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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 carbamazepine-10,11-epoxide in human serum and plasma

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Abstract

Objectives: A reference measurement procedure (RMP) using isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) was developed and validated with the aim of accurately measuring carbamazepine-10,11-epoxide concentrations in human serum and plasma.
Methods: To establish traceability to SI units, the absolute content of the reference material was determined using quantitative nuclear magnetic resonance (qNMR) spectroscopy. As sample preparation a protein precipitation protocol followed by a high dilution step was established. Chromatographic separation from carbamazepine and potential metabolites was achieved using a C18 stationary phase. Selectivity, specificity, matrix effects, precision and accuracy, inter-laboratory equivalence, and uncertainty of measurement were evaluated based on guidelines from the Clinical and Laboratory Standards Institute, the International Conference on Harmonization, and the Guide to the Expression of Uncertainty in Measurement.
Results: The RMP demonstrated very good selectivity and specificity, showing no evidence of a matrix effect. This enabled accurate quantification of carbamazepine-epoxide in the concentration range of 0.0400–12.0 µg/mL. The intermediate precision was found to be less than 2.1 %, and the repeatability coefficient of variation (CV) ranged from 1.2 to 1.8 % across all concentration levels. Regarding accuracy, the relative mean bias varied from 1.4 to 2.5 % for native serum levels and from 1.4 to 3.5 % for Li-heparin plasma levels. The measurement uncertainty for single measurements ranged from 1.6 to 2.1 %.
Conclusions: In this study, we introduce a new LC-MS/MS-based candidate RMP for accurately measuring carbamazepine-10,11-epoxide in human serum and plasma. This novel method offers a traceable and dependable platform, making it suitable for standardizing routine assays and assessing clinically relevant samples.

Keywords: carbamazepine-10,11-epoxide; isotope dilution-liquid chromatography-tandem mass spectrometry; qNMR; reference measurement procedure; traceability; SI units

Introduction

Carbamazepine-10,11-epoxide ($C_{15}H_{12}N_2O_3$, molecular weight=252.3 Da, conversation factor from µg/mL to molar unit [µmol/L]=4.0) is the main metabolite of the anti-convulsive drug carbamazepine. The epoxide is produced by oxidation of carbamazepine by CYP3A4 in the liver [1]. It is then further metabolized to the trans-diol 10,11-dihydro-10,11-hydroxycarbamazepine by an epoxide hydrolase [2–5]. Unlike the secondary metabolite carbamazepine-10,11-epoxide shows similar anti-convulsive effects as the parent drug by blocking sodium and calcium channels. Carbamazepine-10,11-epoxide occurs in concentrations of up to 50 % of the parent drug depending on how carbamazepine is administered [6, 7]. Drugs like primidone and phenobarbital decrease the half-life time of the parent drug and valproic acid and lamotrigine inhibits the epoxide hydrolase, both leading to a prolonged half-life time [2, 8–10]. Since carbamazepine-10,11-epoxide causes side effects similar to but more severe than the parent drug, namely double vision, dizziness, tiredness, and ataxia [11],

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the epoxide is also part of the Therapeutic Drug Monitoring (TDM) and the ratio of the parent drug and metabolite is important for optimal medication with carbamazepine. The therapeutic range of carbamazepine-10,11-epoxide is not well defined and is between 0.400 and 4.00 µg/mL, with toxic effects occurring above 9.00 µg/mL [12].

To ensure optimal comparison between laboratories and assays, RMPs play a vital role to assign values that are free of systematic errors (i.e., measurement bias) to calibrators. Therefore, primary calibrators with defined concentrations are prepared and used to determine the concentration of industrial master calibrators, which are then used to define the analyte in patient samples. To qualify as a "reference measurement laboratory" according to ISO 15195, the laboratory must implement RMPs of a higher order and the results produced must be accurate and traceable to national or international reference materials [13].

qNMR spectroscopy is a well-established primary method (primary ratio method) to determine the mass fraction (absolute content; g/g) of an analyte in a single, non-destructive experiment along with the potential of complete structure elucidation, when required using a plethora of 1D- and 2D-pulse sequences [14, 15]. Owing to powerful structure elucidation characteristics, the linear response to the amount of the analyte and direct traceability to the kilogram via the qNMR internal standards (ISTDs), this technique provides unparalleled ability for determination of the amount of a particular analyte. The highest order qNMR ISTDs are traceable directly to NIST benzoic acid 350b (coulometric) and/or NIST PS1 (benzoic acid; first primary qNMR standard) [16]. These commercially available qNMR ISTDs are available from Sigma Aldrich (TraceCert Standards) and from Wako (TraceSure standards). Additionally, as per the latest IUPAC Technical Report, qNMR has been stated as a potential primary reference measurement procedure ideally suited for the characterization of primary reference materials [17].

Little to no data is available for the half-life time of carbamazepine-10,11-epoxide, however early publications suggest, that with 4–8 h it clears faster from the circulation than its parent drug carbamazepine [18]. As stated, typical plasma concentrations associated with regular carbamazepine dosages range between 0.400 and 4.00 µg/mL.

Chromatographic methods are preferred for the simultaneous monitoring of carbamazepine and its epoxide [19]. There are many research methods published using LC-MS/MS [20–24] and at least two commercial IVD-CE certified kit solutions are on the market. Immunoassays tailored to measure the epoxide metabolite of carbamazepine are not present on the market [25, 26], however carbamazepine immunoassays usually show a cross-reactivity to this compound. For example, the PETINIA carbamazepine assay measures carbamazepine-10,11-epoxide almost quantitatively, with a cross reactivity of more than 90 % [25–27]. This leads to clinically unclear raised carbamazepine drug level readings, especially when carbamazepine is measured in patients with concomitant use of other drugs and health conditions that lead to increased carbamazepine-10,11-epoxide concentrations [25–28]. Consequently, for the TDM of carbamazepine and carbamazepine-10,11-epoxide chromatographic methods with separate readings for these two compounds are preferred in clinical use.

The acceptable measurement imprecision (k=1) associated with these figures of merit is 7.4 % if the pharmacokinetic approach by Fraser and a dosing interval of 8 h is assumed. Using the approach by Glick, based on the therapeutic range only, the allowable imprecision raises to 9.0 %. Since RMP uncertainties should not exceed one-third of desired routine uncertainties [29], the maximal acceptable measurement uncertainty (k=1) should range between 2.5 and 3.0 %.

Herein, we outline a novel candidate RMP for carbamazepine-10,11-epoxide that meets the requirements of the ISO 15193 guideline [30]. To facilitate the reproduction of the candidate RMP by other laboratories, details are described in three supplementary 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 detailed description on the LC-MS/MS procedure methodology can be found in the Supplementary Material 1.

Chemicals and reagents

LC-MS grade methanol (CAS 67-56-1) and formic acid were (CAS 64-18-6) 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), carbamazepine (CAS 298-46-4, Art. No. PHR1067, Lot No. LRAC1961) and 1,3,5-trimethoxybenzene (CAS 621-23-8, Art. No. 74599, Lot No. BCBW3670) were purchased from Sigma Aldrich (Taufkirchen, Germany). Isopropanol (HPLC grade) was bought from Riedel-de Haën (Seelze, Germany). The standard carbamazepine-10,11-epoxide (CAS 36507-30-9, Art. No. MM0076.01, Lot Nr. W988078) was bought from LGC Mikromol (Teddington, UK) and its ISTD [$^{2}H_{8}$]-carbamazepine-10,11-epoxide (CAS 36507-30-9 unlabeled, Art. No. C3529, Lot No. TM-ALS-13-234-P1) was purchased from Alsachim (Illkirch-Graffenstaden, France). The secondary metabolite 10,11-dihydro-10-hydroxycarbamazepine (CAS 29331-92-8, Art. No. 18467, Lot No. 0489614) was purchased from Cayman (Ann Arbor, Michigan US) and 10,11-dihydro-10,11-dihydroxycarbamazepine (CAS 58955-93-4, Art. No. D449040, Lot No. 1-NWW-177-2) was bought from TRC Canada (North

York, Canada). 5 mm NMR tubes were obtained from Eurisotop GmbH (Hadfield, United Kingdom). 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 (Li-heparin, K2-EDTA and K3 EDTA) was obtained from anonymized, leftover patient samples. Water was purified using a Millipore Milli Q 3 UV system from Merck (Darmstadt, Germany).

General requirements for laboratory equipment

Certified and calibrated equipment was used. 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). Positive displacement pipettes were used to measure organic solvents and serum. Volumetric glassware (class A volumetric flasks) that fulfilled the criteria of ISO 1042 and USP were used to prepare stock and spike solutions.

qNMR for determination of the purity of the standard materials

qNMR experiments were performed on a Jeol 600 MHz NMR (Jeol Ltd, Tokyo, Japan) equipped with a helium-cryoprobe head. A single-pulse ^{1}HNMR was utilized for the quantitation (epoxide ring protons; δ=4.36 ppm; 2H; 1,3,5-trimethoxybenzene as qNMR ISTD; DMSO-d_6 as solvent) with an inter-scan delay of 70 s. The epoxide resonance was utilized as the quantitative signal because it is the only signal well-separated from the 1H-signals of reactants from which is synthesized, viz., carbamazepine, 10,11-dihydrocarbamazepine, 10,11-dihydro-5*H*-dibenzo[b,f]azepine-5-carboxamide, iminostilbene, 5-cyano-5*H*-dibenzo[b,f]azepine and 1-phenyl-1*H*-indole. We also measured 2D-TOCSY in order to eliminate the presence of unknown impurities. Even the aforementioned hypothetical presence of these molecules would not interfere with our qNMR methodology. Further details about NMR acquisition and processing parameters are available in the Supplementary Material 2 (Table 1, Figures 1 and 2).

Preparation of calibrators and quality control samples

Two independent calibrator stock solutions were prepared by weighing 16 mg of carbamazepine-10,11-epoxide in tin boats on a microbalance (XPR2, Mettler Toledo, Columbus, Ohio, USA) and dissolving it in 5 mL DMSO using volumetric flasks. The concentrations of the stock solutions were calculated considering the purity of the reference material determined by qNMR (99.7±0.2 %) and the amount weighed, resulting in stock solutions with a concentration of 3.20 µg/mL. These stock solutions were further diluted with DMSO to prepare working solutions, which were used together with the stock solutions to prepare eight calibrator spike solutions. Final matrix-based calibrators, uniformly distributed from 0.0400 to 12.0 µg/mL (0.159–47.6 µmol/L, see Figure 1), were then prepared by a volumetric 1+99 dilution (v/v) of the calibrator spike solutions in native human serum matrix.

Using a third independent stock solution, four levels of matrix-based quality control (QC) levels were prepared following the same procedure as described for the calibrator levels. The QC concentrations were set at four crucial control points: above the lower limit of quantification, below and within the therapeutic reference range, and at the laboratory alert level (refer to Figure 1). Final concentrations of the QC levels were 0.120, 0.450, 1.50 and 6.00 µg/mL.

Internal standard stock solution

For the preparation of [$^{2}H_8$]-carbamazepine-10,11-epoxide ISTD stock solution, 1 mg was dissolved by a volumetric addition of DMSO to obtain a concentration of 1,000 µg/mL. The stock solution was stored at −20 °C. [$^{2}H_8$]-carbamazepine-10,11-epoxide ISTD working solution was freshly prepared each day by mixing 95 µL of DMSO with 5 µL of the ISTD stock solution, followed by the addition of 3,900 µL Milli-Q water to reach a final concentration of 1.25 µg/mL.

Table 1: MS/MS parameters of carbamazepine-10,11-epoxide and its ISTD.

Analyte		Precursor ion, *m/z*	Product ion, *m/z*	Dwell time, ms	DP, V	CE, V
Carbamazepine-10,11-epoxide	Quantifier	253.1	180.1	50	88	36
	Qualifier		210.1	50	88	21
[$^{2}H_8$]-carbamazepine-10,11-epoxide	Quantifier	261.1	188.1	50	88	36
	Qualifier		218.1	50	88	21

DP, declustering potential; CE, collision energy; ISTD, internal standard; V, volts.

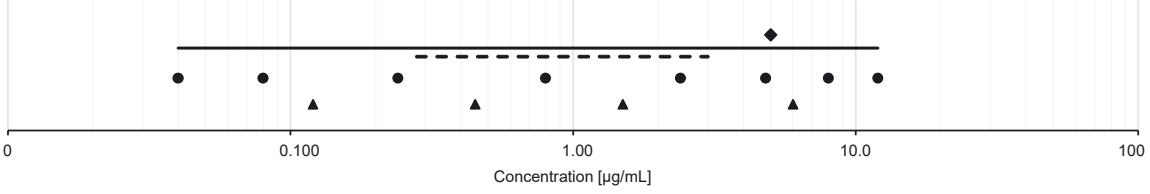

Figure 1: Schematic overview of carbamazepine-10,11-epoxide calibrator and control levels chosen to allow optimal coverage of measurement and therapeutic ranges. Black circles, calibration samples 1–8; black triangles, QC levels 1–4; black diamond, alert level; black line, measurement range; dashed line, therapeutic reference range. Conversion factor µg/mL to µmol/L: 4.0.

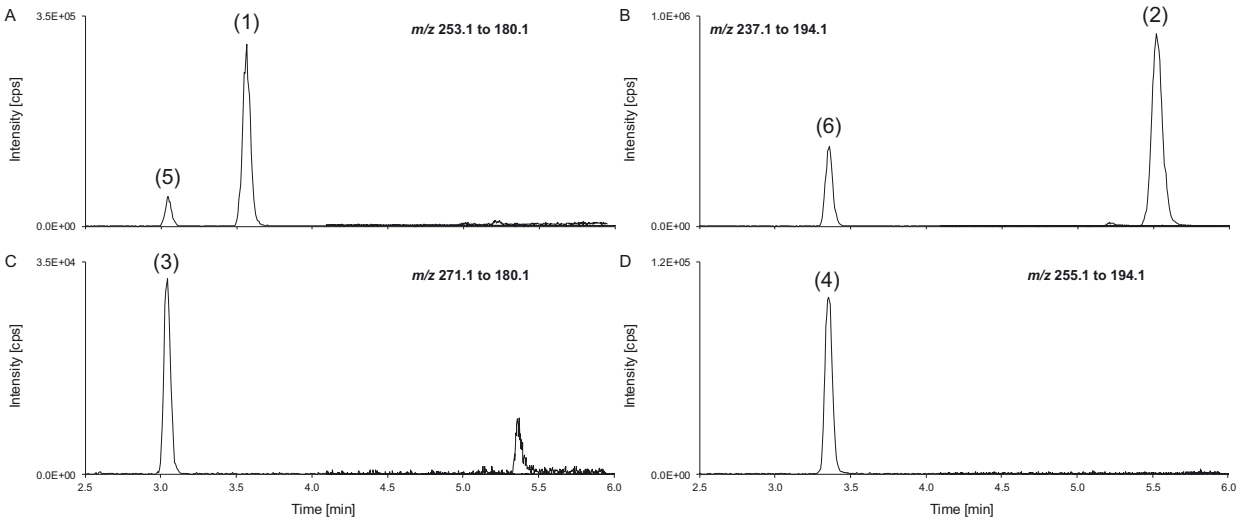

Figure 2: LC-MS/MS readouts of a native human serum sample containing 0.800 µg/mL of each carbamazepine-10,11-epoxide (peak 1), carbamazepine (peak 2), 10,11-dihydro-10,11-dihydroxycarbamazepine (peak 3), 10,11-dihydro-10-hydroxycarbamazepine (peak 4). SRM ion traces of (A) carbamazepine-10,11-epoxide (peak 1); peak 5 shows the in-source dehydration of 10,11-dihydro-10,11-dihydroxycarbamazepine to oxcarbazepine. (B) Carbamazepine (peak 2); peak 6 showing in-source fragmentation of 10,11-dihydro-10-hydroxycarbamazepine to carbamazepine. (C) 10,11-Dihydro-10,11-dihydroxycarbamazepine. (D) 10,11-Dihydro-10-hydroxycarbamazepine.

Sample preparation

Native human serum, surrogate serum, and plasma (Li-heparin, K2--EDTA, and K3-EDTA) can be used as sample matrix. In a 2 mL tube (Eppendorf Safe-Lock Tubes), 100 µL of ISTD working solution was mixed with 50 µL of sample specimen (native sample/calibrator/QC). 1,000 µL 75 % methanol (v/v) was then added for protein precipitation. After centrifugation, 20 µL of the supernatant was mixed with 980 µL Milli-Q water (1+19 [v/v]).

Liquid chromatography mass spectrometry

The chromatographic separation was performed on an Agilent 1290 Infinity II LC system (Santa Clara, California, USA) consisting of a binary pump, a vacuum degasser, an autosampler set at 7 °C, and a column compartment maintained at 40 °C. A chromatographic gradient with a total run time of 9 min at a flow rate of 0.6 mL/min was developed to separate the target analyte carbamazepine-10,11-epoxide from the parent drug carbamazepine and the secondary metabolites (10,11-dihydro-10-hydroxycarbamazepine and 10,11-dihydro-10,11-dihydroxycar-bamazepine) using a Luna Omega Polar C18 column (100x3 mm, 3 µm, Lane Cove, Australia). 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). Contamination of the MS was reduced by using a diverter valve to switch the eluent flow until 0.5 min and from 7.0 min to the waste.

Carbamazepine-10,11-epoxide was detected in multiple reaction monitoring (MRM) mode using an AB Sciex Triple Quad 6500+ or a Q-Trap 6500+ MS (Framingham, Massachusetts, USA) with a Turbo V ion source operating in positive electrospray ionization mode (ESI+ mode). An ion spray voltage of 5,000 V and a temperature of 400 °C were applied. As curtain gas, collision gas, ion gas source 1, and ion gas source 2 nitrogen was used and was set at 45 psi, 11 psi, 50 psi, and 60 psi, respectively. For all mass transitions the collision cell entrance potential was set at 10 V and the collision cell exit potential at 15 V. A second specific mass transition (qualifier) was monitored to eliminate the possibility of interference from other substances present in native matrix samples. This was achieved by comparing the quantifier/qualifier ratios of neat System Suitability Test (SST) samples and native matrix samples. The difference between the ratios should not exceed 20 %. The SRM transitions and the remaining MS settings are summarized in Table 1.

System suitability test

System sensitivity, chromatographic performance, and potential carry-over were evaluated by performing an SST prior to each analysis. The concentration levels of SST sample 1 and 2 were equivalent to the corresponding analyte concentration of processed calibrator level 1 and 8, respectively. To pass the SST, the signal-to-noise ratio of the quantifier transition for SST sample 1 had to be ≥10. The retention time of both SST samples had to be within 3.6±0.5 min. Moreover, the carry-over effect was evaluated by injecting SST sample 2 followed by injecting two solvent blanks. The analyte peak area observed in the blank after the injection of SST sample 2 had to be ≤20 % of the analyte peak area of the calibrator 1 level.

Calibration, structure of analytical series and data processing

The final calibration function was generated by measuring the calibration levels in increasing concentration at the beginning and the end of the analytical series. A quadratic regression of the area ratios of the analyte and ISTD (y) against the analyte concentration (x) was used to obtain the function $y=a_0+a_1x+a_2x^2$ using both calibrations. Data

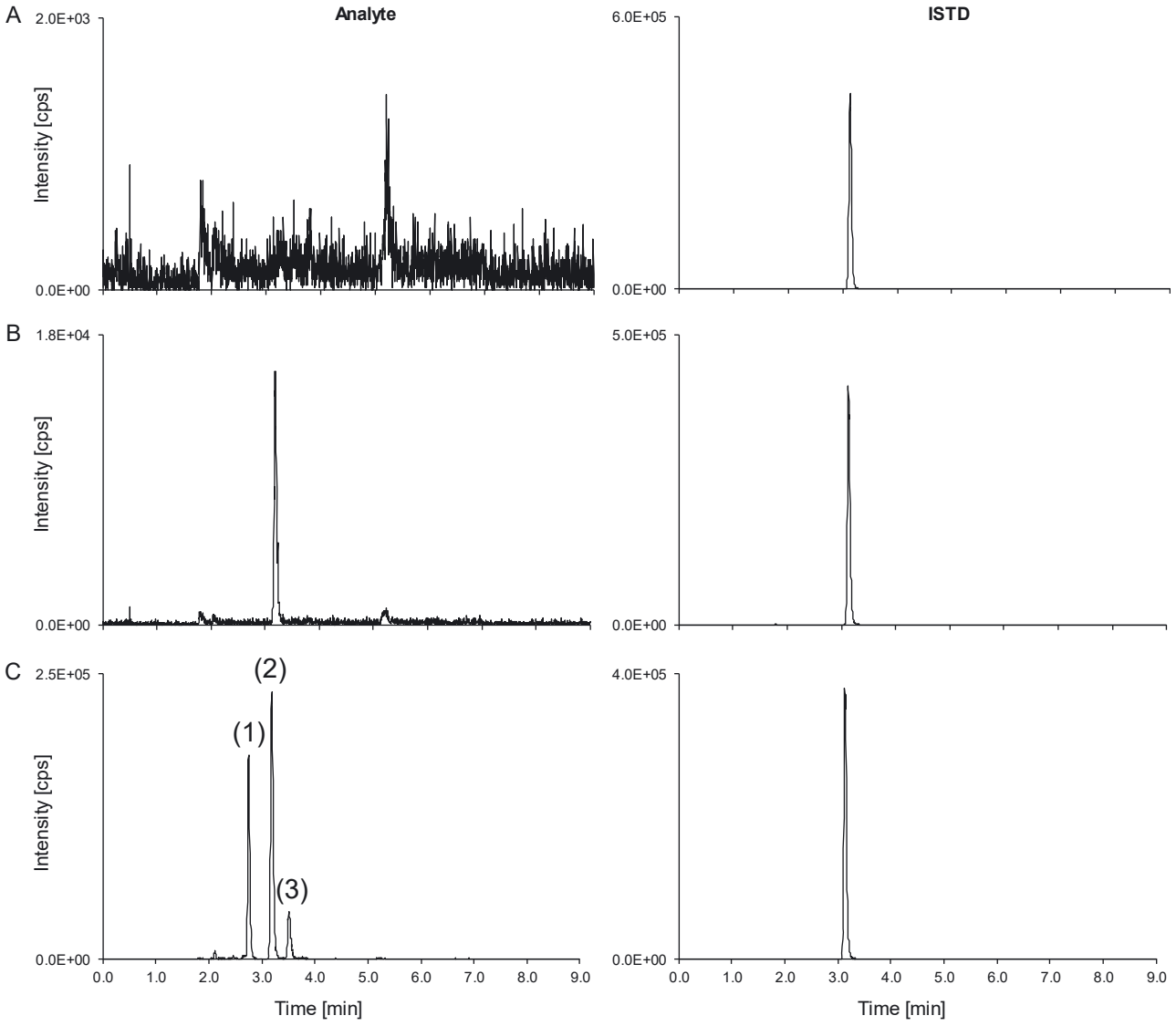

Figure 3: Carbamazepine-10,11-epoxide LC-MS/MS derived analytical readouts. (A) Chromatogram of a matrix blank showing the analyte SRM ion trace (left) and the ISTD SRM ion trace (right). (B) Chromatogram of the lowest calibrator level with a concentration of 0.0400 µg/mL spiked in native serum; analyte (left) and ISTD (right). (C) Patient sample with a concentration of 0.678 µg/mL, showing peaks of (1) dehydration of 10,11-dihydro-10,11-dihydroxycarbamazepine to oxcarbazepine (2) carbamazepine-10,11-epoxide and (3) unknown interference; analyte (left) and ISTD (right). ISTD, internal standard.

processing was performed using Analyst® software (version 1.6.3 or higher) with the IntelliQuant algorithm. Carbamazepine-10,11-epoxide and [$^{2}H_{8}$]-carbamazepine-10,11-epoxide eluted at a retention time of 3.6 min and were integrated within a 30 s window. Peak integration included a smoothing factor of 3 and a peak splitting factor of 2. The noise percent was set to 90 % with a base sub window of 0.5 min.

Method validation

Assay validation and determination of measurement uncertainty were performed based on existing validation guidelines such as Clinical & Laboratory Standards Institute's C62A *Liquid Chromatography-Mass Spectrometry Methods* [31], the International Council of Harmonization's (ICH) guidance document *Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2 (R1)* [32], the *Guide to the expression of uncertainty in measurement* (GUM) [33]. A more detailed description on the estimation of measurement uncertainty was published previously by Taibon et al. [34].

Selectivity: Baseline separation of carbamazepine-10,11-epoxide, carbamazepine (*m*/*z* 237.1 to 194.1), 10,11-dihydro-10-hydroxycarbamazepine (*m*/*z* 255.0 to 194.1), 10,11-dihydro-10,11-dihydroxycarbamazepine (*m*/*z* 271.1 to 180.1) was analyzed in native human serum, surrogate serum, and Li-heparin plasma at a concentration level of 0.800 µg/mL. Moreover, analyte free matrices were checked for possible interferences at the expected retention time of carbamazepine-10,11-epoxide.

Deuterated ISTD was spiked into analyte-free matrices to determine any residual unlabeled analyte, which had to be ≤20 % of the lower limit of the measurement interval (LLMI).

Matrix effects and specificity: A post-column infusion experiment was performed with a neat solution of carbamazepine-10,11-epoxide at a concentration of 10 ng/mL infused into the HPLC column effluent at a flow rate of 7 µL/min. Processed matrix samples (native human serum, surrogate serum, and native plasma [Li-heparin, K2-EDTA, and K3-EDTA]) were then injected and the change in signal intensity at the expected retention time was monitored.

A quantitative matrix effect (ME) experiment was conducted by comparing calibration curves in different sample matrices (neat solution [Milli-Q-water], native human serum, surrogate serum, and native Li-heparin plasma) in terms of slopes and coefficients of determination (n=6, sample preparations) [35]. To exclude MEs, it was required that the confidence intervals (CI) of the slopes overlap, and the coefficients of determination are ≥0.99. Additionally, recoveries of calibrator samples in surrogate matrix, native serum and Li-heparin plasma were determined using the neat calibrator levels as standards.

To further demonstrate that individual ion traces of the candidate RMP can be recorded without ion yield influence from the matrix entering the ion source at the same timepoint as the target analyte, a comparison of the absolute areas of the analyte and its ISTD was performed [36]. The analyte and ISTD solutions were spiked after protein precipitation into neat solution, native human serum, surrogate serum, and native Li-heparin plasma for three levels over the working range (0.450, 1.50, 6.00 µg/mL). All samples were prepared in five replicates. Mean peak areas of the analyte and ISTD of the matrix samples were compared to the neat samples and ion enhancement or suppression was evaluated. To account for the measurement imprecision and the error propagation of the independent measurements the deviation decision limit was set to ±10 %.

Linearity: The linearity of the method was evaluated by extending the calibration range to ±20 % at the upper and at the lower end of the measuring range in native human serum. This involved preparing two additional calibrator levels (0.0320 and 14.4 µg/mL) and determining the coefficients of determination and residuals for each calibration (n=6, sample preparations). The coefficients of determination and the residuals were required to be ≥0.99 and randomly distributed, respectively.

In addition, to prove the linearity of the assay, the recovery and linear relationship of serially diluted samples were determined. Therefore, calibrator level 1 was mixed with calibrator level 8 to obtain nine additional samples 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). A linear relationship must be observed between the measured concentration and the dilution factor, resulting in a coefficients of determination of ≥0.99. Furthermore, recovery was reported as the percentage of recovery of the measured concentration relative to the nominal concentration of the sample pools.

Lower limit of the measuring interval and limit of detection: Native human serum samples spiked at the quantitation limit were prepared. The LLMI corresponded to the lowest calibrator level (0.0400 µg/mL). Samples were prepared in five replicates. The recovery, bias, and precision of the analyte was determined and required to fall within the ranges defined in the accuracy and precision experiment.

To estimate the limit of detection (LOD), the approach of Armbruster et al. [37] was used. Therefore, the limit of blank was calculated using 10 independent matrix blank samples as follows: $LOB=mean_{blank}+1.645(SD_{blank})$. The LOD was then estimated using 10 replicates of calibrator level 1, which was used as the low concentration sample: $LOD=LOB+1.645(SD_{low\ concentration\ sample})$.

Precision and accuracy: Precision and accuracy was determined in a multi-day validation experiment as previously described [34, 38–42]. In brief, four spiked native serum and native Li-heparin plasma samples (0.120, 0.450, 1.50 and 6.00 µg/mL) covering the measuring range as well as two native patient serum samples were prepared over five days in triplicate for part A and B and injected twice (n=12 measurements per day and n=60 measurements per 5 days). Each day and part was independently calibrated. To account for varying conditions, sample preparation for parts A and B was carried out by one operator each, and two different column batches were used. Data evaluation was done performing an ANOVA-based variance component analysis using Biowarp, an internal statistic program based on the VCA Roche OpenSource software package in R [43].

As there were no secondary reference materials available accuracy of the candidate RMP was assessed using the above mentioned spiked human serum samples as well as two spiked high concentrated levels (14.0 and 20.0 µg/mL; dilution 1 and 2). All samples were prepared in triplicates for each part A and part B (n=6, sample preparations, two operators) on one day and percentage of recovery of the measured concentration relative to the spiked concentration determined.

Sample stability: The stability of processed native serum samples was evaluated by measuring samples again after 7 days of storage at 7 °C and comparing them to freshly prepared serum samples. Additionally, the stability of frozen native serum calibrators and QC levels stored at −20 °C was investigated after 36 days. The recoveries were calculated by comparing the measured value with freshly prepared serum samples. The Total Error (TE) was used as acceptance criteria and was estimated based on the results from precision and trueness experiment, resulting in a TE of ±6 %. Stability can be guaranteed 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 two independent laboratories: A method-comparison study was performed to examine the agreement of the candidate RMP between two independent laboratories (Site 1 at Dr. Risch Ostschweiz AG, Buchs, Switzerland; and Site 2 at Roche Diagnostics GmbH, Penzberg, Germany). A total of 140 samples were measured, including 70 native serum and 30 native plasma patient samples, 10 native patient pools, and 30 spiked samples. In addition, a three-day precision experiment was performed at Site 2 using the same system setup with following modifications: an ultra-microbalance XP6U/M (Mettler Toledo) and aluminum weighing boats were used for the preparation of stock solutions.

Uncertainty of measurements: Measurement uncertainty was determined according to the GUM [33] and Taibon et al. [34] and considered the following steps: purity of the reference material, weighing of the analyte, preparation of stock, working, spike and calibrator solutions, preparation of the ISTD solution, sample preparation of the calibrators, measurement of the calibrators and generation of the calibration curve, preparation and measurement of the unknown samples as well as evaluation of the sample results. The estimation of the uncertainty in the preparation of the calibrators was performed as a type B evaluation. All other aspects were evaluated as type A evaluation. The total

measurement uncertainty of the whole approach was estimated as a combination of type A and type B uncertainties.

Results and discussion

Traceability to SI units

Traceability to the SI unit of mass (kilogram), the most important parameter for a reference measurement method, has been established by the utilization of 1,3,5-trimethoxybenzene as a qNMR ISTD which is directly traceable to NIST PS1 (primary qNMR standard). Furthermore, traceability to the SI unit of amount of substance (mole) is also included owing to NIST PS1. Since Planck's constant is now the main parameter for defining kilogram and Avogadro's constant for mole, qNMR methodology is best suited to fulfill both the traceability chains. Additionally, as per the latest IUPAC Technical Report, qNMR has been stated as a potential primary RMP ideally suited for the characterization of primary reference materials [17]. Six individual experiments (Supplementary Figure 2; Table 1) involving six individual weightings, yield a final absolute content value of 99.7±0.2 % (k=1). The extended uncertainty (k=2) can be obtained by multiplying the above-mentioned uncertainty by a factor of 2.

Selectivity

Baseline separation of carbamazepine-10,11-epoxide, carbamazepine, 10,11-dihydro-10-hydroxycarbamazepine, and 10,11-dihydro-10,11-dihydroxycarbamazepine was achieved on a reversed phase column (Phenomenex Luna Omega Polar C18) showing resolutions greater than 2.0 in all tested matrices (Figure 2). The chromatogram of different matrices (pools of analyte-free native human serum, surrogate serum, and human Li-heparin plasma) showed no signals at the expected retention time.

In some patient samples, a signal was observed during the method comparison study at a retention time of −0.4 min relative to carbamazepine-10,11-epoxide as well as at a retention time of +0.3 min relative to carbamazepine-10,11-epoxide (Figure 4). The signal at +0.3 min occurred only in two of 110 serum samples, whereas the other signal was observed in all native serum and plasma samples. The signal eluting prior to the target analyte may be explained by the in-source dehydration of the metabolite 10,11-dihydro-10,11-dihydroxycarbamazepine to oxcarbazepine, a congener isobaric to carbamazepine-10,11-epoxide (schematically shown in Figure 3). The chemical nature of the isobaric component eluting later than the target analyte was not identifiable. Since all signals are baseline separated the quantification of carbamazepine-10,11-epoxide is not affected (Figure 4). The labelled ISTD is considered suitable for the intended use as it did not contain any unlabeled analyte.

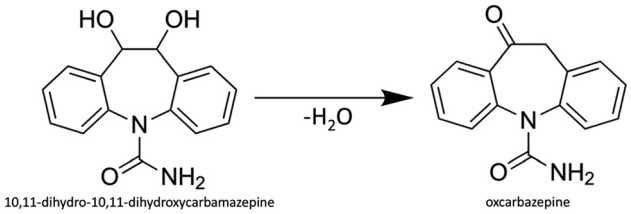

Figure 4: Schematic overview of the in-source dehydration of 10,11-dihydro-10,11-dihydroxycarbamazepine to oxcarbazepine.

Matrix effect and specificity

The sample preparation protocol was based on protein precipitation followed by a high dilution step to minimize possible matrix effects. Matrix independency was demonstrated by the post column infusion experiment, as no change in the ionization field was observed at the expected retention times in native human serum, surrogate serum, and native plasma matrix (Li-heparin, K2-EDTA and K3-EDTA plasma).

Moreover, matrix effects were investigated by comparing slopes and coefficients of determination of calibration curves in different matrices. The slopes of carbamazepine-10,11-epoxide (n=6, sample preparations) were 0.867 (95 % CI 0.854 to 0.879) for neat solution, 0.876 (95 % CI 0.862 to 0.890) for native serum matrix, 0.874 (95 % CI 0.858 to 0.890) for surrogate serum matrix, and 0.878 (95 % CI 0.863 to 0.892) for Li-heparin plasma matrix. Since the 95 % CIs overlap, meaning that they are not significantly different, a matrix effect can be excluded. The coefficients of determination were 1.00 regardless of the calibration matrix. The relative bias for all matrices tested ranged from −0.8 to 2.3 % with CVs of ≤3.2 % compared to neat solution, except for calibrator 1 (0.0400 µg/mL) in surrogate serum, which showed a CV of 5.7 %.

Comparing analyte peak areas, ISTD peak areas as well as area ratios of matrix samples with neat samples no matrix effects were observed. Carbamazepine-10,11-epoxide and [$^2H_8$]-carbamazepine-10,11-epoxide peak areas showed mean relative recoveries to neat samples from 94 to 105 % and from 96 to 104 %, respectively. The corresponding comparison of mean area ratios ranged from 97 to 102 % (Table 2).

Table 2: Matrix effect data of carbamazepine-10,11-epoxide of three different matrices compared to neat analyte solution. 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.

Carbamazepine-10,11-epoxide conc. µg/mL		Analyte		ISTD		Ratio	
		Mean, %	95 % CI, %	Mean, %	95 % CI, %	Mean, %	95 % CI, %
Level 1	Native serum	105	102 to 108	104	101 to 107	101	99 to 102
0.450 µg/mL	Surrogate serum	100	97 to 103	98	96 to 100	102	99 to 105
	Native plasma	99	97 to 102	98	96 to 101	101	100 to 102
Level 2	Native serum	102	100 to 105	101	100 to 102	101	99 to 103
1.50 µg/mL	Surrogate serum	98	96 to 101	96	94 to 98	102	100 to 103
	Native plasma	99	96 to 102	99	97 to 101	100	98 to 102
Level 3	Native serum	99	96 to 102	102	100 to 103	98	95 to 100
6.00 µg/mL	Surrogate serum	94	91 to 96	96	93 to 99	97	95 to 99
	Native plasma	96	93 to 99	98	96 to 101	98	96 to 99

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

Linearity

The linearity was evaluated by analyzing six native serum calibration curves with an extended measuring range of 20 % at both lower and higher concentrations, resulting in a linearity over the range from 0.0320 to 14.4 µg/mL. The residuals were not randomly distributed in a linear regression model, therefore a quadratic model was applied. The coefficients of determination were 1.00 for all individual calibration curves.

Based on the selected regression model, the dilution series was evaluated and showed a linear dependence with a coefficient of determination of 1.00. The relative deviation (n=6, sample preparations) ranged from −0.2 to 2.4 % with CVs of ≤2.5 %.

Lower limit of the measuring interval and limit of detection

The LLMI was determined using samples spiked with the concentration of the lowest calibrator (0.0400 µg/mL). The relative bias showed a deviation of 2.1 % and CV of 4.0 %. The LOD was estimated to be 0.0111 µg/mL. The LOD's intensity is approximately one-fourth of the average peak height of calibrator 1.

Accuracy and precision

To estimate precision, accuracy, and trueness, spiked native human serum and Li-heparin plasma samples were used at four concentration levels (0.120, 0.450, 1.50, 6.00 µg/mL). Moreover, two high-concentrated native human serum samples (14.0 and 20.0 µg/mL) were diluted with native human serum prior to the sample preparation to define the accuracy of high-concentration samples (dilution 1 and 2).

Intermediate precision and repeatability were determined using an ANOVA-based variance component analysis including variances as between-day, -calibration, -preparation, and -injection and resulted in CVs ≤2.1 and ≤1.8 %, respectively, regardless of the concentration levels and matrix. The measurement variability of patient samples was found to be indistinguishable from spiked materials (Table 3).

Trueness was evaluated by comparing the mean measured concentrations to the nominal sample concentrations. The mean bias (n=6, sample preparations, two operators) of native human serum samples ranged from 1.4 to 2.5 %, while Li-heparin plasma samples showed a mean bias between 1.4 and 3.5 %. Diluted samples (dilution 1 and 2) showed comparable results ranging from 3.2 to 3.4 % (Table 4).

Stability

The stability of processed samples at 7 °C was verified for 6 days. The mean recovery was 99.4 %. The stability of spiked calibrator and QC samples stored at −20 °C was evaluated for 35 days with a mean recovery of 103.2 %.

Equivalence of results between two independent laboratories

Equivalence between two independent laboratories was demonstrated performing a method comparison study with 140 samples (native serum and Li-heparin plasma patient

Table 3: Precision performance parameters for carbamazepine-10,11-epoxide quantification using the candidate RMP (n=60 measurements).

Variance source	Serum samples CV, %					
	Level 1 0.120 µg/mL	Level 2 0.450 µg/mL	Level 3 1.50 µg/mL	Level 4 6.00 µg/mL	Patient sample 1 0.502 µg/mL	Patient sample 2 2.27 µg/mL
Intermediate precision	**2.0**	**1.9**	**1.6**	**1.3**	**1.6**	**1.7**
Between-day	0.0	0.0	0.7	0.0	0.5	0.4
Between-calibration	0.9	1.4	0.3	0.6	0.8	0.6
Repeatability	**1.7**	**1.3**	**1.4**	**1.2**	**1.4**	**1.5**
Between-preparation	0.2	0.1	0.5	0.3	0.0	0.0
Between-injection	1.7	1.3	1.3	1.2	1.4	1.5

	Plasma samples CV, %			
	Level 1 0.120 µg/mL	Level 2 0.450 µg/mL	Level 3 1.50 µg/mL	Level 4 6.00 µg/mL
Intermediate precision	**2.0**	**2.1**	**1.5**	**1.4**
Between-day	0.7	0.0	0.1	0.0
Between-calibration	0.7	1.3	0.0	0.6
Repeatability	**1.8**	**1.6**	**1.4**	**1.3**
Between-preparation	0.4	0.0	0.0	0.7
Between-injection	1.7	1.6	1.4	1.1

CV, coefficient of variation; RMP, reference measurement procedure. Conversion factor µg/mL to µmol/L: 4.0. The coefficients of variation for repeatability and intermediate precision, which were determined from the individual variances, are printed in bold.

Table 4: Bias and 95 % CI of native serum and native Li-heparin plasma samples (n=6) of carbamazepine-10,11-epoxide. The mean bias and corresponding confidence intervals were calculated using the individual sample bias of n=6 preparations.

	Concentration, µg/mL	Serum		Plasma	
		Mean bias, %	95 % CI, %	Mean bias, %	95 % CI, %
Level 1	0.120	1.4	−0.2 to 3.0	1.4	0.8 to 2.0
Level 2	0.450	2.5	1.5 to 3.4	3.5	2.3 to 4.7
Level 3	1.50	1.4	0.1 to 2.7	1.9	0.7 to 3.2
Level 4	6.00	1.9	1.0 to 2.8	1.6	0.2 to 2.9
Dilution 1	14.0	3.4	2.8 to 4.0	–	–
Dilution 2	20.0	3.2	2.6 to 3.8	–	–

CI, confidence interval. Conversion factor µg/mL to µmol/L: 4.0.

samples, patient pools, and spiked samples). From these samples, five samples were below the calibration range and two samples could not be measured due to insufficient sample volume and were therefore excluded from the analysis (total n=133 samples). Passing–Bablok regression analysis showed a very good agreement between the two laboratories, resulting in a regression equation with a slope of 1.01 (95 % CI 1.00 to 1.02) and an intercept of −0.002 (95 % CI −0.003 to 0.009) (Figure 5A). The Pearson correlation coefficient was found to be ≥0.999. The plot of the relative Bland–Altman analysis confirmed the good agreement between the two laboratories and showed that the data scatter was independent of analyte concentration with a 2S agreement of 7.1 % (lower limit CI interval of −7.7 to −5.6 %, upper limit CI interval of 6.5–8.7 %). The resulting bias was found to be 0.5 % with a 95 % CI interval from ranging from −0.2 to 1.1 % (Figure 5B).

Precision evaluation at Site 2 showed a mean intermediate precision CV ≤3.4 % and a repeatability CV ≤3.2 % comparable to Site 1. Due to a laboratory error within day three, level 4 was evaluated with n=30 measurements, while all other samples were evaluated with n=36 measurements. The data scatter was in good agreement with the error propagation between the two independent measurements and the method is transferable regardless of the laboratory equipment or personnel.

Uncertainty of results

Total measurement uncertainties and expanded measurement uncertainties for single measurement ranged from 1.6 to 2.2 % and from 3.2 and 4.5 %, respectively (Table 5). The derived total measurement uncertainty is multiplied by a coverage factor of k=2 to obtain an expanded uncertainty that represents a confidence level of 95 %, assuming normal distribution.

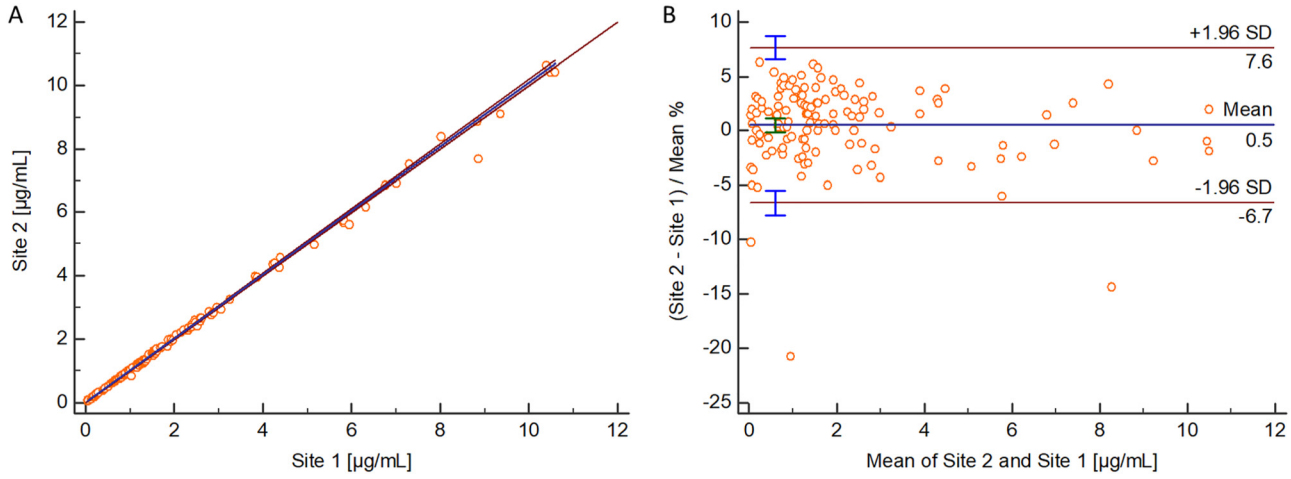

Figure 5: Results from the carbamazepine-10,11-epoxide method comparison study performed between two independent laboratories. (A) Passing–Bablok regression plot including the Pearson regression analysis for the method comparison study of the RMP (n=133 samples) between the independent laboratories (Site 1: Risch; Site 2: Roche). Passing–Bablok regression analysis resulted in a regression equation with a slope of 1.01 (95 % CI 1.00 to 1.02) and an intercept of −0.002 (95 % CI −0.003 to 0.009). The Pearson correlation value was ≥0.999. (B) Bland–Altman plot for the method comparison study of the RMP (n=133 samples) between two independent laboratories (laboratory 1: Risch site, and laboratory 2: Roche site). The inter-laboratory measurement bias was 0.5 % (95 % CI interval from −0.2 to 1.1 %) and the 2S interval of the relative difference was 7.1 % (lower limit CI interval from −7.6 to −5.6 %, upper limit CI interval from 6.5 to 8.7 %).

Table 5: Overview of measurement uncertainty for carbamazepine-10,11-epoxide quantification with the candidate RMP in serum samples.

	Level					
	Level 1 0.120 µg/mL	**Level 2 0.450 µg/mL**	**Level 3 1.50 µg/mL**	**Level 4 6.00 µg/mL**	**Patient sample 1 5.02 µg/mL**	**Patient sample 2 2.27 µg/mL**
Type B uncertainty: calibrator preparation, CV, %	**0.83**	**0.83**	**0.83**	**0.78**	**0.83**	**0.83**
Characterization of reference material	0.2	0.2	0.2	0.2	0.2	0.2
Preparation of						
Stock solution	0.31	0.31	0.31	0.31	0.31	0.31
Working solution	0.43	0.43	/	/	0.43	/
Spike solution	0.56	0.56	0.55	0.49	0.56	0.55
Matrix based calibrator	0.83	0.83	0.83	0.78	0.83	0.83
Type A uncertainty: mean of measurement results, CV, %	**2.0**	**1.9**	**1.6**	**1.3**	**1.6**	**1.7**
Total measurement uncertainty (k=1), CV, %	**2.1**	**2.2**	**1.8**	**1.6**	**1.8**	**1.9**
Expanded measurement uncertainty (k=2), CV, %	**4.3**	**4.5**	**3.6**	**3.2**	**3.7**	**3.7**

CV, coefficient of variation. Conversion factor µg/mL to µmol/L: 4.0. The total measurement uncertainty of the whole approach for target value assignment estimated as a combination of the uncertainty of calibrator preparation (type B uncertainty) and uncertainty of the precision experiment (type A uncertainty) are given in bold.

Discussion

The presented ID-LC-MS/MS based candidate RMP allows the quantification carbamazepine-10,11-epoxide in human serum and plasma. The method design based on routine measurements and targeted therapeutic analyte concentrations in human serum and plasma specimens. Protein precipitation followed by dilution was found to be more effective than liquid–liquid extraction or solid-phase extraction. Baseline separation from the metabolites and the drug carbamazepine was achieved using a reversed phase chromatography and detection was performed using a tandem MS with an ESI.

Since no primary reference material is available, material characterized by qNMR were utilized to calibrate the assay. qNMR characterization allowed an unbroken traceability chain from the RMP to SU units (kg and mole) to be established.

The preparation of calibrator solutions was presented with transparency, including associated uncertainty terms. Concentrations of calibrator and control solutions were carefully optimized to adequately cover the measurement range sufficiently. The pipetting scheme including the choice of pipet volumes and number of pipetting steps was optimized to minimize the uncertainty contributions.

The development of the method involved selecting a stationary phase, optimizing mobiles phase composition gradient, and ion source, as well as sample preparation. This included selecting optimal pipettes for fluid handling, determining equilibration times for protein precipitation, diluting samples into the linear range of the MS detector, establishing an optimal calibration and control scheme, and preparing calibrator and control materials with careful pipetting.

The validation study confirmed that the developed analytical method met the sensitivity, selectivity, and reproducibility requirements for an RMP. In addition, the absence of MEs was evaluated using a calibration slope comparison and an ion yield attenuation experiment. The measurement uncertainty for multiple measurements (n=6) was found to be within the desired target measurement uncertainty of 5.0 % (k=2). The developed method showed in general better measurement uncertainties (≤2.2 %) than intra/inter day CVs of published methods. In these the CV ranged from 0.4 to 12.0 % [20–24]. Moreover, the presented candidate RMP seems to be less affected by MEs than the cited studies, ≤3 % compared to ≤9.9 % [20] and ≤4 % [21].

The method transfer to a second independent laboratory showed that it is possible without significant increase in bias between laboratories. This proves that the RMP, including the preparation of the calibration solutions, and the samples preparation protocols, is robustly designed. In addition, the method comparison study showed that the method is suitable for processing a high-volume patient sample in a relatively short time.

Conclusions

We presented a LC-MS/MS-based candidate RMP for the detection of carbamazepine-10,11-epoxide in human serum and plasma. It provides a traceable and reliable platform for the standardization of routine assays and evaluation of clinically relevant samples.

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

Informed consent: Not applicable.

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

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

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

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