**Supplemental Material 1:**

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

**Short title:** An ID-LC-MS/MS-based candidate RMP for the quantification of lamotrigine

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***Test instruction***

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1. Summary of Test Principle and Scope of Application

The present procedure describes an isotope-dilution LC-MS/MS method for quantitative determination of lamotrigine in human serum and plasma. Analyte detection in the range of 0.600 to 24.0 µg/mL is performed in MRM (multiple reaction monitoring) mode.

Samples (calibrators, QC and other samples) are prepared by adding internal standard (ISTD) and incubating them on a lab shaker for 15 minutes at 37 °C. Proteins are removed by means of precipitation and subsequent centrifugation. Analysis is run on an Agilent 1290 Infinity II LC system coupled to a Sciex QTrap 6500+ / TripleQuad 6500+ mass spectrometer, operated in ESI (electrospray ionisation) positive mode. Chromatographic separation is achieved on an Agilent Zorbax Eclipse XDB-C18 column (100 x 3 mm, 3.5 µm). Mobile phase A consists of water with 2 mM ammonium acetate and 0.1 % formic acid, mobile phase B of methanol / 2 mM ammonium acetate in water 95+5 (v+v) with 0.1 % formic acid.

Calibration is performed by calculating the peak area ratios of analyte to ISTD:

The response factors are plotted against the calibrator concentrations and the unknown samples are determined by means of their specific peak area ratios.

1. Safety Precautions

**CAUTION:** Observe universal precautions, wear laboratory coats, safety glasses, and protective gloves during all steps of this method. Consider all serum / plasma specimen received for analysis as potentially positive infectious. Handling of biological materials in an microbiological safety cabinet is recommended. All areas in which biological material was handled have to be disinfected after work is completed. Any residual sample material as well as any plastic or glassware that contacts biological material has to be disposed by autoclaving; liquid waste has to be chemically deactivated. If no autoclave is available, residual biological sample material and contaminated material is to be disposed of in infectious waste containers.

**CAUTION:** For all chemicals, reagents and solvents used in this reference measurement procedure „Material safety data sheets“ (MSDSs) have to be considered and can be viewed e.g. on the Sigma Aldrich Homepage (<http://www.sigmaaldrich.com/sigma-aldrich/home.html>) or on the VWR Homepage (<http://www.vwr.com/index.htm>).

1. Equipment and Instrumentation

|  |  |
| --- | --- |
| Analytical instrument | e.g. Mass spectrometer Sciex QTrap 6500+ / TripleQuad 6500+ coupled to an Agilent 1290 Infinity II LC system.\*)  The Agilent 1290 Infinity II LC system consists of a binary pump (e.g. G7120A), a column temperature control device (e.g. G7116B), and a thermostatted autosampler (e.g. G7167B).  Data evaluation is performed with the Sciex Analyst® software.  \*) Alternatively, any conventional triple-quadrupole mass spectrometer equipped with an ESI source and upstream HPLC as inlet system (with comparable resolution, sensitivity and mass range) can be used, if the system suitability test has been passed successfully |
| Analytical column | Agilent Zorbax Eclipse XDB-C18, 100 x 3 mm, 3.5 µm (Art. No. 961967-302) |
| Pipettes | e.g. Eppendorf Research Plus variable  10 – 100 µL, with epT.I.P.S.® 2 – 200 µL (Art. No. 30073436) 20 – 200 µL, with epT.I.P.S.® 2 – 200 µL (Art. No. 30073436) 100 – 1000 µL, with epT.I.P.S.® 50 – 1000 µL (Art. No. 30073479) 500 – 5000 µL, with epT.I.P.S.® 0.1 – 5 mL (Art. No. 30000978)  Eppendorf Multipette® M4  with Combitips advanced®, 5.0 mL (Art. No. 0030089456)  Eppendorf Multipette® E3 / E3x with Combitips advanced®, 0.1 mL (Art. No. 0030089405), 0.2 mL (Art. No. 0030089413), 0.5 mL (Art. No. 0030089421), 1.0 mL (Art. No. 0030089642), 2.5 mL (Art. No. 0030089804), 5.0 mL (Art. No. 0030089456)  Gilson Microman® E  1 - 10 µL, with capillary pistons CP10 (Art. No. F148312) 10 – 100 µL, with capillary pistons CP100 (Art. No. F148314) 50 – 250 µL, with capillary pistons CP250 (Art. No, F148014) 100 – 1000 µL, with capillary pistons CP1000 (Art. No. F148560) |
| Analytical balance | e.g. Sartorius Genius M235-0CE  Mettler Toledo AT261 Delta Range |
| Microbalance | e.g. Mettler Toledo XP6U/M  Mettler Toledo XPR2 |
| Centrifuge | e.g. Eppendorf 5430R with rotor FA-45-48-11  Eppendorf 5427R with rotor FA-45-30-11 |
| Thermomixer | e.g. Eppendorf Thermomixer C with thermoblock for 1.5 mL and 5 mL tubes |
| Vortex | e.g. Scientific Industries, Vortex Genie® 2 |
| Ultrasonic bath | e.g. Bandelin Sonorex Digital 10P |
| Reaction tubes | e.g. Eppendorf Safe-Lock Tubes, 1.5 mL (Art. No. 0030120086),  2 mL (Art. No. 0030120094)  Eppendorf Tubes 5 mL (Art. No. 0030119380) |
| HPLC vials / caps | e.g. Wicom 2 mL glass vials (Art. No. WIC42700)  with PTFE / silicone seal snap cap (Art. No. WIC44770) or Wicom 2 mL glass vials (Art. No. WIC41205) with PTFE / silicone seal screw caps (Art. No. WIC43959) |
| Volumetric flasks | e.g. Blaubrand® borosilicate glass,  USP certified: 5 mL, NS 10/19 (Art. No. 36938), 10 mL, NS 10/19 (Art. No. 36943) |
| Measuring cylinder | e.g. Blaubrand® borosilicate glass, 100 mL (Art. No. 32138),  1000 mL (Art. No. 32162) |
| Screw cap bottles | e.g. Duran® Youtility borosilicate glass, 1 L, with PP screw caps GL45  (Art. No. 218815457) Duran® borosilicate glass , 100 mL, with PP screw cap GL 45 (Art. No. 218012458) Duran® borosilicate glass, 25 mL, with PP screw caps GL25 (Art. No. 218011453) |
| Screw cap vials | e.g. Wicom 8 mL glass vials (Art. No. WIC41500) with PTFE / rubber seal screw cap (Art. No. SEE53421) |
| Glass pipettes | e.g. Poulten & Graf GmbH, FORTUNA®, 1 mL measuring pipette  (Art. No. 11421904) |
| Spatulas | e.g. LevGo smartSpatula® micro, PP (Art. No. 17231) |
| Weighing boats | e.g. Säntis Analytical tin boats, various volumes  (Art. No. SA76982802, SA76983102) |
| Weighing dishes | e.g. VWR aluminium oval micro weigh dish (Art. No. 611-1358)  WHEATON® aluminium weighing dishes (VWR Art. No. WHEA370792) |
| Pasteur pipettes | e.g. Brand® Pasteur pipettes (glass), 1.5 mL (Art. No. 747715) with suction cups, silicone (HuberLab, Art. No. 15.2172.02) |

1. Preparation of Reagents, Calibration Standards, Controls and all other Materials

All listed chemicals can be purchased from different vendors / manufacturers, provided that the corresponding qualities are available. Certified reference materials which fit the intended use should be applied if available. A given certified reference material is supported by documentation containing source of material, measurement results, and metrological traceability. If a different material is used, the determination of the absolute content including measurement uncertainty by qNMR analysis is mandatory.

For all chemicals, reagents, and solvents used in this reference measurement procedure, material safety data sheets (MSDS) have to be considered. All reagents / solutions have to be clearly labelled and signed with hazard symbols, if applicable.

The laboratory balance used must be calibrated and certified according to the manufacturer's specifications. The minimum sample weight has to be determined according to USP guidelines (USP Chapters 41 and 1251). Each weighing process has to be in accordance with the determined minimum sample weight.

Pipettes used have to be calibrated and certified by the manufacturer. Calibration and re-calibration have to comply with the manufacturer's requirements.

For pipetting organic solvents and serum / plasma, only use positive displacement pipettes, such as Gilson Microman® E, Eppendorf Multipette® E3 / E3x, or Eppendorf Multipette® M4.

Volumetric flasks have to be of the highest quality and have to fulfill requirments of ISO 1042 and/or USP. Glassware with ISO and/or USP certificates is mandatory.

* 1. Chemicals and Reagents

| **Material** | **Vendor / Manufacturer** |
| --- | --- |
| Lamotrigine CAS No. 84057-84-1 | Sigma-Aldrich Supelco Cat. No. PHR1392 |
| [13C3D3]-Lamotrigine CAS No. 1246815-13-3 | Brunschwig Cat. No. TRCL173253-1mg |
| Methanol absolute, LCMS grade CAS No. 67-56-1 | Biosolve Cat. No. 136841 |
| Dimethylsulfoxide, ACS reagent, ≥ 99.9 % CAS No. 67-68-5 | Sigma-Aldrich Cat. No. 472301 |
| Water, ultrapure | In-house: Millipore Milli-Q System Direct-Q 3UV |
| Ammonium acetate. LCMS grade CAS No. 631-61-8 | Sigma-Aldrich LiChropur Cat. No. 73594 |
| Formic acid, LCMS grade CAS No. 64-18-6 | Biosolve Cat. No. 0006914139BS |
| 2-Propanol, HPLC grade CAS No. 67-63-0 | Riedel-de Haën Cat. No. 34863 |
| Analyte-free human serum, normal (matrix) | Merck Millipore Cat. No. « S1-Liter » |
| TDM free human serum (surrogate matrix) | Roche ID No. 12095432001 |

* 1. Preparation of Reagents

If the volume of solutions for sample preparation or chromatographic analysis is not sufficient, a whole multiple of the given volume has to be prepared as described. Final volumes should be combined prior to use.

Volumetric flasks, measuring cylinders and screw cap bottles are cleaned with methanol or acetone and / or with Milli-Q water before use (dependent on the eluent that will be used). The use of a dishwasher is not suitable for USP volumetric flasks.

* + 1. Mobile Phase

|  |  |  |
| --- | --- | --- |
| **2 mM Ammonium acetate in water:** | | Weigh 154 ± 2 mg ammonium acetate on an analytical balance into a beaker. Measure 1 L water in a 1 L measuring cylinder. Transfer the ammonium acetate to the eluent bottle by rinsing the beaker with a portion of the water. Mix the solution until dissolved. |
| **Eluent A:** | **2 mM Ammonium acetate + 0.1 % formic acid** | |
|  | Measure 950 mL 2 mM ammonium acetate solution in a 1 L measuring cylinder and transfer the solution into an eluent bottle. Add 950 µL formic acid. | |
|  | After mixing, the solution may be degassed in an ultrasonic bath for 5 min at 50 % intensity (optional). | |
| **Eluent B:** | **MeOH / 2 mM ammonium acetate 95+5 (v+v) + 0.1 % formic acid** | |
|  | Measure 950 mL methanol in a 1 L measuring cylinder. Measure 50 mL 2 mM ammonium acetate solution in a 100 mL measuring cylinder and combine both solutions in an eluent bottle. Add 1000 µL formic acid. | |
|  | After mixing, the solution may be degassed in an ultrasonic bath for 5 min at 50 % intensity (optional). | |

Store eluents A and B at room temperature for a maximum of four weeks.

* + 1. Autosampler Wash Solution

|  |  |
| --- | --- |
| **75 % MeOH** (1+3, v+v) | Measure 750 mL methanol and 250 mL Milli-Q water in a 1 L measuring cylinder and mix both solutions in a 1 L screw cap bottle (glass). |

Store autosampler wash solution at room temperature for maximum of 6 months.

* + 1. Seal Wash Solution

|  |  |
| --- | --- |
| **20 % 2-Propanol** (1+4, v+v) | Measure 200 mL 2-propanol and 800 mL Milli-Q water in a 1 L measuring cylinder and mix both solutions in a 1 L screw cap bottle (glass). |

Store seal wash solution at room temperature for maximum of four weeks.

* + 1. Protein Precipitation Solution

|  |  |
| --- | --- |
| **75 % MeOH** (3+1, v+v) | Measure 750 mL methanol and 250 mL Milli-Q water in a 1 L measuring cylinder and mix both solutions in a 1 L screw cap bottle (glass). |

Store protein precipitation solution at room temperature for maximum of six months. It is recommended to use smaller aliquots of the prepared solution for sample preparation to avoid contamination of the solution.

* 1. Preparation of Calibration Standards

As primary reference material lamotrigine (Sigma-Aldrich Supelco, Cat. No. PHR1392) is used. The absolute content of analyte needs to be determined by qNMR (including measurement uncertainty).

Calibrator stock / working solutions and spike solutions are prepared in volumetric flasks. Glass Pasteur pipettes should be used for adding solvent to the flask. Weigh lamotrigine substance in disposable weighing boats on a microbalance.

**Note:** Final calibrator concentrations are allowed to vary for a maximum of ± 10% from the target value. Purity has to be either included by weighing more in order to achieve the target concentration, or by multiplying the weighed amount with the purity factor. Final concentrations in matrix have to be adjusted.

If other pipettes or glassware than indicated are used, the measurement uncertainty must be recalculated.

* + 1. Lamotrigine Stock Solutions

Two calibrator stock solutions are prepared independently.

|  |  |
| --- | --- |
| **Material** | **Target** |
| Lamotrigine | approx. 60 mg |
| DMSO | approx. 10 mL |

Place an aluminium weighing dish on the balance (optional) and tare. More than one weighing boat may be necessary for weighing lamotrigine substance. Place the weighing boat(s) on the aluminium dish or directly on the balance and determine the weight of the boat(s). Tare the balance before adding the substance.

**Note:** If multiple individual weighing steps are preferred, this has to be considered for the calculation of measurement uncertainty.

|  |  |
| --- | --- |
| **Stock solution 1:** | Weigh 30 ± 1 mg lamotrigine in the disposable weighing boat(s) and transfer it to a 5 mL volumetric flask. Fill to the mark with DMSO. Make sure all analyte is completely dissolved before using the solution.  c = 6.0 mg/mL (± 0.2 mg/mL) |
| **Stock solution 2:** | Weigh 30 ± 1 mg lamotrigine in the disposable weighing boat(s) and transfer it to a 5 mL volumetric flask. Fill to the mark with DMSO. Make sure all analyte is completely dissolved before using the solution.  c = 6.0 mg/mL (± 0.2 mg/mL) |

Prepare lamotrigine stock solutions for calibrators freshly.

* + 1. Lamotrigine Working Solutions

|  |  |
| --- | --- |
| **Material** | **Target** |
| Lamotrigine stock solution 1 | approx. 500 µL |
| Lamotrigine stock solution 2 | approx. 500 µL |
| DMSO | approx. 10 mL |

|  |  |
| --- | --- |
| **Working solution 1:** | Pipet 500 µL lamotrigine stock solution 1 into a 5 mL volumetric flask. Fill to the mark with DMSO and mix thoroughly.  Pipette to be used: Eppendorf Multipette® E3/E3x, Combitip advanced® 0.5 mL  c = 0.60 mg/mL (± 0.02 mg/mL) |
| **Working solution 2:** | Pipet 500 µL lamotrigine stock solution 2 into a 5 mL volumetric flask. Fill to the mark with DMSO and mix thoroughly.  Pipette to be used: Eppendorf Multipette® E3/E3x, Combitip advanced® 0.5 mL  c = 0.60 mg/mL (± 0.02 mg/mL) |

Prepare lamotrigine working solutions for calibrators freshly.

* + 1. Calibrator Spike Solutions

|  |  |
| --- | --- |
| **Material** | **Target** |
| Lamotrigine stock solution 1 | approx. 1775 µL |
| Lamotrigine stock solution 2 | approx. 3000 µL |
| Lamotrigine working solution 1 | approx. 500 µL |
| Lamotrigine working solution 2 | approx. 1400 µL |
| DMSO | approx. 34 mL |

Prepare calibrator spike solutions as outlined in the following table.

All calibrator spike solutions are prepared in 5 mL volumetric flasks. Calibrator spike solutions 1, 3, 5, and 7 are prepared from stock / working solution 1, spike solutions 2, 4, 6, and 8 are prepared from stock / working solution 2. After pipetting the respective stock or working solution, fill the volumetric flask with DMSO to the mark and mix thoroughly.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Calibrator spike solution | Solution | V Solution [µL] | V flask  [mL] | Conc. [µg/mL] | Pipette to be used |
| 1 | Lamotrigine working solution 1 | 500 | 5 | 60 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 0.5 mL |
| 2 | Lamotrigine working solution 2 | 1000 | 5 | 120 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 1.0 mL |
| 3 | Lamotrigine stock solution 1 | 150 | 5 | 180 | Gilson Microman® E, 50 – 250 µL |
| 4 | Lamotrigine stock solution 2 | 250 | 5 | 300 | Gilson Microman® E, 50 – 250 µL |
| 5 | Lamotrigine stock solution 1 | 375 | 5 | 450 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 0.5 mL |
| 6 | Lamotrigine stock solution 2 | 750 | 5 | 900 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 1.0 mL |
| 7 | Lamotrigine stock solution 1 | 1250 | 5 | 1500 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 2.5 mL |
| 8 | Lamotrigine stock solution 2 | 2000 | 5 | 2400 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 2.5 mL |

* + 1. Serum Calibrator Solutions

|  |  |
| --- | --- |
| **Material** | **Target (5 mL aliquots)** |
| Analyte-free serum | approx. 45 mL |
| Lamotrigine calibrator spike solutions | 50 µL each |
| DMSO | 50 µL |

Serum calibrators are prepared as 5 mL aliquots.

Prepare nine 5 mL Eppendorf tubes and pipet 4950 µL analyte-free serum into each. Add 50 µL of the respective calibrator spike solution 1 – 8 to the corresponding Eppendorf tube. For the zero sample (calibrator 0), add 50 µL DMSO to the serum.

|  |  |  |  |
| --- | --- | --- | --- |
| Calibrator | V Serum [µL] | V Spike solution [µL] | V DMSO [µL] |
| 1 – 8 | 4950 | 50 | - |
| Zero | 4950 | - | 50 |
| Pipette to be used | Eppendorf Multipette® E3 / E3x, Combitip advanced® 5 mL | Gilson Microman® E, 10 – 100 µL | Gilson Microman® E,  10 – 100 µL |

Incubate the serum calibrator samples on a Thermomixer for 15 minutes at 37 °C and 500 rpm.

Aliquots of at least 75 µL can be aliquoted in 1.5 mL Eppendorf tubes and stored at – 20 °C for later use.

Serum calibrators are stable for 28 days when stored at – 20 °C.

**Note:** For one sample preparation, 50 µL serum calibrator is used and an aliquot of 75 µL is sufficient. If more than one sample preparation is to be performed, aliquots of higher volumes can be prepared.

The final concentrations of serum calibrator solutions are given in the following table. Note that the exact concentration depends on the purity of the analyte, the exact weighing, and the volume displaced by the weighing boat(s) used (see chapter 7).

|  |  |
| --- | --- |
| **Serum Calibrator** | **Concentration [µg/mL]** |
| Zero (Cal 0, matrix blank) | 0.000 |
| Cal 1 | 0.600 |
| Cal 2 | 1.20 |
| Cal 3 | 1.80 |
| Cal 4 | 3.00 |
| Cal 5 | 4.50 |
| Cal 6 | 9.00 |
| Cal 7 | 15.0 |
| Cal 8 | 24.0 |

**Note:** The nominal concentrations of calibrator samples are entered into the instrument software with five significant figures.

* 1. Control Samples

Quality Control (QC) stock / working solutions and spike solutions are prepared in volumetric flasks. Glass Pasteur pipettes should be used for adding solvent to the flask. Weigh lamotrigine substance in disposable weighing boats on a microbalance.

**Note:** Final quality control concentrations are allowed to vary for a maximum of ± 10% from the target value. Purity has to be either included by weighing more in order to achieve the target concentration, or by multiplying the weighed amount with the purity factor. Final concentrations in matrix have to be adjusted.

* + 1. QC Stock solution

Prepare one QC stock solution.

|  |  |
| --- | --- |
| **Material** | **Target** |
| Lamotrigine | approx. 25 mg |
| DMSO | approx. 5 mL |

Place an aluminium weighing dish on the balance (optional) and tare. More than one weighing boat may be necessary for weighing lamotrigine substance. Place the weighing boat(s) on the aluminium dish or directly on the balance and determine the weight of the boat(s). Tare the balance before adding the substance.

|  |  |
| --- | --- |
| **QC Stock solution:** | Weigh 25 ± 1 mg lamotrigine in the disposable weighing boat(s) and transfer it to a 5 mL volumetric flask. Fill to the mark with DMSO. Make sure all analyte is completely dissolved before using the solution.  c = 5.0 mg/mL (± 0.2 mg/mL) |

Prepare the lamotrigine QC stock solution freshly.

* + 1. QC Working solution

|  |  |
| --- | --- |
| **Material** | **Target** |
| QC stock solution | approx. 500 µL |
| DMSO | approx. 5 mL |

|  |  |
| --- | --- |
| **QC Working solution:** | Pipet 500 µL QC stock solution 1 into a 5 mL volumetric flask. Fill to the mark with DMSO and mix thoroughly.  Pipette to be used: Eppendorf Multipette® E3 / E3x, Combitip advanced® 0.5 mL  c = 0.5 mg/mL (± 0.02 mg/mL) |

Prepare the lamotrigine QC working solution freshly.

* + 1. QC Spike Solutions

|  |  |
| --- | --- |
| **Material** | **Target** |
| QC stock solution | approx. 2800 µL |
| QC working solution | approx. 2900 µL |
| DMSO | approx. 14.3 mL |

Prepare QC spike solutions as outlined in the following table.

QC spike solutions are prepared in 5 mL volumetric flasks from the working / stock solution as indicated below.

After pipetting the respective stock or working solution, fill the volumetric flask with DMSO to the mark and mix thoroughly.

| QC spike solution | Solution | V Solution [µL] | V flask [mL] | Conc. [µg/mL] | Pipette to be used |
| --- | --- | --- | --- | --- | --- |
| 1 | QC working solution | 900 | 5 | 90 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 1.0 mL |
| 2 | QC working solution | 2000 | 5 | 200 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 2.5 mL |
| 3 | QC stock solution | 800 | 5 | 800 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 1.0 mL |
| 4 | QC stock solution | 2000 | 5 | 2000 | Eppendorf Multipette® E3 / E3x, Combitip advanced® 2.5 mL |

* + 1. Serum QC Solutions

|  |  |
| --- | --- |
| **Material** | **Target (5 mL aliquots)** |
| Analyte-free serum | approx. 20 mL |
| QC spike solutions | 50 µL each |

Serum QC samples are prepared as 5 mL aliquots.

Prepare four 5 mL Eppendorf tubes and pipet 4950 µL analyte-free serum into each. Add 50 µL of the respective QC spike solution 1 – 4 to the corresponding Eppendorf tube.

|  |  |  |
| --- | --- | --- |
| QC Sample | V Serum [µL] | V Spike solution [µL] |
| 1 – 4 | 4950 | 50 |
| Pipette to be used | Eppendorf Multipette® E3 / E3x,  Combitip advanced® 5 mL | Gilson Microman® E,  10 – 100 µL |

Incubate the serum QC samples on a Thermomixer for 15 minutes at 37°C and 500 rpm.

Aliquots of at least 75 µL can be aliquoted in 1.5 mL Eppendorf tubes and stored at -20°C for later use.

Serum calibrators are stable for 28 days when stored at -20°C.

**Note:** For one sample preparation, 50 µL serum QC sample is used and an aliquot of 75 µL is sufficient. If more than one sample preparation is to be performed, aliquots of higher volumes can be prepared.

The final concentrations of serum QC solutions are given in the following table. Note that the exact concentration depends on the purity of the analyte, the exact weighing, and the volume displaced by the weighing boat(s) used (see chapter 7).

|  |  |
| --- | --- |
| **Serum QC** | **Concentration [µg/mL]** |
| QC 1 | 0.900 |
| QC 2 | 2.00 |
| QC 3 | 8.00 |
| QC 4 | 20.0 |

**Note:** The nominal concentrations of QC samples are entered into the instrument software with five significant figures.

* 1. Internal Standard Solution

Commercially available isotope-labelled material from other vendors / manufacturers can be used, if they are available in the desired isotopic composition.

* + 1. Internal Standard Stock Solution

|  |  |
| --- | --- |
| **Material** | **Target** |
| [13C3D3]-Lamotrigine | approx. 1 mg |
| DMSO  Target concentration | approx. 1 mL  1.0 mg/mL ± 0.1 mg/mL |

Prepare a 1 mg/mL solution of [13C3D3]-lamotrigine by pipetting DMSO directly into the manufacturer's container to dissolve the whole amount of [13C3D3]-lamotrigine (approximately 1 mg). In order to reach the target concentration, the volume of the added solvent has to be adapted according to the purity and amount of the ISTD in the container. Make sure the substance is completely dissolved.

Prepare aliquots of the final solution of e.g.330 µL in HPLC vials with screw caps and store the ISTD stock solution at ‑20°C for later use.

* + 1. Internal Standard Working Solution

|  |  |
| --- | --- |
| **Material** | **Target** |
| [13C3D3]-Lamotrigine stock solution | approx. 5 µL |
| DMSO | approx. 100 µL |
| Milli-Q water | approx. 4 mL |

Add 5 µL ISTD stock solution to 95 µL DMSO, mix, and add 3900 µL Milli-Q water:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Dilution | Solution | V Solution [µL] | V DMSO [µL] | Conc. [µg/mL] |
| Intermediate solution | ISTD stock solution | 5 | 95 | 50 |
| Pipette |  | Gilson Microman E, 1 – 10 µL | Gilson Microman E, 10 – 100 µL |  |
| Dilution | Solution | V Solution [µL] | V H2O [µL] | Conc. [µg/mL] |
| ISTD working solution | Intermediate solution | 100 | 3900 | 1.25 |
| Pipette |  |  | Eppendorf Research, 1 – 5 mL |  |

Prepare the ISTD working solution freshly.

**Note:** The amount of internal standard working solution is sufficient for analysing 35 samples. If more samples are to be prepared, the amounts given may be pipetted multiple times in an appropriate bottle.

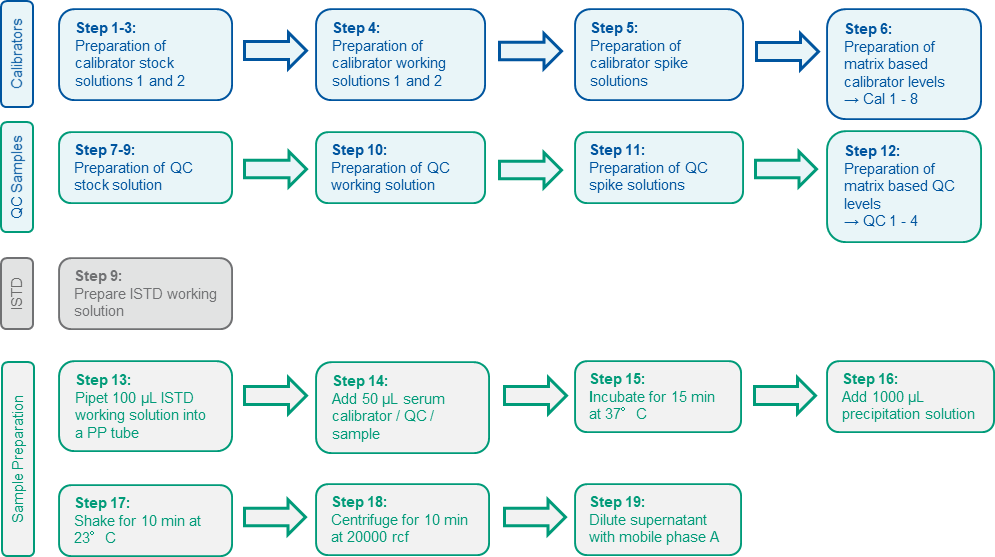
1. Specimen Collection, Storage and Handling Procedure

Serum, plasma (Lithium Heparin plasma, K2-/ K3-EDTA plasma) and analyte-free serum as surrogate matrix can be used as sample matrix. Serum / plasma samples should be transported and stored at below – 20 °C. Avoid several freeze-thaw cycles.

**CAUTION:** Consider all serum / plasma specimen received for analysis as potentially infectious. Handling of biological materials in a microbiological safety cabinet is recommended. All areas in which biological material was handled have to be disinfected after work is completed. Any residual sample material as well as any plastic or glass ware that contacts biological material has to be disposed by autoclaving; liquid waste has to be chemically deactivated. If no autoclave is available, residual biological sample material and contaminated material is to be disposed of in infectious waste containers.

|  |  |
| --- | --- |
| **Material** | **Target per sample** |
| Native sample, calibrator, control | 50 µL |
| Internal standard working solution | 100 µL |
| Precipitation solution (75 % MeOH) | 1000 µL |
| Mobile phase A | 1880 µL |

A schematic illustration of the sample preparation process is given below:



|  |  |  |
| --- | --- | --- |
| **Step** | **Volume** | **Pipette to be used** |
| Addition of ISTD | | |
| ISTD working solution | 100 µL | Eppendorf Research, 10 – 100 µL  alternatively Eppendorf Multipette® E3 / E3x, Combitip advanced® 0,5 mL, dispense mode |
| Serum calibrator / QC sample / native sample | 50 µL | Gilson Microman E, 10 – 100 µL |
|  | Incubate on Thermomixer for 15 min, 37°C, 500 rpm | |
| Protein precipitation | | |
| Precipitation solution (75 % MeOH) | 1000 µL | Eppendorf Multipette® M4,  alternatively Eppendorf Multipette® E3 / E3x, Combitip advanced® 5.0 mL, dispense mode |
|  | Shake on Thermomixer for 10 min, 23°C, 2000 rpm | |
|  | Centrifuge for 10 min, 4°C, 20 000 rcf | |
| Use supernatant for further dilutions. | ~1000 µL |  |

Transfer 100 µL ISTD working solution to a 2 mL Eppendorf tube. Add 50 µL serum calibrator / QC sample / native sample and incubate the solution for 15 minutes on a thermomixer at 37 °C, 500 rpm. For protein precipitation, 1000 µL precipitation solution (75 % methanol) are added, incubated for 10 minutes on a thermomixer at 23°C, 2000 rpm. The samples are then centrifuged for 10 minutes at 4°C, 20 000 rcf, and the supernatant is further diluted with mobile phase A as outlined in the table below:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Dilution step | V sample [µL] | V mobile phase A [µL] | V final [µL] | Dilution factor |
| Dilution step 1 | 70 | 980 | 1050 | 15 |
| Pipette to be used | Gilson Microman® E,  10 – 100 µL | Eppendorf Research, 100 – 1000 µL, alternatively Eppendorf Multipette® E3 / E3x, Combitip advanced® 5,0 mL, dispense mode |  |  |
| Dilution step 2 | 100 | 900 | 1000 | 10 |
| Pipette to be used | Eppendorf Research, 10 – 100 µL | Eppendorf Research, 100 – 1000 µL, alternatively Eppendorf Multipette® E3 / E3x, Combitip advanced® 5,0 mL, dispense mode |  |  |
| Final dilution factor |  |  |  | 150 |

The processed samples are stable for 14 days when stored at 7 °C.

**Sample Dilution**:

Highly concentrated samples (>24.0 µg/mL) are diluted 1+1 (v+v) or 1+19 (v+v) with analyte free serum matrix to a concentration within the working range (0.600 – 24.0 µg/mL) before sample preparation.

Pipetting scheme:

|  |  |  |  |
| --- | --- | --- | --- |
| Dilution factor | V sample [µL] | V serum [µL] | V total [µL] |
| 2 | 75 | 75 | 150 |
| Pipette | Gilson Microman® E, 10 – 100 µL | Gilson Microman® E, 10-100 µL |  |
| Dilution factor | V sample [µL] | V serum [µL] | V total [µL] |
| 20 | 10 | 190 | 200 |
| Pipette | Gilson Microman® E, 1 – 10 µL | Gilson Microman® E, 50-250 µL |  |

1. Procedure Operating Instructions, System Suitability Test (SST) and the Structure of Analytical Series

General instructions for handling and operating the LC-MS system are given in the manufacturer's manual.

* 1. LCMS Parameters
     1. HPLC Parameters

The HPLC parameters of the method are given as follows:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| HPLC system: | e.g. Agilent 1290 Infinity II | | | |
| Column: | Agilent Zorbax Eclipse XDB-C18, 100 x 3 mm, 3.5 µm | | | |
| Mobile Phase A: | 2 mM Ammonium acetate + 0.1 % formic acid | | | |
| Mobile Phase B: | MeOH / 2 mM ammonium acetate 95+5 (v+v) + 0.1 % formic acid | | | |
| Flow rate: | 0.6 mL/min | | | |
| Gradient profile: | Time [min] | % Mobile Phase A | % Mobile Phase B |  |
|  | 0.0 | 100 | 0 |  |
|  | 1.0 | 100 | 0 |  |
|  | 1.9 | 55 | 45 |  |
|  | 2.9 | 55 | 45 |  |
|  | 3.8 | 0 | 100 |  |
|  | 6.0 | 0 | 100 |  |
|  | 6.1 | 100 | 0 |  |
|  | 8.0 | 100 | 0 |  |
| Column temperature: | 40 °C | | | |
| Injection volume: | 5 µL | | | |
| Autosampler temperature: | 7°C | | | |
| Needle wash: | Flush port, at least 4 sec., Standard wash mode | | | |
| Needle wash solution: | 75 % MeOH | | | |

* + 1. MS Parameters

|  |  |  |
| --- | --- | --- |
| Mass spectrometer: | e.g. Sciex QTrap 6500+ / TripleQuad 6500+ | |
| Ionisation mode: | ESI positive | |
| Scan mode: | MRM | |
| Source parameters: | Curtain gas (CUR) | 45 psi |
|  | Collision gas (CAD) | 11 psi |
|  | Voltage (IS) | 5000 V |
|  | Temperature (TEMP) | 400 °C |
|  | Ion source gas (GS1) | 50 psi |
|  | Ion source gas (GS2) | 60 psi |
|  | Entrance potential (EP) | 10 V |
| Valco Valve (optional): | Waste: 0.0 – 0.8 min, 5.0 – 8.0 min | |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Analyte | Mass transition [Da] | Dwell time [ms] | DP [V] | CE [V] | CXP [V] |
| Lamotrigine quantifier | 256.1 → 211.0 | 50 | 89 | 35 | 15 |
| Lamotrigine qualifier | 256.1 → 145.0 | 50 | 89 | 55 | 15 |
| [13C3D3]-Lamotrigine quantifier | 262.0 → 217.0 | 50 | 89 | 35 | 15 |
| [13C3D3]-Lamotrigine qualifier | 262.0 → 148.0 | 50 | 89 | 55 | 15 |

All settings and conditions in section 6.1.2 are only standard values and may vary from device to device.

**Method transfer to another device / tuning procedure:**

|  |  |  |  |
| --- | --- | --- | --- |
| Analyte solution: | Lamotrigine, 10 ng/mL in mobile phase A / mobile phase B 1+1 (v+v) | | |
| MS profile: | Manual tuning mode, no LC flow | | |
| Ionisation mode: | ESI positive | | |
| Syringe pump flow: | 7 µL/min | | |
| Source parameters: | as described above | | |
| Parent mass: | 256.1 Da | Product ions: | m/z 211.0, 145.0 |

* 1. System Suitability Test (SST)

The SST samples consist of lamotrigine in mobile phase A and are used to evaluate the chromatographic performance (retention time), the sensitivity (S/N ratio), and possible carry-over effects of the system setup. For each analysis run, the system suitability test must be passed.

* + 1. Preparation of SST samples

**SST stock solution**

|  |  |
| --- | --- |
| **Material** | **Target** |
| Lamotrigine | approx. 10 mg |
| DMSO | approx. 10 mL |

Place an aluminium weighing dish on the balance (optional) and tare. Place the weighing boat on the aluminium dish or directly on the balance and determine the weight of the boat. Tare the balance before adding the substance.

**Note:** Final SST concentrations are allowed to vary for a maximum of ± 10% from the target value. Purity has to be either included by weighing more in order to achieve the target concentration, or by multiplying the weighed amount with the purity factor. Final concentrations have to be adjusted.

|  |  |
| --- | --- |
| **SST Stock solution:** | Weigh 10 ± 0.5 mg lamotrigine in the disposable weighing boat and transfer it to a 10 mL volumetric flask. Fill to the mark with DMSO. Make sure all analyte is completely dissolved before using the solution.  c = 1.00 mg/mL (± 0.05 mg/mL) |

Prepare aliquots of the SST stock solution of e.g. 330 µL in HPLC vials with screw caps and store the SST stock solution at – 20 °C for later use.

**SST samples**

|  |  |
| --- | --- |
| **Material** | **Target** |
| Lamotrigine stock solution | approx. 10 µL |
| DMSO | approx. 1 mL |
| Mobile phase A | approx. 4 mL |

Prepare two SST samples. The concentrations of lamotrigine in SST 1 and SST 2 correspond to the final dilutions of calibrator 1 and calibrator 8, respectively.

|  |  |  |
| --- | --- | --- |
| SST 1: | Lamotrigine in mobile Phase A, 0.174 ng/mL | (corresponds to calibrator 1) |
| SST 2: | Lamotrigine in mobile Phase A, 6.96 ng/mL | (corresponds to calibrator 8) |

The lamotrigine SST stock solution is diluted with DMSO and mobile phase a as indicated below. Three dilutions are prepared, the final solutions are diluted from dilution solution 2 and 3, respectively.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Dilution 1: | |  |  |  |  |  |
| Solution | conc. [µg/mL] | Target conc. [µg/mL] | V Stock solution [µL] | V DMSO [µL] | V final [µL] | Dilution factor |
| Stock solution | 1000 | 10 | 10 | 990 | 1000 | 100 |
| Pipette |  |  | Gilson Microman® E, 1 - 10 µL | Gilson Microman® E, 100 – 1000 µL |  |  |
| Dilution 2: | |  |  |  |  |  |
| Solution | conc. [µg/mL] | Target conc. [µg/mL] | V Dilution 1 [µL] | V mobile phase A [µL] | V final [µL] | Dilution factor |
| Dilution 1 | 10 | 0.1 | 10 | 990 | 1000 | 100 |
| Pipette |  |  | Gilson Microman® E, 1 - 10 µL | Eppendorf Research, 100 – 1000 µL |  |  |
| Dilution 3: | |  |  |  |  |  |
| Solution | conc. [µg/mL] | Target conc. [µg/mL] | V Dilution 2 [µL] | V mobile phase A [µL] | V final [µL] | Dilution factor |
| Dilution 2 | 0.1 | 0.001 | 10 | 990 | 1000 | 100 |
| Pipette |  |  | Gilson Microman® E, 1 - 10 µL | Eppendorf Research, 100 – 1000 µL |  |  |
| **SST 1:** |  |  |  |  |  |  |
| Solution | conc. [µg/mL] | Target conc. [µg/mL] | V Dilution 3 [µL] | V mobile phase A [µL] | V final [µL] | Dilution factor |
| Dilution 3 | 0.001 | 0.000174 | 174 | 826 | 1000 | 5.7 |
| Pipette |  |  | Eppendorf Research, 20 – 200 µL | Eppendorf Research, 100 – 1000 µL |  |  |
| **SST 2:** |  |  |  |  | |  |
| Solution | conc. [µg/mL] | Target conc. [µg/mL] | V Dilution 2 [µL] | V mobile phase A [µL] | V final [µL] | Dilution factor |
| Dilution 2 | 0.1 | 0.00696 | 70 | 936 | 1006 | 14.4 |
| Pipette |  |  | Eppendorf Research, 10 – 100 µL | Eppendorf Research, 100 – 1000 µL |  |  |

SST 1 and SST 2 are used to confirm the sensitivity and chromatographic performance in every sequence. SST 1 is used to evaluate the signal to noise ratio (S/N), SST 2 to check for carry-over effects.

The retention time and S/N are calculated using the Analyst software. The lamotrigine peak height is divided by the background noise of the chromatographic system. To determine the background noise, the software calculates the standard deviation of a defined background region (1.0 – 2.5 min).

Evaluation and acceptance criteria:

|  |  |
| --- | --- |
| SST 1: | S/N must be ≥ 10 |
| SST 2: | Evaluate the blank injection following SST 2. The peak area must be ≤ 20 % of the peak area of SST 1. |
| Analyte retention time in both SST samples: 3.3 min ± 0.5 min. | |

**Note:** If the retention time of the analyte is shifted, the data processing method has to be adjusted accordingly.

* 1. System Maintenance

After completion of the measurement and for storage of the analytical column, the LCMS system including the analytical column should be flushed with pure organic solvent and water, e.g. methanol / water 80+20 (v+v).

* 1. Calibration

Calibration is performed in bracketing mode and calibrator levels are measured in increasing concentration. Both calibration lines are used to generate the final calibration function.

The calibration curve is generated from the area ratios analyte / ISTD from eight calibrators (Cal 1 – Cal 8), using a linear fit with a 1/x2 weighting, not forcing the curve through the origin.

The calibration functions are obtained by linear regression of the area ratios of the analyte and internal standard (y) against the analyte concentration (x), resulting in the function *y = a x + b*.

* 1. Structure of Analytical Series

|  |  |
| --- | --- |
| Blank | (mobile phase A), at least two injections |
| SST 1 | (lamotrigine in mobile phase A, 0.174 ng/mL) |
| SST 2 | (lamotrigine in mobile phase A, 6.96 ng/mL) |
| Blank SST 1 | (mobile phase A) |
| Blank SST 2 | (mobile phase A) |
| Zero | (matrix blank, Cal 0) |
| Blank | (mobile phase A), at least two injections |
| Calibrators 1 - 8 | (inject in increasing concentration) |
| Blank | (mobile phase A), at least two injections |
| QC samples 1 - 4 | (inject in increasing concentration) |
| Blank | (mobile phase A), at least two injections |
| Samples\*, \*\* |  |
| Blank | (mobile phase A), at least two injections |
| QC samples 1 - 4 | (inject in increasing concentration) |
| Blank | (mobile phase A), at least two injections |
| Calibrators 1 - 8 | (inject in increasing concentration) |
| Blank | (mobile phase A), at least two injections |

Sequences setup may vary due to specific measurement requirements.

\* If reference values should be assigned, the number of sample preparations is dependent on the desired measurement uncertainty (n = x). Additionally, samples should be measured at least on two different days.

\*\* If a method comparison study has to be performed or complaint samples have to be measured, samples are prepared (n= 1) and measured.

1. Data Processing and Calculation of Measurement Results

Data evaluation is performed using the Analyst® software, version 1.6.2 or higher, employing the IntelliQuant algorithm.

The lamotrigine and [13C3D3]-lamotrigine signal show a retention time of 3.3 min and are integrated within a 30.0 sec window. Peak integration includes a smoothing factor of 3 and peak splitting factor of 2. The noise percent is set to 90 % with a base sub window of 0.5 min and may be adapted down to 60 % for peaks with an area equal to or below calibrator 2, if necessary. The minimum peak height is set to equal three times the background noise individually for every sequence. The background region for determining the baseline noise is set at the interval of 1.0 – 2.5 min in the quantitation method. Settings in the quantitation method are only default values that may vary from device to device or due to the instrument and equipment condition and may be adjusted accordingly.

Calibration is performed in bracketing mode and calibrator levels are measured in increasing concentration. Both calibration lines are used to generate the final calibration function.

The calibration curve is generated from the area ratios analyte / ISTD from eight calibrators (Calibrator 1 – Calibrator 8), using a linear fit with a 1/x2 weighting, not forcing the curve through the origin.

The calibration functions are obtained by linear regression of the area ratios of the analyte and internal standard (y) against the analyte concentration (x), resulting in the function *y = a x + b*.

For calculating the nominal concentrations of calibrators and QC samples, the purity of the analyte, the exact weighing, and the volume displaced by the weighing boat(s) used are taken into account.

Calculation of the volume of the used weighing boat(s):

The weight of the weighing boat(s) used is determined prior to use and divided by the density of the material (e.g. tin). The resulting determined volume of the weighing boat(s) is subtracted from the nominal volume of the measuring flask.

**Note:** The volume of the weighing boat(s) is only considered if the resulting bias is greater than 0.1 %, e.g. > 5.0 µL for a 5 mL flask.

Example:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Weighing boat(s) | Material | Density [g/mL] | Weight [mg] | Volume [µL] |
| 2 Tin boats 10x4x4.5 mm | Tin | 7.265 | 34.536 | 4.754 |

The volume of the stock solution in a 5 mL flask is therefore 5 mL – 4.754 µL = 4.9952 mL.

**Note:** If multiple individual weighing steps are preferred, this has to be considered for the calculation of measurement uncertainty.

The nominal concentrations of calibrator and QC samples are entered into the instrument software with five significant figures.

The *reportable result* is reported in µg/mL with 3 significant figures. Measurement uncertainty SD (µg/mL) is reported with 3 significant figures and the CV (%)with 1 decimal place.

For the assignment of reference or target values, the reportable result is calculated as the arithmetic mean of n = x sample preparations, in which sample preparations are performed over at least two days. Measurement uncertainty is calculated and reported as total uncertainty, given by the formula:

(unccal) uncertainty of calibrator preparation

(uncmean) uncertainty of n= x sample preparations

Results of a method comparison study or complaint sample (n= 1) are reported as the reportable result with the measurement uncertainty determined within the validation.

(unccal) uncertainty of calibrator preparation

(uncprec) uncertainty of precision experiment within the validation

**Note:** If other pipettes or glassware than indicated are used, the uncertainty must be recalculated.

1. Reporting of Results

All analysis requests are controlled by a sample management system. Each sample / sample set to be measured has to be requested using a specific order form which includes:

* identification of source and type of sample (native samples are anonymized)
* sample receipt and latest date of measurement
* sample storage place
* reference measurement procedure employed

Within the corresponding report, results are presented as follows:

* short description on reference measurement procedure used
* observations of unusual properties of sample
* observations as unusual features of the measurement procedure or use of modifications
* results are reported with sample name / identification name, numerical value, measurement unit and measurement uncertainty if required

All data (e.g. sequences, raw data, and calculations) have to be checked in four-eyes principle, except for calculations done by a validated tool, e.g. Biowarp. The final report has to be signed by the operator and approver.

1. Abbreviations and Definitions

|  |  |
| --- | --- |
| CE | Collision energy |
| CXP | Cell exit potential |
| Da | Dalton |
| DP | Declustering potential |
| ESI | Electrospray ionisation |
| HPLC | High performance liquid chromatography |
| ID | Isotope dilution |
| ISTD | Internal standard |
| LCMS | Liquid chromatography coupled with mass spectrometry |
| MeOH | Methanol |
| Milli-Q water | Ultrapure water (18.2 MΩ cm-1) from Millipore MilliQ System Direct-Q 3UV |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometer |
| NS | Normschliff (standard taper joint) |
| PSI | Pound per square inch |
| SST | System Suitability Test |
| TDM | Therapeutic drug monitoring |
| QC | Quality Control |