**SUPPLEMENTAL MATERIAL 2**

**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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***Characterization of reference material by quantitative nuclear magnetic resonance (qNMR) measurements***

Quantitative nuclear magnetic resonance (qNMR) measurements were performed in six replicates using the International System of Units (SI units) traceable internal standard (ISTD) for the quantification of the absolute content of gabapentin.

The following formula was utilized to calculate the absolute content of levetiracetam:

**Equation S1:**

*Px*: Purity of the analyte (LEV) as mass fraction

*Pstd*: Purity of the qNMR internal standard (ISTD) as mass fraction

*Ix*: Integral of the analyte

*Istd*: Integral of the ISTD

*Nx*: No. of analyte protons

*Nstd*: No. of internal qNMR standard protons

*Mx*: Molecular weight of the analyte

*Mstd*: Molecular weight of the ISTD

*mx*: Mass of analyte

*mstd*: Mass of ISTD

The following values are common to all the qNMR measurements:

Molecular Weight of levetiracetam = 170.2089 g/mol; Molecular weight of the qNMR internal standard ISTD (1,3,5-Trimethoxybenzene = 168.1898 g/mol; Purity of the qNMR internal standard as mass fraction = 99.96 ± 0.13% (k = 2); Number of Protons taken into account for the ISTD = 3 and for levetiracetam = 1.

Procedure for sample preparation and processing parameter:

Levetiracetam (Supplemental Table 1) and the qNMR standard (Supplemental Table 1) were weighed together in a glass vial on an Ultra-microbalance (XPR6U), from Mettler Toledo. DMSO-d6 (approx. 700 µL) was added to the glass vial, and after a short vortexing, the solution was transferred to a 5 mm NMR tube. The NMR measurements were performed on a JEOL 600 MHz NMR spectrometer (Jeol Ltd, Tokyo, Japan) equipped with an ultra-cool probe head, which provides 4-5 times sensitivity enhancement compared to normal room-temperature probe heads. NMR measurements were performed at 298K/300 K utilizing 64k data points and the number of scans were limited to 64 with automatic receiver gain conditions. Single-Pulse-1H{13C}NMR (Supplemental Figure 1) was utilized for the quantitation with an inter-scan delay of 60 seconds. Processing was performed with an exponential window function (line broadening = 0.3 Hz), followed by manual phase and baseline corrections. A small singlet impurity integral was subtracted from the integral area calculation of the levetiracetam resonance.

Table 1 : qNMR calculation of the purity of Levetiracetam

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Levetiracetam source | Measurement No. | Mass of Levetiracetam weighed (mx) | Mass of qNMR ISTD weighed (mstd) | Integral of the qNMR ISTD (Istd) | Integral of the analyte (Levetiracetam) | Purity of Levetiracetam as mass fraction (Px) % |
| Sigma | 1 | 8.7218 | 2.6336 | 0.9151 | 0.9993 | 100.07 |
| Sigma | 2 | 8.4888 | 2.6272 | 0.9393 | 0.9992 | 99.91 |
| Sigma | 3 | 8.1177 | 3.0086 | 1.1256 | 0.9991 | 99.84 |
| Sigma | 4 | 8.5058 | 2.3685 | 0.8444 | 0.9992 | 100.00 |
| Sigma | 5 | 8.4798 | 3.0163 | 1.0805 | 0.9989 | 99.80 |
| Sigma | 6 | 8.9656 | 3.6277 | 1.2267 | 0.9989 | 99.99 |



Supplemental Figure 1: qNMR spectrum indicating the protons utilized for the quantification of levetiracetam

A picture containing diagram

Description automatically generated

Supplemental Figure 2: Overlay of the 6 spectra acquired under the aforementioned quantitative conditions.