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

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**Abstract**

**Objectives:** To develop an isotope dilution-liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based candidate reference measurement procedure (RMP) for levetiracetam quantification in human serum and plasma.
**Methods:** Quantitative nuclear magnetic resonance spectroscopy (qNMR) was used to characterize the RMP material to ensure traceability to SI units. To quantify levetiracetam, an LC-MS/MS method was optimized using a C8 column for chromatographic separation following protein-precipitation-based sample preparation. Spiked matrix samples of serum and plasma were used to test selectivity and specificity. Matrix effects were determined by performing a post-column infusion experiment and comparing standard line slopes. Precision and accuracy were evaluated over 5 days. Measurement uncertainty was evaluated according to the Guide to the Expression of Uncertainty in Measurement (GUM).
**Results:** The RMP was proven to be highly selective and specific with no evidence of a matrix effect, allowing for quantification of levetiracetam within the range of 1.53–90.0 µg/mL. Intermediate precision was <2.2% and repeatability was 1.1–1.7% across all concentrations. The relative mean bias ranged from −2.5% to −0.3% across all levels and matrices within the measuring range. Diluted samples were found with a mean bias ranging from −0.1 to 2.9%. The predefined acceptance criterion for measurement uncertainty was met and determined for individual measurements independently of the concentration level and sample type to be ≤4.0% (k=2).
**Conclusions:** We present a novel LC-MS/MS)-based candidate RMP for levetiracetam in human serum and plasma. Its expanded measurement uncertainty of ≤4.0% meets the clinical needs in levetiracetam monitoring. Utilizing qNMR to characterize levetiracetam reference materials allowed metrological traceability to SI units.

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

## Introduction

The unbroken chain of traceability of routine measurements to reference measurement procedures (RMPs) is understood as a cornerstone of global comparability of laboratory analysis results. In laboratory medicine standardization, in accordance with ISO 17511 and ISO 15195, RMPs enable the laboratory analysis of measurement value relationships without bias contributions from calibration value assignments and differences between individual assays.

Levetiracetam ($C_8H_{14}N_2O_2$, molecular weight=170.2 Da, conversion factor from µg/mL to molar unit [µmol/L]=5.9), a chiral pyrrolidinone derivative, is a broad-spectrum antiepileptic drug (AED) that is widely used for the prophylaxis and treatment of focal and generalized epilepsy and for the treatment of seizures in palliative care [1–3]. By binding to synaptic vesicle protein SV2A, levetiracetam interferes with neurotransmitter signaling in the brain and inhibits rapidly firing neurons [2, 4]. A widely accepted therapeutic range for levetiracetam is 10–40 µg/mL [5]. Above 50 µg/mL, undesired

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side effects, such as somnolence, asthenia, and dizziness, occur more frequently.

Although evidence for the mechanism of levetiracetam metabolism, a frequent cause for alterations in drug clearance, is lacking [5], monitoring is said to be beneficial for patients in whom the pharmacokinetics and pharmacodynamics of levetiracetam may be altered, e.g., in pediatric, critically ill, or elderly patients, or pregnant women [1, 5, 6]. A large cohort study in Korean patients with epilepsy found that even weight-adjusted levetiracetam dosing leads to a broad range of serum levels, with 42% of patients outside the therapeutic range [7]. Monitoring levetiracetam serum levels is also recommended for patients receiving concomitant therapy with enzyme-inducing AEDs, owing to resulting changes in levetiracetam pharmacokinetics [1, 8]. In addition, co-administration of levetiracetam and methotrexate has been reported to decrease methotrexate clearance, resulting in potentially toxic accumulation of methotrexate [9].

Liquid chromatography coupled with UV detection (LC-UV) or tandem mass spectrometry (LC-MS/MS) has been successfully marketed and CE (Conformitè Europëenne)-certified, as *in vitro* diagnostic (IVD-CE) assays for routinely monitoring levetiracetam. IVD-CE certified immunoassays have also been launched successfully [10, 11]. Furthermore, a multitude of LC-MS/MS assays have also been published in research settings [12–14]. None of the IVD-CE certified solutions make a statement on traceability to higher metrological order [15]. Neither was one of these methods intended to server as RMP candidates for levetiracetam. In general, routine LC-MS/MS methods might be less specific due to possible cross-reactivity, matrix effects or isobaric congener. Since they are often designed as multi-analyte methods, resulting in reduced precision due to limitations in the data points recorded. The overall ion yield is usually lower in a multi-analyte method than for RMPs where only the analyte of interest is monitored. Among others, these effects can lead to higher inaccuracy and precision of routine methods compared to RMPs.

Since it is an ongoing challenge for medical laboratories and the *in vitro* diagnostic industry to prove the traceability of applied methodologies to a metrological higher order [16], that is to reference materials and RMPs, their lack of public availability in the scientific field represents an unmet need. An RMP must have measurement uncertainty well below the routine requirement since error propagation in the traceability chain leads to an inescapable increase of the random error from reference to routine method. It is generally accepted that an RMP should have only one-third of the measurement uncertainty of the routine method [17].

In therapeutic drug monitoring (TDM) measurement, uncertainty goals can either be derived from pharmacokinetic-based calculations or from the therapeutic range. Following the associated estimation protocols by Glick, Burnett, or Fraser, levetiracetam routine TDM measurement uncertainties should not exceed 6% for therapeutic-range-derived goals and approximately 12% according to the pharmacokinetic model by Fraser [18]. In a recent investigation into proficiency testing, routine levetiracetam measurements from more than 70 laboratories led to an inter-laboratory measurement uncertainty of 5–15% for both LC-MS/MS and enzyme immunoassays [11]. Correcting for the inter-laboratory expansion factor by applying the average inter-/intra-laboratory variance component factor of 0.8 [19] this range translates into an intra-laboratory uncertainty range of approximately 4–12%. It is evident that this is in good agreement with the data derived from the theoretical therapeutic range or pharmacokinetic modelling approaches. For the establishment of the levetiracetam RMP, this means that the analytical specification from the routine establishment of the parameter requires a measurement uncertainty of less than 2%, equivalent to an expanded uncertainty of 4%. Regarding systematic errors, it is a paradigmatic must that the bias associated with an RMP must be nil [20]. This can be achieved by thorough and transparent reference material characterization, e.g., by the mass balance approach or by quantitative nuclear magnetic resonance spectroscopy (qNMR).

qNMR is an effective method of determining absolute quantities of analytes [21, 22] and establishing direct traceability to SI units via National Institute of Standards and Technology (NIST) traceable primary standards, e.g., benzoic acid 350b or PS1 [23]. Owing to its operational ease and non-destructive nature, qNMR is quickly gaining acceptance by national metrological institutes for measuring absolute amounts in primary reference standards. Isotope-dilution-LC-MS/MS (ID-LC-MS/MS)-based candidate RMPs utilizing qNMR for the value assignment of the reference material have been developed for the steroid hormone androstenedione [24] and immunosuppressive drugs [25].

Herein, we describe a novel candidate RMP for levetiracetam that meets the requirements of the ISO 15193 guideline. In order to facilitate the reproduction of the candidate RMP, it is described in detail in three supplemental documents focusing on the technical implementation of the procedure, the qNMR-based reference material characterization, and the calculation of measurement uncertainty.

## Materials and methods

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

## Chemicals and reagents

LC-MS grade reagents 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 631-31-8, LC-MS grade), isopropanol (CAS 67-63-0, high-performance liquid chromatography [HPLC] grade), and levetiracetam (CAS 102767-28-2, Cat. No. PHR1447, Lot LRAC2807) were obtained from Sigma-Aldrich (Taufkirchen, Germany). The internal standard [$^2H_6$]-levetiracetam (CAS 1133229-30-7, Cat. No. C597, Lot ES-ALS-14-088-P2) was purchased from Alsachim (Illkirch-Graffenstaden, France). DMSO-$d_6$ (CAS 2206-27-1) and qNMR internal standard 1,3,5-trimethoxybenzene (TraceCert®, Lot Nr. BCBW3670, CAS 621-23-8) were obtained from Sigma-Aldrich Germany.

Native human serum (Art. No. S1-LITER) was obtained from Merck (Darmstadt, Germany), TDM-free human serum (multi-individual pooled; surrogate matrix, ID No. 12095432001) from Roche Diagnostics GmbH (Mannheim, Germany), native plasma matrix (lithium-heparin [Li-heparin], $K_2$-EDTA and $K_3$-EDTA) from anonymized, residual patient samples, and water was purified using a Millipore Milli-Q 3 UV system from Merck (Darmstadt, Germany). Pools were prepared from the patient samples in accordance with the Declaration of Helsinki.

## qNMR for determination of the purity of the standard materials

qNMR measurements were performed on a JEOL 600 MHz NMR spectrometer with a He-cooled Cryoprobe. Single-pulse 1H{13C} NMR (Supplemental Material 2, Figure 1) was utilized for the quantitation. Additional details about qNMR acquisition and FID (free induction decay) processing parameters can be found in Supplemental Material 2.

## Preparation of calibrators and quality control (QC) samples

In brief, two calibrator stock solutions were prepared for the subsequent preparation of spike solutions and the final matrix-based calibrator levels. Per stock solution, 90 mg levetiracetam was weighed on a micro balance (XPR2, Mettler Toledo) and dissolved in 5 mL DMSO in a volumetric flask to produce concentrations of 18.0 mg/mL. The concentration of each primary stock solution was calculated based on the purity of the reference material (99.9% ± 0.1%, determined by qNMR) and the amount of levetiracetam added.

Each primary stock solution was used to prepare working solutions of 1.80 mg/mL, from which calibrator spike solutions of different concentrations were obtained. Final matrix-based calibrators (eight levels [Cal 1–8]), uniformly distributed from 1.53–90.0 µg/mL (8.99–529 µmol/L), (Figure 1) were prepared by a 1 + 99 dilution (v/v) into human serum matrix.

Four levels of matrix-based QC samples (QC levels [QC 1–4]) were prepared in the same way as the calibrator levels, using an independent third primary stock solution. The concentrations for the control levels were set at four critical points: above the limit of quantitation, below and within the therapeutic range, and at the laboratory alert level (Figure 1). Final concentration levels were 2.40, 7.50, 20.0 and 60.0 µg/mL, respectively. All samples (spiked and native material) were stored at –20 °C for a maximum of 27 days. All liquids were allowed to reach room temperature prior to use.

## Internal standard (ISTD) solution

For the preparation of the ISTD stock solution, [$^2H_6$]-levetiracetam was dissolved in DMSO to obtain a 1,000 µg/mL solution and stored at –20 °C for a maximum of 6 months. The ISTD working solution was prepared by a twofold dilution of the ISTD stock solution (40 µL DMSO added to 60 µL ISTD stock solution) followed by the addition of 3,900 µL Milli-Q water to achieve a final concentration of 15 µg/mL.

## Sample preparation

ISTD working solution (100 µL) was transferred into a 2 mL tube (Eppendorf Safe-Lock Tubes) and 50 µL of the sample specimen (either native sample, calibrator or QC material) were 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, Hamburg, Germany). Proteins were precipitated by adding 75% methanol (v/v) followed by an incubation on a thermomixer for 10 min (2000 rpm, 23 °C). Then, proteins were separated by centrifugation at 4 °C and 20,000 rcf for 10 min. Subsequently, 10 µL supernatant were diluted 1 + 99 (v/v) using mobile phase A in an HPLC vial (Wicom, Heppenheim, Germany).

## Liquid chromatography-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. A Waters SymmetryShield RP8 (100 × 3 mm, 3.5 µm, Milford, Massachusetts, USA) was used for chromatography. Mobile phases consisted of 2 mM ammonium acetate in Milli-Q water with 0.1% formic acid (A) and methanol:2 mM ammonium acetate in Milli-Q-water 95:5 (v/v) with 0.1% formic acid (B).

Measurements were performed at a flowrate of 0.6 mL/min within a total runtime of 10 min. The injection volume was 5 µL. Contamination of the mass spectrometer was reduced using a divert valve to switch the eluent flow until 0.6 min and from 7.0 min to the waste.

Levetiracetam was detected in multiple reaction monitoring mode using an AB Sciex Triple Quad 6500+ mass spectrometer (Framingham, Massachusetts, USA) with a Turbo V ion source operating in positive electrospray ionization (ESI) mode. An ion spray voltage of 4,500 V and a temperature of 300 °C were applied. Curtain gas, collision gas, and ion gas source 1 and source 2 were set at 35, 8, 55 and 55 psi, respectively. For all mass transitions, a declustering potential of 65 V and a dwell time of 20 ms were applied. Several analyte specific mass transitions were tested and optimized regarding intensity and reproducibility resulting in following transitions: *m/z* 171.1 → 69.1 (quantifier) and *m/z* 171.1 → 98.0 (qualifier). Monitoring the quantifier/qualifier ratio in native matrix samples compared to the quantifier/qualifier ratio of neat SST samples allowed checking for interfering substances within matrix samples.

Table 1 shows an overview of the selected reaction monitoring transitions and the remaining compound-dependent MS settings.

## System suitability test (SST)

An SST was performed prior to each analysis to check the sensitivity and chromatographic performance of the system. Concentration levels of

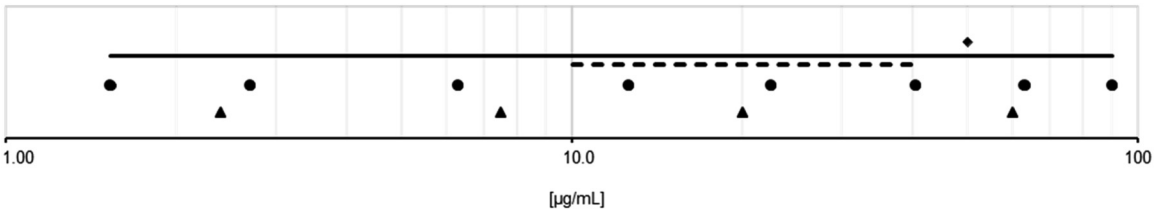


**Figure 1:** Schematic overview of levetiracetam calibrator and control levels chosen to allow optimal coverage of measurement and therapeutic reference ranges. Black circles, calibration samples 1–8; black triangles, QC levels 1–4; black diamond, alert level; black line, measurement range; dotted black line, therapeutic reference range. Conversion factor µg/mL to µmol/L: 5.9.

**Table 1:** MS/MS parameters of levetiracetam and its ISTD.

| Analyte | | Precursor ion, *m/z* | Product ion, *m/z* | EP, V | CE, V | CXP, V |
|---|---|---|---|---|---|---|
| Levetiracetam | Quantifier | 171.1 | 69.1 | 10 | 40 | 8 |
| | Qualifier | | 98.0 | 8 | 30 | 12 |
| [$^2H_6$]-levetiracetam | Quantifier | 177.1 | 74.1 | 10 | 46 | 9 |
| | Qualifier | | 104.0 | 10 | 21 | 15 |

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

SST1 and SST2 corresponded to the analyte concentration within the processed calibrator levels 1 and 8, respectively. To pass the SST, a signal-to-noise ratio of the quantifier transition of ≥10 for SST1 and retention time of 2.9 ± 0.5 min for SST1 and SST2 were required. Carry-over effects were examined by injecting the high-concentration sample SST2, followed by two solvent blanks. In addition, the analyte peak area observed in the first blank after the injection of the SST2 sample must be ≤20% of the analyte peak area of SST1 or calibrator level 1.

## Calibration and structure of analytical series, and data processing

The assay was calibrated using the calibrators described in section "Preparation of calibrators and quality control (QC) samples". The calibration curve was generated from the area ratios analyte/ISTD for calibrators 1–8 and using a quadratic fit with a $1/x^2$ weighting. The curve was not forced through the origin. For processing of the raw data file, Analyst® software (version 1.6.3 or higher) was used with the IntelliQuant algorithm. The levetiracetam and [$^2H_6$]-levetiracetam signals showed a retention time of 2.9 min and were integrated within a 30 s window. Peak integration included a smoothing factor of 3 and a peak splitting factor of 3. 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 & Laboratory Standard Institute Guidelines *C62A Liquid Chromatography-Mass Spectrometry Methods* [26], the International Conference on Harmonization guidance document *Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2 (R1)* [27], and the *Guide to the Expression of Uncertainty in Measurement* [28].

**Selectivity:** Selectivity was determined by spiking levetiracetam and ISTD [$^2H_6$]-levetiracetam in native serum, surrogate serum, and native Li-heparin plasma. To examine possible interfering matrix signals for analyte quantifier and qualifier transitions, matrices were checked at the expected retention time. In addition, matrices were spiked with the deuterated ISTD solution to evaluate residual unlabeled analyte within the stable isotope-labelled ISTD.

**Matrix effects/specificity:** To determine possible matrix effects, a qualitative post-column infusion experiment was performed based on the comparison of standard line slopes [29] and a comparison of absolute areas of analyte and ISTD [30].

In the qualitative post-column infusion experiment, a neat solution of the analyte (10 ng/mL levetiracetam in mobile phase A) was infused at a flow rate of 15 µL/min via a T-piece into the HPLC post-column eluent before entering the MS/MS system, to generate a stable analyte background signal. Processed matrix samples (native human serum), surrogate serum, and native plasma (Li-heparin, $K_2$-EDTA, and $K_3$-EDTA) were then injected.

A comparison of standard line slopes was performed by comparing the following four matrices: neat solution (mobile phase A), a native human serum pool, surrogate serum, and Li-heparin plasma. For this, calibrator levels were prepared as described in section "Preparation of calibrators and quality control (QC) samples". Slopes and coefficients of determination were compared. In addition, the calibrator samples in surrogate matrix, native serum, and Li-heparin 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.

Furthermore, a comparison of absolute areas of analyte and ISTD was performed. Analyte and ISTD solutions (analyte concentration 7.50, 20.0, and 60.0 µg/mL) were spiked in the four matrices, listed above, after protein precipitation. All samples were prepared in five replicates.

**Linearity:** The linearity of the method was checked for an extended calibration range of ±20% (1.22–108 µg/mL) in native human serum. The calibrators were prepared in sixfold, and the peak area ratio of analyte and the corresponding ISTD were plotted against the respective concentrations. Correlation coefficients and residuals for each curve were determined, and the regression model was chosen accordingly.

The linearity of the method was proven based on 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 must show a linear dependency. Recovery was reported as the percentage of recovery of the measured concentration relative to the nominal concentration of the sample pool.

**Lower limit of measuring interval (LLMI) and limit of detection (LOD):** Precision and accuracy at the quantitation limit (QL; matched with the lowest calibrator level [1.53 µg/mL]) were measured using spiked samples in the anticipated QL concentration range. Samples were prepared fivefold for the determination of recovery, bias, and precision.

The limit of detection (LOD) was estimated by determining the mean and SD of blank matrix samples (100 data points at the retention time of the analyte, 0.2 min time window, 10 independent samples from the precision experiment) and calculating LOD as the mean +3 SD with the mean peak height of calibrator 1 analysis serving as quantification reference.

**Precision and accuracy:** A 5-day validation experiment, as described in Taibon et al. [31], was performed to evaluate precision and accuracy of the developed method. Total method variability was estimated by an ANOVA-based variance component analysis. On each day, four spiked serum and Li-heparin plasma samples (2.40, 7.50, 20.0, and 60.0 µg/mL) and two native patient serum samples (6.26 and 36.5 µg/mL), which were close to the medical decision point (therapeutic reference range 10–40 µg/mL [5]), were prepared in triplicate for part A and B and injected twice (n=12 measurements per day; n=60 measurements 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 the VCA Roche Open-Source software package in R [32].

Since there are no secondary reference materials available, accuracy was assessed using four spiked human serum and plasma samples with the following concentrations: 2.40, 7.50, 20.0, and 60.0 µg/mL. Dilution integrity was performed using two spiked serum samples (levetiracetam-free human serum) at concentration levels of 99.0 and 150 µg/mL. All samples were prepared in triplicate for each 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 stability of the processed samples on the autosampler was investigated at 7 °C after 7 days. For this purpose, samples from calibrator and QC levels from the linearity experiment were used. Stability of matrix-based calibrator and control material stored at −20 °C was evaluated after 28 days. Recoveries were calculated by comparing the measured value with freshly prepared samples. The total error (TE) was used as an acceptance criterion and calculated based on the results from precision and trueness experiment, resulting in a TE of ±6%. Stability can be ensured for a measurement interval of 2–28 days (x) for x – 1 day, and for a measurement interval of >4 weeks (y) for y – 1 week.

**Equivalence of results between independent laboratories:** To assess the agreement of the RMP between two independent laboratories (Site 1 at Dr. Risch Ostschweiz AG, Buchs, Switzerland; and Site 2 at Roche Diagnostics GmbH, Penzberg, Germany), a method-comparison study was performed including 80 native serum and 10 native plasma patient samples, 10 native patient pools, and 30 spiked native patient samples. The RMP was transferred to Site 2 and the system was setup as described within Supplemental Material 1. The LC-MS system and laboratory equipment in Sites 1 and 2 were identical, and calibrators were prepared independently as described above.

**Uncertainty of measurements:** Measurement uncertainty was determined according to GUM [28] and the following parameters were evaluated: (I) uncertainty estimation of qNMR target value assignment of the primary reference material, (II) uncertainty estimation of the preparation of calibrator materials, and (III) estimation of uncertainty of the LC-MS/MS method. Uncertainty measurements were calculated as described in Taibon et al. [31]. Details can be found in Supplemental Material 3.

# Results

## Traceability to SI units

Regarding traceability to the SI-unit kilogram, a qNMR ISTD directly traceable the primary standard NIST PS1, has been applied for qNMR measurements [23]. Sixfold experiments (Supplemental Material 2, Figure 2), involving six individual weightings of the analyte and the qNMR ISTD, yielded for the calibrator material a final levetiracetam content value of 99.9 ± 0.1% (k=1).

## Selectivity

The developed solvent gradient combined with the reversed-phase column (Waters SymmetryShield RP8, 100 × 3 mm, 3.5 µm) allowed to place levetiracetam at a retention time of 2.9 min well separated from polar and apolar matrix components (Figure 2). Selectivity was determined by analyzing sample pools of analyte-free human serum, surrogate serum, and human Li-heparin plasma. No signals were observed at the expected retention time. Moreover, no levetiracetam signal was detected in the [$^2H_6$]-levetiracetam ISTD ion trace.

Measuring native patient samples within the method comparison study no interferences were observed in the retention time window of levetiracetam. In a few samples a peak occurred at 4.9 min. However, this peak does not interfere with the analyte.

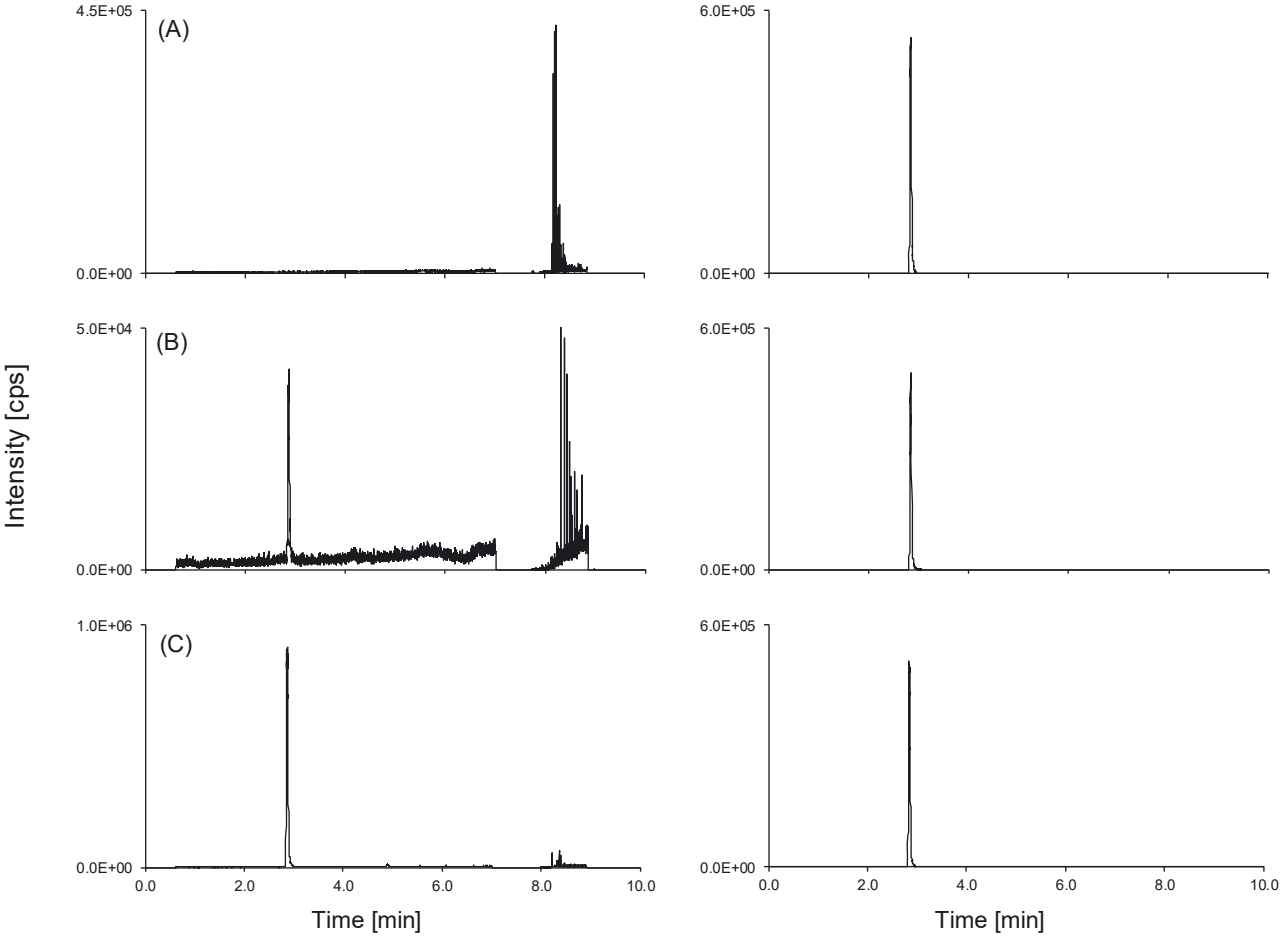


**Figure 2:** Levetiracetam LC-MS/MS derived analytical readouts. (A) Chromatogram of a matrix blank showing the analyte SRM ion trace (left) and the ISTD SRM ion trace (right). (B) Chromatogram of the lowest calibrator level with a concentration of 1.53 µg/mL spiked in native serum; analyte (left) and ISTD (right). (C) Pooled patient sample with a concentration of 36.5 µg/mL; analyte (left) and ISTD (right).ISTD, internal standard.

## Matrix effect

High dilution after protein precipitation, as detailed in the protocol, minimized matrix-dependent effects caused by salts, proteins, and phospholipids. No suppression or enhancement occurred at the expected retention time of levetiracetam independent from the matrix.

Slopes and coefficients of determination of calibrators in different matrices were compared. Slopes were found to be 0.0512 (95% CI 0.0509 to 0.0515) for native serum matrix, 0.0513 (95% CI 0.0508 to 0.0518) for neat solution, 0.0507 (95% CI 0.0503 to 0.0510) for surrogate serum matrix, and 0.0507 (95% CI 0.0504 to 0.0511) for plasma matrix. Correlation coefficients were 1.00 independent of the matrix used for calibration. Furthermore, calibrator samples in surrogate serum, native serum, and Li-heparin plasma were evaluated as controls (n=6 sample preparations) by applying the neat calibration as standard. Relative bias for all matrices and levels ranged from −1.6 to 1.5% with coefficients of variation (CVs) of less than 2.4%.

Additionally, matrix effects were determined according to Matuszewski et al. [30]. No matrix effect was observed for all levels, with mean values of 95–104% for the analyte and the corresponding ISTD (Table 2). The mean area ratios were 99–100% with the 95% CI interval ranging from 97–102%. Thus, the compensating effect of the labelled ISTD was confirmed.

## Linearity

Linearity was determined by analyzing six native serum calibration curves, which were adjusted to extend the desired measuring range by 20% at both lower and higher concentrations. The residuals were randomly and equally distributed in a quadratic regression model. The correlation coefficients were r=1.00 for all individual calibration curves.

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

| Levetiracetam conc., µg/mL | | Analyte | | ISTD | | Ratio | |
|---|---|---|---|---|---|---|---|
| | | Mean, % | 95% CI, % | Mean, % | 95% CI, % | Mean, % | 95% CI, % |
| 7.50 (Level 1) | Native serum | 103 | 101–105 | 104 | 103–105 | 99 | 98–101 |
| | Surrogate serum | 97 | 96–98 | 98 | 96–100 | 99 | 98–100 |
| | Native plasma | 98 | 97–99 | 98 | 97–100 | 99 | 97–101 |
| 20.0 (Level 2) | Native serum | 100 | 98–101 | 100 | 99–101 | 100 | 99–102 |
| | Surrogate serum | 96 | 95–97 | 97 | 96–98 | 99 | 98–101 |
| | Native plasma | 96 | 96–97 | 97 | 96–97 | 100 | 98–101 |
| 60.0 (Level 3) | Native serum | 99 | 98–100 | 100 | 99–101 | 99 | 98–101 |
| | Surrogate serum | 95 | 94–97 | 96 | 95–97 | 100 | 98–101 |
| | Native plasma | 95 | 94–96 | 95 | 93–97 | 100 | 99–101 |

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, where set 2 corresponds to the respective matrix samples and set 1 to the neat samples. No matrix effect is present if ME=100%. CI, confidence interval; ISTD, internal standard; ME, matrix effect.

The linearity of the method was confirmed using serially diluted samples. The measurement results showed a linear dependence with a correlation coefficient of 1.00. The relative deviation (n=6) ranged from 0.4 to 3.0% and the CV was ≤1.5%.

## Lower limit of measuring interval (LLMI) and limit of detection (LOD)

The LLMI was determined using spiked samples at the concentration of the lowest calibrator level (1.53 µg/mL). Relative bias showed a deviation of 0.7% while the CV was 2.2%. The LOD was estimated as 0.130 µg/mL with an intensity corresponding approximately to one tenth of the average calibrator 1 peak height.

## Accuracy and precision

For the validation of precision and accuracy, spiked serum and plasma samples at four concentration levels were used. Each sample was prepared sixfold from two different operators on five different days. Prior to sample preparation, two high-concentration samples were diluted with native blank matrix and sample preparation performed as described.

The evaluation of precision was performed in a multi-day validation experiment. Variability components between injections, between preparations, between calibrations, and between days were determined using an ANOVA-based variance component analysis to assess the overall variability of the methods. Dependency of intermediate precision, including variances as between-day -calibration, -preparation, and -injection from the matrix, was <2.2%. Repeatability CV showed a range of 1.1–1.7% across all concentration levels; patient sample measurement variability was found indistinguishable from spiked materials (Table 3). The evaluation of the trueness within this experimental setup was evaluated by comparing the measured data to calculated sample concentrations. The mean bias (n=6) showed slightly negative results for samples in serum and plasma, ranging from –2.5%

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

| Matrix, sample | Nominal concentration, µg/mL | Repeatability | | Intermediate precision | |
|---|---|---|---|---|---|
| | | SD, µg/mL | CV, % | SD, µg/mL | CV, % |
| **Serum** | | | | | |
| Spike level 1 | 2.40 | 0.039 | 1.6 | 0.040 | 1.7 |
| Spike level 2 | 7.50 | 0.126 | 1.7 | 0.135 | 1.8 |
| Spike level 3 | 20.0 | 0.236 | 1.2 | 0.278 | 1.4 |
| Spike level 4 | 60.0 | 0.659 | 1.1 | 0.775 | 1.3 |
| **Plasma** | | | | | |
| Spike level 1 | 2.40 | 0.039 | 1.6 | 0.042 | 1.8 |
| Spike level 2 | 7.50 | 0.129 | 1.7 | 0.163 | 2.2 |
| Spike level 3 | 20.0 | 0.256 | 1.3 | 0.281 | 1.4 |
| Spike level 4 | 60.0 | 0.750 | 1.3 | 0.823 | 1.4 |
| **Serum** | | | | | |
| Patient sample 1 | 6.26 | 0.086 | 1.4 | 0.101 | 1.6 |
| Patient sample 2 | 36.5 | 0.433 | 1.2 | 0.490 | 1.3 |

CV, coefficient of variation; RMP, reference measurement procedure; SD, standard deviation. Conversion factor µg/mL to µmol/L: 5.9.

to −0.3%, whereas the mean bias of diluted samples ranged from −0.1 to 2.9% (Table 4). The found deviations from zero are within the range of the measurement uncertainty. Hence, within the limitations of this experiment, no conclusion can be drawn, if the found numbers are deviating from zero are true systematic errors. Consequently, the total measurement uncertainty of individual measurement campaigns is understood as acceptance goal of this procedure.

### Stability

Autosampler stability of processed samples (7 °C) was demonstrated for 6 days. The recovery was 96–101%. The stability of pure spike solutions and spiked control samples (−20 °C) was 27 days, and the recovery was 100–106% compared with the original value.

### Equivalence of results between independent laboratories

The 130 samples (native serum and plasma patient samples, patient pools, and spiked samples) were analyzed at Sites 1 and 2. Two samples were outside the calibration range (one above and one below), therefore they were excluded from the analysis. A repeated measurement by diluting high concentrated sample was not possible since it was a leftover sample, and the volume was not sufficient. 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 0.99 to 1.00) and an intercept of 0.0208 (95% CI −0.11 to 0.14) (Figure 3A). The Pearson correlation coefficient was found to be ≥0.999. The relative Bland–Altman analysis plot also showed a good agreement between the two laboratories (Figure 3B). The data scatter is independent from the analyte concentration with a 2S agreement of 6.5% (lower limit CI interval from −7.6 to −5.6%, upper limit CI interval from 5.4 to 7.4%). Since the performance evaluation in Site 2 yielded an intermediate precision comparable to Site 1 (better than 3.0%), the data scatter was in good agreement with the error propagation between the two independent measurements. The result bias in the patient cohort was −0.1% and not statistically significantly different from zero (95% CI interval from −0.7 to 0.5%). Hence, both data scatter and data bias indicate that the proposed levetiracetam RMP is transferable between independent laboratories.

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

| | Concentration, µg/mL | Serum | | Plasma | |
|---|---|---|---|---|---|
| | | Mean bias, % | 95% CI, % | Mean bias, % | 95% CI, % |
| Level 1 | 2.40 | −1.7 | −2.5 to −0.9 | −2.5 | −4.3 to −0.6 |
| Level 2 | 7.50 | −1.9 | −3.4 to −0.3 | −0.6 | −3.3 to 2.1 |
| Level 3 | 20.0 | −0.3 | −1.2 to −0.7 | −0.4 | −2.0 to 1.3 |
| Level 4 | 60.0 | −0.6 | −2.3 to 1.2 | −1.3 | −1.9 to −0.6 |
| Dilution 1 | 99.0 | −0.1 | −2.0 to 1.8 | – | – |
| Dilution 2 | 150 | 2.9 | 0.5 to 5.3 | – | – |

CI, confidence interval. Conversion factor µg/mL to µmol/L: 5.9.

### Uncertainty of results

The estimation of uncertainty in the preparation of the calibrators was performed as a type B evaluation. All other aspects, e.g., calibration, sample preparation, and measurement and evaluation of the sample result, were evaluated as type A uncertainty in the precision experiment described above. Total measurement uncertainties and expanded measurement uncertainty for levetiracetam for single measurements were ≤2.0% and ≤4.0%, respectively, independent of the concentration level and the nature of the sample (Table 5) meeting the requirements defined in the introduction. The derived total uncertainty is multiplied by a coverage factor of k=2 to obtain an expanded uncertainty. The coverage factor corresponds to a confidence level of 95%, assuming a normal distribution.

## Discussion

The ID-LC-MS/MS-based levetiracetam RMP candidate for drug quantification in human serum and plasma presented here is suitable for its intended use. Its expanded measurement uncertainty of ≤4.0% meets the clinical needs in levetiracetam monitoring. Utilizing qNMR to characterize levetiracetam reference materials allowed metrological traceability to SI units.

The RMP candidate was designed using considerations for routine levetiracetam measurements. Owing to the relatively high drug concentration in serum or plasma, protein precipitation followed by dilution was considered advantageous over liquid–liquid extraction or solid-phase extraction for sample preparation before the chosen analytical method of tandem mass spectrometry with ESI and reversed-phase chromatography. All elements of the method were carefully evaluated during the design phase of

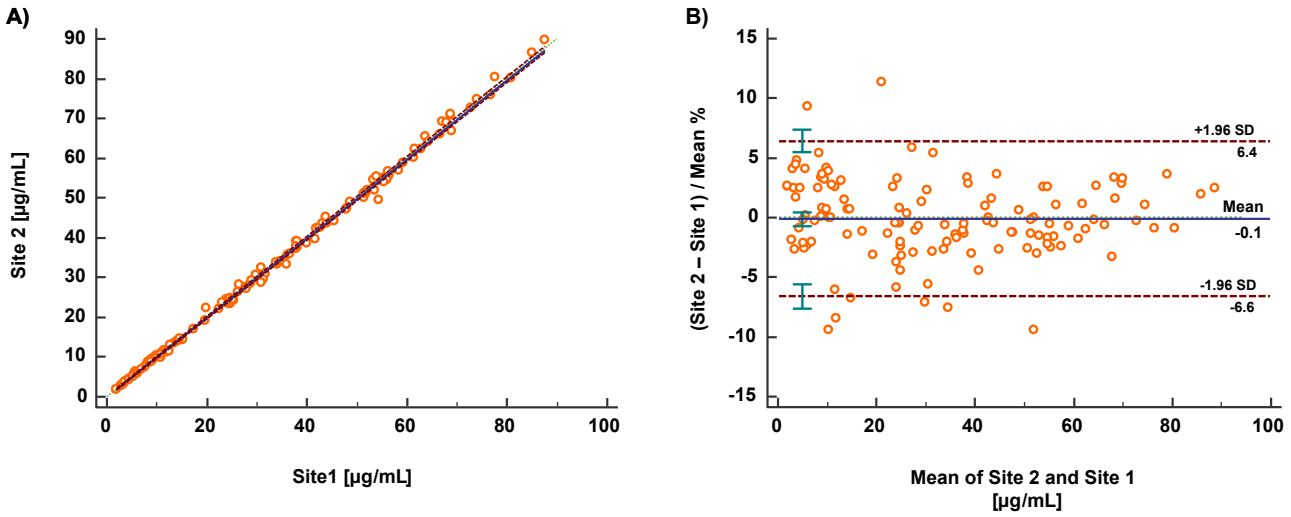


**Figure 3:** Results from the patient sample-based levetiracetam method comparison study performed between two independent laboratories. (A) Passing–Bablok regression plot including the Pearson regression analysis for the method comparison study of the RMP (n=128 patients) between the independent laboratories (Site 1: Risch; Site 2: Roche). Passing–Bablok regression analysis resulted in a regression equation with a slope of 1.00 (95% CI 0.99 to 1.00) and an intercept of 0.0208 (95% CI −0.11 to 0.14). The Pearson correlation value was ≥0.999. (B) Bland–Altman plot for the method comparison study of the RMP (n=128 patients) between two independent laboratories (Laboratory 1: Risch site, and Laboratory 2: Roche site). The interlaboratory measurement bias was −0.1% (95% CI interval from −0.7 to 0.5%) and the 2S interval of the relative difference was 6.5% (lower limit CI interval from −7.6 to −5.6%, upper limit CI interval from 5.4 to 7.4%).

**Table 5:** Combined measurement uncertainty for a single measurement in serum samples.

| | Sample name | | | | | |
|---|---|---|---|---|---|---|
| | **Spike level 1** | **Spike level 2** | **Spike level 3** | **Spike level 4** | **Native patient sample 1** | **Native patient sample 2** |
| Concentration, µg/mL | 2.40 | 7.50 | 20.0 | 60.0 | 6.00 | 36.0 |
| **Type B uncertainty: calibrator preparation, CV %** | **0.87** | **0.83** | **0.82** | **0.78** | **0.84** | **0.78** |
| Characterization of reference material | 0.12 | 0.12 | 0.12 | 0.12 | 0.12 | 0.12 |
| Preparation of: | | | | | | |
| Stock solution | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 | 0.26 |
| Working solution | 0.46 | 0.46 | – | – | – | – |
| Spike solution | 0.62 | 0.56 | 0.55 | 0.48 | 0.58 | 0.48 |
| Matrix-based calibrator | 0.87 | 0.83 | 0.82 | 0.78 | 0.84 | 0.78 |
| **Type A uncertainty: intermediate precision, CV %** | **1.7** | **1.8** | **1.4** | **1.3** | **1.6** | **1.3** |
| **Total measurement uncertainty, CV %** | **1.9** | **2.0** | **1.6** | **1.5** | **1.8** | **1.6** |

CV, coefficient of variation. Conversion factor µg/mL to µmol/L: 5.9.

the project, and a prototype method was evaluated prior to method validation.

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 experiment. Optimization of sample preparation comprised fluid handling, including selection of optimal pipettes; protein precipitation with equilibration times; dilution into the linear range of the MS detector; establishment of an optimized calibration and control step scheme; and optimized preparation of calibrator and control materials, including pipetting.

The absence of matrix effects was proven by calibration slope comparison in addition to ion yield attenuation experiments. The validation study showed that the method meets requirements for an RMP for levetiracetam in terms of sensitivity, selectivity, and reproducibility.

Independent of the concentration level and the specimen type the procedure led to total measurement uncertainties of ≤2.0%, which is sufficiently low to monitor

routine operations. Recent publications of multi-analyte measurement procedures designed to be utilized in clinical settings reported at QC level inter-assay uncertainties ranging from 2.1 to 6.5% [33–35]. These figures of merit match well the data derived from proficiency testing, were uncertainties between 4 and 12% were reported [11].

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 preparation of the calibration solutions and the sample preparation protocols are robustly designed. The platform comparison study established that the method is suitable for processing a high volume of patient samples in a relatively short time. This gives the user confidence in this RMP for the evaluation of routine samples with unclear results. Consequently, the method fulfills both the requirement to take a leading role in the traceability chain and the requirement to perform method comparison studies and check problematic routine samples.

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