter-individual measurement value relationships without bias contributions between test providers. In the field of therapeutic drug level measurements (TDM), currently only a few of several hundred utilized medications have been made traceable to higher order references. To expand methodological traceability in TDM, the authors founded an establishment initiative that has developed candidate reference measurement procedures (RMPs) in accordance with ISO 15193 for several drugs. This work serves to communicate the reference method candidate for gabapentin.

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

Gabapentin ($C_9H_{17}N_1O_2$, molecular weight = 171.2 Da, conversion factor to molar unit [umol/l] = 5.8), as GABA analog an amino acid derivative, has antiepileptic and analgesic properties and is used for the treatment of epilepsy and nerve pain. It acts through binding to voltage-gated calcium channels. It is a widely recognized and commonly used drug with a medical history stretching back almost three decades [1]. First approved as an antiepileptic treatment, gabapentin is currently indicated for use as adjunctive therapy in the treatment of partial onset epileptic seizures and for the management of postherpetic neuralgia in adults [1, 2]. In addition, gabapentin is prescribed off-label for a variety of conditions including pain syndromes [3–5], movement disorders [6], alcohol use disorder [7], and neuropsychiatric disorders [8–10].

The generally accepted therapeutic range for gabapentin in epilepsy is between 2.00 and 20.0 µg/mL (steady state serum trough value), and values exceeding 25.0 µg/mL are associated with toxic side effects such as drowsiness, dizziness, ataxia and viral infections [11, 12]. There is considerable inter-individual variability in the dose required to achieve an optimal response, possibly influenced by dose-related saturable drug absorption [13, 14]. Although therapeutic drug monitoring (TDM) is recommended for gabapentin, it is only routinely implemented for epilepsy [12, 14–16]. However, the optimal therapeutic range in other conditions may differ from that of epilepsy [14, 17], and there are also recent concerns about drug misuse and mortality associated with gabapentin overdose or combination with opioids [11, 18, 19]. Rapid and reliable quantitative determination of gabapentin in human serum or plasma is therefore necessary to ensure appropriate therapeutic doses and to monitor usage, across individuals and indications.

An ongoing challenge for analytical laboratories is to ensure the accuracy of their results for any given measurand, given the variation in methodology, instruments, and reagents used across different sites [20]. Thus, the gold standard for accurate measurement and minimum inter-laboratory variability requires the use of an RMP and higher order reference materials [21]. Liquid chromatograph-tandem mass spectrometry (LC-MS/MS) procedures have, for many years, provided the cornerstone for RMPs in laboratory medicine. However, while multiple LC-MS/MS methods to quantitate gabapentin in human blood have been reported in the literature [22–27] no effort was undertaken to provide an RMP for gabapentin until now.

Herein, we report a novel method for the measurement of gabapentin, using quantitative nuclear magnetic resonance (qNMR) spectroscopy to determine the purity of the standard materials plus isotope-dilution LC-MS/MS (ID-LC-MS/MS) to accurately measure serum and plasma gabapentin concentrations. ID-LC-MS/MS is considered as gold standard and has been proposed by the Joint Committee for Traceability in Laboratory Medicine and the International Federation for Clinical Chemistry and Laboratory Medicine as the key methodology to improve clinical assay standardization [28].

When establishing a reference method, the associated measurement uncertainty must be significantly lower than the allowable uncertainty in routine operation. This figure of merit can be estimated either from the therapeutic range to be monitored or pharmacokinetic consideration-based calculations. Following estimation protocols by Glick, Burnett, or Fraser as outlined in 2001 by Steele and colleagues [29] for several drugs, gabapentin routine TDM measurement uncertainties should not exceed 9% with the therapeutic range derived goals and should stay below approximately 13% if the pharmacokinetic model by Fraser is considered. A recent investigation into the inter-laboratory measurement uncertainty in proficiency testing did show that gabapentin routine services exhibited inter-laboratory uncertainties between 8 and 20% [30]. Correcting for the inter-laboratory expansion factor by applying the average inter-laboratory/intra-laboratory variance component factor of 0.8 [31], this range translates into an intra-laboratory uncertainty range of approximately 6.4–16.0%. This is in good agreement with the data derived from the theoretical therapeutic range or pharmacokinetic modelling approaches.

To allow for error propagation along the traceability chain, Braga and Panteghini recently suggested that an RMP is granted only one third of the measurement uncertainty of the routine method [32]. For gabapentin, this means that a measurement uncertainty below 3% equalling an expanded uncertainty of 6% is a major design goal. Regarding systematic errors, it is a paradigmatic must that the bias associated with a reference measurement procedure must be nil [33]. This can be achieved by thorough and transparent reference material characterization e.g., either by the mass balance approach or by qNMR.

The established non-destructive, primary ratio method of qNMR spectroscopy provides a linear response to the amount of analyte and allows direct traceability to International System (SI) units via qNMR internal standards (ISTDs), which are directly traceable to primary reference material (e.g. National Institute of Standards and Technology [NIST] benzoic acid 350b or NIST PS1 primary standard for qNMR [34]) and can be used to determine the mass fraction (absolute content; g/g) of a calibration standard in a single experiment [35, 36]. Since there is no primary reference material for gabapentin, we developed an in-house qNMR protocol for target value assignment. As such, the

combination of qNMR and ID-LC-MS/MS is ideally suited for application as an RMP for the measurement of gabapentin in human blood samples.

Within the following paragraphs we will therefore describe a novel candidate RMP for gabapentin that meets the requirements of the guidelines ISO 15193. To ease the reproduction of the RMP candidate by other laboratories, it is – in addition to the main body of the paper – described in detail in supplement documents focusing on the technical implementation of the procedure (Supplemental Material 1), the qNMR-based reference material characterization (Supplemental Material 2), and the calculation of measurement uncertainty (Supplemental Material 3).

Materials and methods

A detailed operation procedure including a full list of materials and equipment used as well as a step-by-step instruction for the application of the method is given within the 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). Dimethyl sulfoxide (DMSO; CAS 67-68-5, ACS reagent, ≥99.99%), ammonium acetate (CAS 631-61-8, LC-MS grade), isopropanol (CAS 67-63-0, high-performance liquid chromatography [HPLC] grade) and D_2O (CAS 7789-20-0) were purchased from Sigma-Aldrich (Taufkirchen, Germany).

qNMR ISTD DSS-d6 (TraceSure, Lot Nr. APL6177) was purchased from WAKO FUJIFILM (Neuss, Germany). 5 mm 8-inch NMR tubes were purchased from Sigma-Aldrich and/or Eurisotop GmbH (Hadfield, United Kingdom).

Native human serum was obtained from Merck (Darmstadt, Germany), TDM-free human serum (multi-individual pooled, surrogate matrix) was obtained from Roche Diagnostics GmbH (Mannheim, Germany), native plasma matrix (lithium-heparin [Li-Hep], dipotassium [K2]-ethylene diamine tetraacetic acid [EDTA], and tripotassium [K3]-EDTA) was obtained from anonymized patient samples, and water was purified using a Millipore Milli-Q 3 UV system from Merck. The patient samples were anonymized residual samples, and pools prepared from them, in accordance with the Declaration of Helsinki.

The standard of gabapentin (CAS 60142-96-3, Cat. No. PHR1049, Lot LRAB7794), gabapentin-lactam (CAS 64744-50-9, Cat. No. Y0001281), was purchased from Sigma-Aldrich (Taufkirchen, Germany), and the deuterated ISTD [$^2H_6$]-gabapentin HCl (CAS 1432061-73-8, Art. No. C467, Lot JA-ALS-14-094-P1), was purchased from Alsachim (Illkirch Graffenstaden, France).

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 determination of the purity of the standard material

qNMR measurements were performed on a JEOL 600 MHz NMR with a He-cooled Cryoprobe. Single-pulse-$^1H\{^{13}C\}$NMR (Supplemental Figure 1) was utilized for the quantitation (NH_2CH_2; δ= 2.39 ppm, 2H). Additional details about NMR acquisition and processing parameters can be found in Supplemental 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 executed based on Taibon et al. [37]. A detailed description of the methods used to calculate calibrator level uncertainty can be found in Supplemental Material 3.

In brief, two individual calibrator stock solutions were prepared and further used for the preparation of working and spike solutions. For each stock solution, 50 mg of gabapentin was weighed in tin boats on a microbalance (XPR2, Mettler Toledo) and dissolved in 5 mL 50% methanol in Milli-Q water (v/v) using a volumetric flask to achieve concentrations of 10 mg/mL. The exact concentration of the stock solutions was calculated based on the purity of the reference material (99.6% ± 0.3%, determined by qNMR) and the exact amount weighed.

These primary stock solutions were further diluted with 50% methanol to obtain working solutions with a concentrations of 0.5 mg/mL and 1 mg/mL. Stock and working solutions were used (separately) for further preparation of the eight calibrator spike solutions in 50% methanol (v/v). Final matrix-based calibrators, uniformly distributed from 0.300–48.0 µg/mL (see Figure 1), were prepared with a 1 + 99 dilution (v/v) in human serum matrix.

Four levels of matrix-based QC samples (QC levels) were 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 QC levels were set at four critical control points: above the limit of quantification (0.500 µg/mL), below (1.50 µg/mL) and within (6.00 µg/mL) the therapeutic reference range, and at the laboratory alert level (25.0 µg/mL) [12] (Figure 1). All samples (spiked and native material) were stored at −20 °C for a maximum of 27 days and all liquids were allowed to reach room temperature prior to use.

ISTD solution

For the preparation of the ISTD stock solution [$^2H_6$]-gabapentin HCl was dissolved in the appropriate amount of 50% DMSO in Milli-Q water (v/v) to obtain a 1,000 µg/mL ISTD stock solution and stored at −20 °C until further use. The ISTD working solution was freshly prepared by a twofold dilution of ISTD stock solution: 88 µL 50% DMSO in Milli-Q water (v/v) was mixed with 12 µL of ISTD stock solution, followed by the addition of 3,900 µL Milli-Q water, to achieve a final concentration of 3 µg/mL.

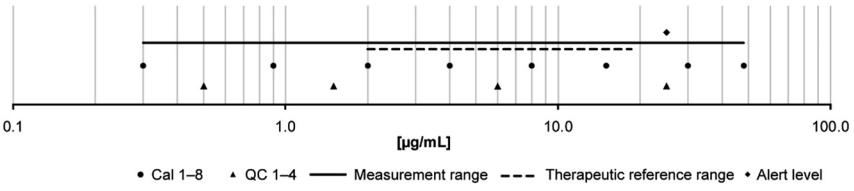

Figure 1: Schematic overview of gabapentin calibrator and control levels chosen to allow optimal coverage of measurement and therapeutic reference range. Cal, calibrator; QC, quality control.

Sample preparation

Native human serum, TDM-free serum (surrogate matrix) and plasma (Li-Hep, K2-EDTA, and K3-EDTA) were used as sample matrix. 100 µL of the ISTD working solution was pipetted 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. To ensure an ISTD equilibration, the sample was mixed briefly on a vortex and then incubated for 15 min (500 rpm, 37 °C) on a thermomixer (Eppendorf). Proteins were precipitated by adding 1,000 µL precipitation solution (75% methanol in Milli-Q water [v/v]), followed by an incubation step on a thermomixer for 10 min (2,000 rpm, 23 °C) and subsequently centrifuged for 10 min at 4 °C and 20,000 rcf. 10 µL of the supernatant was then diluted 1 + 99 (v/v) using mobile phase A in an HPLC vial (Wicom, Heppenheim, Germany).

Liquid chromatograph-mass spectrometry (LC-MS)

Chromatographic separation was performed by an Agilent 1290 Infinity II LC system (Santa Clara, California, USA) equipped with a binary pump, a vacuum degasser, an autosampler at 7 °C, and a column compartment tempered to 40 °C.

An Agilent Zorbax Eclipse XDB-C8 column (100 × 3 mm, 3.5 µm) was used for chromatography as it showed the best peak shapes compared with other reversed-phase columns. 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=4.5 min, 50% A; t=4.6 min, 0% A; t=7 min, 0% A; t=7.1 min, 100% A; t=8.5 min, 100% A. The injection volume was set to 5 µL. A divert valve, switching the eluent flow before 0.8 min and after 5.5 min to the waste, was used to reduce contamination of the mass spectrometer.

Gabapentin was detected in multiple reaction monitoring (MRM) mode using an AB Sciex Triple Quad 6,500+ mass spectrometer (Framingham, Massachusetts, USA), with a Turbo V ion source operating in positive electrospray ionization mode. An ion spray voltage of 5000 V and a temperature of 500 °C were applied. Curtain gas, collision gas, ion gas source 1, and ion gas source 2 were set at 35 psi, 10 psi, 50 psi, and 50 psi, respectively. For all mass transitions, a declustering potential of 57 V and a dwell time of 50 ms were applied. The quantifier transition is the basis of the quantitative method and is associated with a corresponding transition of the ISTD. The additional qualifier allows checking for 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.

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

Analyte		Precursor ion, *m/z*	Product ion, *m/z*	EP, V	CE, V	CXP, V
Gabapentin	Quantifier	172.1	154.1	10	17	15
	Qualifier		137.1	10	26	15
[$^2H_6$]-Gabapentin	Quantifier	178.1	160.1	10	17	15
	Qualifier		143.1	10	26	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, volt.

System suitability test (SST)

An SST was performed prior to each analysis to check the sensitivity of the system, chromatographic performance, and possible carryover effects. Concentration levels of the SST1 and SST2 corresponded to the analyte concentration within the processed calibrator level 1 and 8, respectively.

If the signal-to-noise ratio of the quantifier was ≥10 for SST1 and the retention time for SST1 and SST2 was within 3.6 min (±0.5 min), the SST was considered passed and chromatographic performance was assured. For the investigation of potential carryover effects, the highly concentrated sample SST2 was injected, followed by the injection of two solvent blanks. The analyte peak area at the expected retention time observed in the first blank after the injection of the SST2 sample was required to be ≤20% of the analyte peak area of SST1 (alternatively, 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. Calibrator levels were measured in increasing concentration at the beginning and at the end of the analytical series (bracketing mode) using both calibrations to generate the final calibration function. The final calibration function was obtained by linear regression of the area ratios of the analyte and ISTD (y) against the analyte concentration (x), resulting in the function, $y = ax + b$. The sequence setup is described in Supplemental Material 1.

Data processing

Data evaluation was performed using the Analyst software, Version 1.6.3, employing the IntelliQuant algorithm. Gabapentin and its ISTD

showed a retention time of 3.6 min and were integrated within a 30 s window. For the peak integration, a smoothing and peak splitting factor of 3 were used. The noise percentage was set to 90% with a base sub window of 30 s.

Method validation

Assay validation and determination of measurement uncertainty were performed according to the Clinical and Laboratory Standard Institute Guidelines C62A "Liquid Chromatography-Mass Spectrometry Methods" [38], the International Conference on Harmonization guidance document "Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2 (R1)" [39] and the Guide to the expression of uncertainty in measurement (GUM) [40]. Further details are published in Taibon et al. [37].

Selectivity/specificity: Selectivity was determined by spiking gabapentin, the ISTD [$^2H_6$]-gabapentin HCl, and the relevant impurity gabapentin-lactam in analyte-free human serum and a native human serum pool of five individual donors. The baseline separation of gabapentin and gabapentin-lactam (154.1 $m/z \rightarrow$ 95.1 m/z) was evaluated. To examine possible interfering matrix signals for the analyte quantifier and qualifier transition, an analyte-free human serum pool was checked at the expected retention time. In addition, analyte-free human serum was spiked with deuterated ISTD to evaluate a possible amount of residual unlabeled analyte within the stable isotope labelled ISTD.

Matrix effects: In order to determine possible matrix effects, qualitative post-column infusion, a comparison of standard line slopes [41], and a comparison of absolute areas of analyte and ISTD were performed [42].

In the post-column infusion setting, a neat solution of the analyte (10 ng/mL gabapentin in mobile phase A) was infused with a flow rate of 7 µL/min via a T-piece into the HPLC column effluent prior to entering the MS/MS system to generate a stable analyte background signal. A processed matrix sample was injected and the change in the background signal was measured. Different matrices, such as neat solution (mobile phase A), native human serum, surrogate serum, and plasma (Li-Hep, K2-EDTA, and K3-EDTA) (all matrix pools made up from five individual donors), were analyzed. Any decrease or increase of the MRM analyte signal at the expected retention time would indicate a matrix component-mediated effect on the ionization yield of the gabapentin transitions.

A comparison of standard line slopes was performed comparing the following matrices: neat solution (mobile phase A), native human serum pool, TDM-free human serum, and Li-Hep 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. In addition, the calibrator samples in surrogate matrix, native serum, and plasma were evaluated as controls by applying the neat calibration as standard. 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 also performed. Analyte and ISTD solution were spiked in the four abovementioned matrices after protein precipitation for three levels spread over the working range (1.50 µg/mL, 6.00 µg/mL, and 25.0 µg/mL). The ion enhancement or suppression was evaluated by comparing the peak areas of analyte and ISTD of the matrix samples against the neat samples. All samples were prepared in five replicates.

Linearity: For determination of linearity, calibration curves were prepared in sixfold as described in the 'Preparation of calibrators and quality control (QC) samples' section. Two additional spike solutions were prepared to obtain spiked serum samples for an extended calibration range of ±20%, with final concentrations of 0.240 µg/mL and 60.0 µg/mL. The peak area ratio of analyte to the corresponding ISTD was plotted against the respective analyte concentration (µg/mL). Correlation coefficients and residuals for each curve were determined and the regression model was chosen accordingly. In addition, the linearity of the method was proven on the basis of the recovery of serially diluted samples using the preferred regression model for calculation. 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 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. Measurement results were required to show a linear dependency. Recovery was reported as the percentage of recovery of the measured concentration relative to the nominal concentration of the sample pools.

Lower limit of measuring interval (LLMI): Precision and accuracy at the quantitation limit (QL) were determined by measuring spiked serum matrix samples in the expected concentration range of the QL. The QL matches with the lowest calibrator level (0.602 µg/mL). Samples were prepared in five replicates and recovery, bias and precision determined.

Precision and accuracy: A 5-day validation experiment as described in Taibon et al. [37] was performed to evaluate precision and accuracy of the developed method. Total method variability was estimated using an ANOVA-based variance component analysis including components such as between-injection variability, between-preparation variability, between-calibration variability, and between-day variability. On each day, four spiked serum and Li-Hep plasma samples covering the measuring range (0.500, 1.50, 6.00, and 25.0 µg/mL), as well as two native patient serum samples that were close to the medical decision point (approximately 0.700 and 9.00 µg/mL), were prepared in triplicate for part A and B and injected twice (n=12 measurements per day over 5 days). For each part, an independent calibration curve was generated and used for quantitative analysis. Data evaluation was done with an internal statistic program based on variance component analysis of the VCA Roche Open-Source software package in R [43].

Accuracy was assessed using four spiked human serum and plasma samples (levels 1–4) with the following concentrations: 0.500, 1.50, 6.00, and 25.0 µg/mL. Dilution integrity was performed using two spiked serum samples (gabapentin-free human serum) at concentration levels of 54.0 µg/mL and 60.0 µg/mL (dilution 1 and 2). 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 relative to the spiked concentration.

Sample stability: The stability of the processed samples on the autosampler was investigated at 7 °C after 7 days. For this purpose, calibrator and QC levels from the precision and accuracy assessment described in the 'Precision and accuracy' section were used. Recoveries were calculated by comparing with the measured value of the sample freshly prepared.

Stability of the matrix-based calibrator and control material stored at −20 °C was evaluated after 4 weeks. Recoveries were calculated by comparing with the measured value of the sample freshly prepared.

Equivalence of results between independent laboratories: The agreement of the RMP between two independent laboratories (Laboratory 1: Dr. Risch, Buchs SG; and Laboratory 2: Roche Diagnostics GmbH, Penzberg) was assessed by a method comparison study including 122 native samples provided by Laboratory 2. Samples consisted of native, anonymized serum and plasma patient samples, sample pools, and spiked samples to cover the whole measuring range. A three-day precision experiment was also performed at Laboratory 2 based on the experimental design described in the 'Precision and accuracy' section. Spiked samples were provided by Laboratory 1.

The RMP was transferred to Laboratory 2 and system setup was applied as described in the Supplemental Material 1 with 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 GUM [40] and Taibon et al. [37]; the following parameters were evaluated (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. A detailed description is given within the Supplemental Material 3.

Results and discussion

Traceability to SI units

The use of qNMR-characterized reference standards for calibration of the RMP leads to direct traceability to SI units. Since there are currently no primary reference materials for gabapentin available, traceability was established by using qNMR ISTDs that are directly traceable to primary standards. Therefore, six individual experiments (Supplemental Figure 2 in Supplemental Material 2), involving six individual weightings of the analyte and DSS-d6 were performed yielding a final content value of 99.6 ± 0.3% (coverage factor k=1).

Selectivity/specificity

All elements of the novel method were carefully evaluated during the design phase of the project, and a prototype method was evaluated prior to method validation. The optimization of the LC-MS/MS setup included mobile phase composition and gradient, stationary phase selection, and ion source optimization during the setup of the selected reaction monitoring (SRM) experiment.

Baseline separation of gabapentin and the known interfering compound gabapentin-lactam (see Figure 2) was ensured by the developed gradient combined with the reversed-phase column (Agilent Zorbax Eclipse XDB-C8). The chromatographic resolution (R) was ≥8.5 for all tested matrices, calculated as follows: $R=2 * (t_{R2}-t_{R1})/(w_{b1}+w_{b2})$, where t_{R1} and t_{R2} was the retention time of peaks 1 and 2, respectively, and w_b was the peak width at baseline.

The selectivity was further determined by analyzing sample pools of analyte-free native human serum, surrogate serum, and human plasma (Li-Hep). No signals were detected at the expected retention time. Furthermore, no residual unlabeled analyte was observed in the ISTD [$^2H_6$]-gabapentin.

Matrix effects

To reduce the occurrence of matrix-dependent effects caused by salts, proteins, and phospholipids sample preparation optimization included fluid handling, selection of optimal pipettes, protein precipitation including equilibration times as well as the dilution into the linear range of the MS detector.

The post-column infusion experiment showed no matrix component-mediated effect on the ionization field of gabapentin at the expected retention time for serum and plasma matrix (K2-EDTA plasma, K3-EDTA plasma, and Li-Hep plasma).

For further evaluation of matrix effects, slopes and coefficients of determination of calibrations in different matrices (native serum, surrogate serum, and Li-Hep plasma) were compared against calibrations in neat solution. Slopes (n=6 sample preparations) were found to be 0.253 (95% confidence interval [CI] 0.251 to 0.256) for the native serum matrix, 0.251 (95% CI 0.248 to 0.255) for the neat solution, 0.253 (95% CI 0.251 to 0.256) for the surrogate serum matrix and 0.255 (95% CI 0.251 to 0.258) for the plasma matrix. The CIs of the slopes are overlapping, which leads 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.

Matrix samples (n=6 sample preparations), when calibrated against neat, showed a relative bias for all matrices and levels ranging from −1.2 to 4.2% with CVs of less than 4.4%.

Additionally, matrix effects were evaluated based on Matuszewski et al. [42]. Analyte peak areas, ISTD peak areas, and area ratios from spiked matrix samples were compared with spiked neat samples. Matrix effect values of >100% indicate an ionization enhancement and values <100% indicate an ionization suppression. No matrix effect was observed, with values ranging from 99 to 104% for the

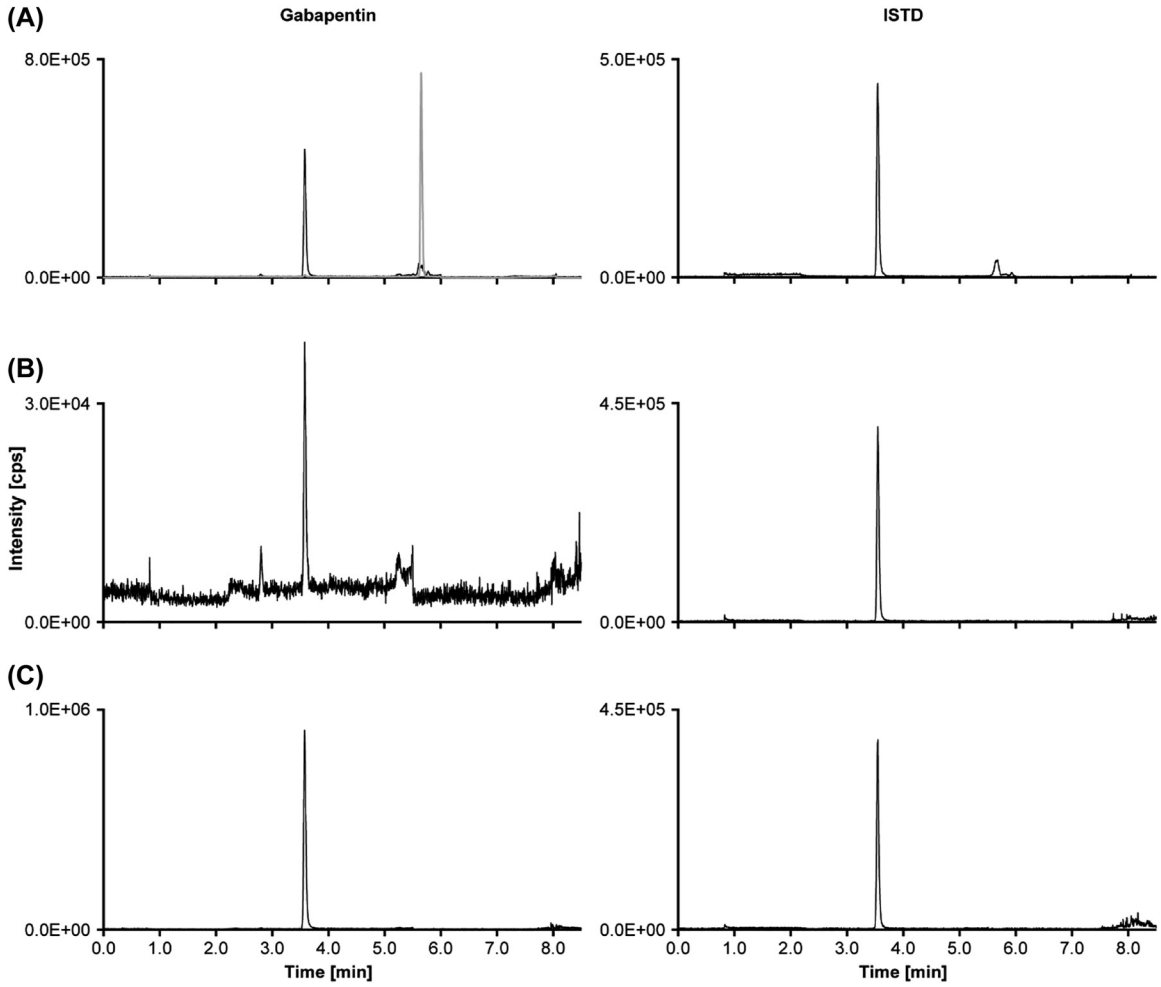

Figure 2: Gabapentin LC-MS/MS derived analytical readouts. Analytes on the left-hand side, gabapentin ISTD always on the right-hand side. (A) Chromatogram of gabapentin (black) and gabapentin-lactam (grey) both with a concentration of 4.00 µg/mL in native serum; (B) lowest gabapentin calibrator peak with a concentration of 0.300 µg/mL in native serum; (C) patient sample pool of five individual patients with a concentration of 9.20 µg/mL. ISTD, internal standard.

analyte and from 103 to 105% for the ISTD in all tested matrices (Table 2). The area ratios were between 96 and 101%. Thus, the compensating effect of the present labelled ISTD is confirmed.

Linearity

Linearity of the method was demonstrated by analyzing calibration curves covering an extended measuring range by ±20% (n=6, sample preparations). Figure 3 shows that residuals were randomly distributed in a linear regression model, thus it was chosen for assay calibration. Correlation coefficients were r ≥0.999 for all individual calibrations. Based on these results, samples 1 to 11 were evaluated and showed a linear dependence with a correlation coefficient of 1.00. The relative deviation ranged from −0.6 to 3.1% and CV was determined to be ≤2.2%.

Lower limit of measuring interval (LLMI)

The LLMI was determined using spiked samples with the concentration of the lowest calibrator level. Relative bias showed a deviation of −2.0% while the CV was found to be 2.6% at a concentration of 0.301 µg/mL.

Precision and accuracy

Precision and accuracy were determined within a multi-day validation experiment using spiked serum and plasma samples, as well as native patient samples.

Table 2: Matrix effect data of three different matrices compared to neat analyte solutions (n=6 sample preparations).

		Analyte		ISTD		Area ratio	
Concentration level		**Mean, %**	**95% CI, %**	**Mean, %**	**95% CI, %**	**Mean, %**	**95% CI, %**
1.50 µg/mL	Native serum	103	101 to 105	103	101 to 105	100	98 to 102
	Surrogate serum	104	102 to 106	103	101 to 105	101	99 to 103
	Native plasma	101	98 to 104	105	103 to 106	96	94 to 99
6.00 µg/mL	Native serum	99	95 to 102	103	100 to 105	96	91 to 101
	Surrogate serum[a]	102	97 to 106	104	103 to 105	98	93 to 102
	Native plasma	101	98 to 105	104	102 to 106	97	93 to 101
25.0 µg/mL	Native serum	101	98 to 103	103	101 to 105	98	94 to 101
	Surrogate serum	102	98 to 105	103	101 to 104	99	96 to 102
	Native plasma	100	96 to 103	103	101 to 104	97	95 to 100

[a]n=4 sample preparations. 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 (%)=set2/set1 x 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.

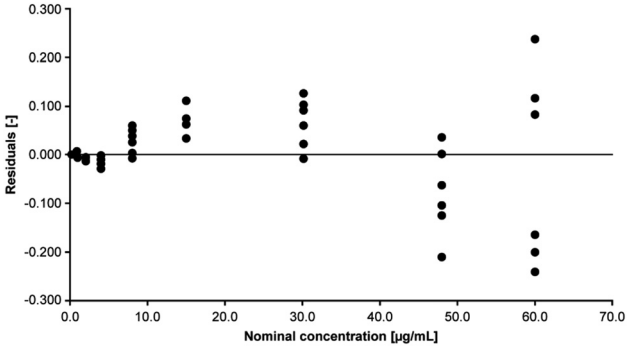

Figure 3: Residuals plot of the calibration model evaluation. A linear model was chosen and six individual sample set preparations were performed to evaluate repeated model performance.

Table 3: Bias and 95% CI of native serum and native Li-Hep plasma samples (n=6).

		Serum		Plasma	
Sample name	**Concentration, µg/mL**	**Mean bias, %**	**95% CI, %**	**Mean bias, %**	**95% CI, %**
Level 1	0.500	−0.8	−2.4 to 0.8	−0.6	−1.1 to −0.1
Level 2	1.50	1.0	−1.0 to 2.9	0.0	−1.4 to 1.5
Level 3	6.00	−0.4	−1.7 to 0.9	0.5	−0.2 to 1.3
Level 4	25.0	−0.1	−0.8 to 0.5	−0.6	−1.7 to 0.6
Dilution 1	54.0	−1.3	−2.1 to −0.5	–	–
Dilution 2	60.0	2.0	−0.1 to 4.1	–	–

CI, confidence interval; Li, lithium. The mean bias and corresponding confidence intervals were calculated using the individual sample biases of n=6 preparations.

Bias was evaluated using three preparations by different operators (n=6). The relative mean bias ranged from −0.8–1.0% for all levels and matrices within the measuring range. Dilution integrity was proven for highly concentrated samples above the measuring range with a bias ranging from −1.3–2.0% (Table 3). The corresponding confidence intervals showed that the results were distributed around the nominal concentration.

Intermediate precision, including variances in between-day -calibration, -preparation and -injection, was found to be less than 2.3% (Table 4), independent of the matrix. Repeatability CV ranged from 0.9–2.0% across all concentration levels (Table 4).

Sample stability

Processed samples were found to be stable at 7 °C for 6 days with recoveries ranging from 99 to 108%. Stability of spiked frozen serum calibrator and QC materials stored at −20 °C was examined after 28 days. Freshly prepared calibrators and QC samples were used to evaluate the stored samples. These were found to be stable for 27 days with recoveries ranging from 96 to 104% compared to the original value.

Equivalence of results between independent laboratories

From the 122 native anonymized patient samples used in the method comparison study, 19 were not considered, as 18 samples were below the limit of quantitation, and one sample was detected as an outlier by the application of the generalized extreme studentized deviate test [44]. Passing-Bablok regression analysis showed very good agreement between the two laboratories and resulted in a regression equation with a slope of 1.00 (95% CI 0.98 to 1.03) and an intercept of −0.05 (95% CI −0.14 to 0.03). Pearson's correlation coefficient was found to be ≥0.996 (Figure 4).

Table 4: Variance component analysis of the gabapentin measurement procedure (n=60 measurements per level).

		Repeatability				Intermediate precision			
		Serum		Plasma		Serum		Plasma	
Sample name	Concentration, µg/mL	SD, µg/mL	CV, %	SD, µg/mL	CV, %	SD, µg/mL	CV, %	SD, µg/mL	CV, %
Spike level 1	0.500	0.00984	2.0	0.00808	1.6	0.0112	2.3	0.00954	1.9
Spike level 2	1.50	0.0227	1.5	0.0253	1.7	0.0232	1.5	0.0253	1.7
Spike level 3	6.00	0.0670	1.1	0.0607	1.0	0.0715	1.2	0.0757	1.3
Spike level 4	25.0	0.253	1.0	0.224	0.9	0.271	1.1	0.261	1.1
Patient sample 1	0.726	0.0135	1.9	–	–	0.0153	2.1	–	–
Patient sample 2	9.20	0.121	1.3	–	–	0.163	1.8	–	–

CV, coefficient of variation; SD, standard deviation.

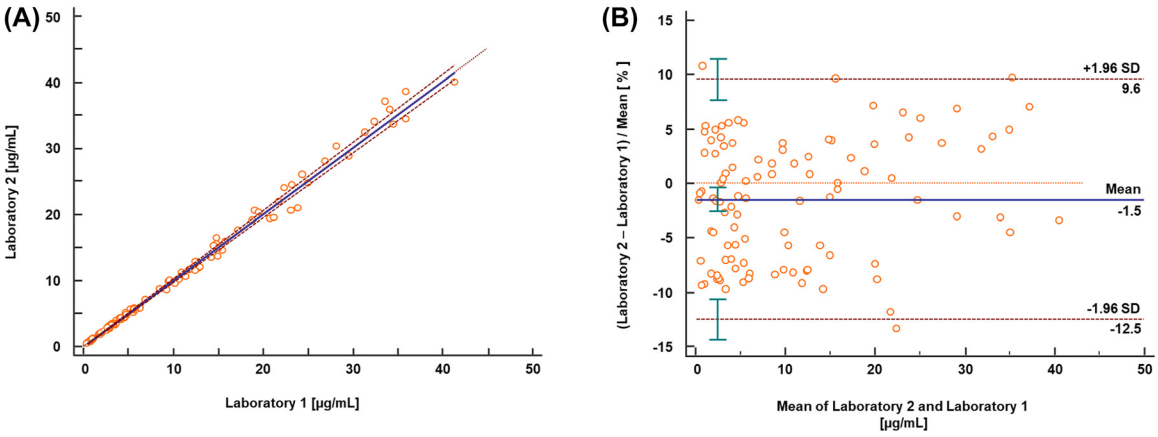

Figure 4: Results from the patient sample-based gabapentin method comparison study performed between two independent laboratories.
(A) Passing–Bablok regression plot including the pearson regression analysis for the method comparison study of the RMP (n=103 patients) between the independent laboratories (Laboratory 1: Risch site, and Laboratory 2: Roche site). The regression analysis resulted in a regression equation y=1.00x−0.05 with a 95% confidence interval (CI) from 0.98 to 1.03 for the slope and from −0.14 to 0.03 for the intercept. The pearson correlation coefficient was 0.996. (B) Bland–Altman plot for the method comparison study of the RMP (n=103 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 −1.5% (95% CI −2.6% to −0.4%) and the ±1.96 SD interval of the relative differences ranged from −12.5 to 9.6% (95% CI 3.8%).

Bland-Altman analysis also showed good agreement between the two laboratories (Figure 4). The data scatter of the relative differences between the laboratories ranged from −12.5 to 9.6 (95% CI 3.8%). The result bias in the patient cohort was −1.5% and statistically significantly different from zero (95% CI interval of the bias range: −0.4% to −2.6%). It is however in the range to be expected from the uncertainty associated with the production of independent calibrator samples. Both data scatter and data bias indicate that the proposed gabapentin RPM is transferable between independent laboratories.

Precision performance evaluated within the second laboratory showed CVs for repeatability and mean precision of ≤2.0 and ≤4.9%, respectively. The variation between-calibration and between-day is higher within laboratory 2 (n=36 measurements per level), resulting in a higher intermediate precision that of laboratory 1 (n=60 measurements per level).

Uncertainty of results

Total measurement uncertainties for single measurements were found to be ≤2.5% (k=1), independent of the concentration level and the nature of the sample (Table 5). To obtain an expanded measurement uncertainty, the derived total uncertainty was multiplied by a coverage factor of k=2, which corresponds to an approximate confidence level of 95% assuming a normal distribution. Determined uncertainties are expressed as CVs for better interpretability.

Table 5: Overview of single measurement uncertainty.

	Sample name					
	Spike level 1	**Spike level 2**	**Spike level 3**	**Spike level 4**	**Patient sample 1**	**Patient sample 2**
Concentration, µg/mL	0.500	1.50	6.00	25.0	0.726	9.20
Type B uncertainty **Calibrator preparation, CV %**	**0.97**	**0.92**	**0.86**	**0.81**	**0.97**	**0.86**
Characterization of reference material	0.30	0.30	0.30	0.30	0.30	0.30
Preparation of:						
Stock solution	0.40	0.40	0.40	0.40	0.40	0.40
Working solution	0.50	0.55			0.50	
Spike solution	0.75	0.69	0.6	0.54	0.74	0.60
Matrix-based calibrator	0.97	0.92	0.86	0.81	0.97	0.86
Type A uncertainty **Intermediate precision, CV %**	**2.3**	**1.5**	**1.2**	**1.1**	**2.1**	**1.8**
Total measurement uncertainty, CV %	**2.5**	**1.8**	**1.5**	**1.4**	**2.3**	**2.0**

CV, coefficient of variation. The total measurement uncertainty of the whole approach for a single measurement estimated as a combination of the uncertainty of calibrator preparation (type B uncertainty) and uncertainty of the precision experiment (type A uncertainty) are given in bold.

Conclusions

The presented ID-LC-MS/MS-based candidate RMP enables the quantification of gabapentin in human serum and plasma. Its design was derived from considerations for routine measurement of gabapentin by LC-MS/MS. Therefore, tandem mass spectrometry with ionization via electrospray (ESI) and reversed-phase chromatography were chosen. Since gabapentin has a relatively high concentration in serum or plasma, protein precipitation followed by dilution was considered advantageous over liquid-liquid extraction or solid-phase extraction.

The presented validation study showed that the developed method meets the requirements for a reference method for gabapentin in terms of accuracy, precision, sensitivity, selectivity and reproducibility. The transfer of the method to a second independent laboratory proved that such a transfer is possible without significant increase in bias between laboratories. This demonstrates that both the established multiple point calibration approach, measured in bracketing mode within the analytical series, and the sample preparation protocols are robustly designed and allow the measurement of low sample volume complaint samples as well as large method comparison studies in a reasonable time frame in addition to the intended use of target value assignment.

Consequently, the method thus fulfills both the requirement to take a leading role in the traceability chain and the requirement to be able to perform method comparison studies and check problematic routine samples.

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