Pregabalin Determination in Hair by Ultra-High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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Pregabalin is an antiepileptic and analgesic drug, commercialized under the name of Lyrica, and generally used to treat neuropathic pain. The determination of pregabalin was performed by using spiked hair samples extracted in methanol and analyzed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry. The validation of a quantification method of pregabalin in hair has been successfully achieved (precision, accuracy, matrix effects and extraction yield). The limit of detection was 0.76 pg/mg and the lower limit of quantification was 2.5 pg/mg. A real case of pregabalin in hair has been analyzed using this method. The result showed a concentration of 540 pg/mg.

Sample preparation

Preparation of the hair

For the method development, drug-free hair samples were used. The hair was washed with water and acetone. After drying with paper, it was cut into little pieces of 2–3 mm and then pulverized for 5 min at 50 Hz at room temperature. Approximately 20 mg of hair sample was put into a glass test tube (10 mL) with 1.8 mL of methanol. After the addition of 20 μL methaqualone 0.1 ng/μL (prepared by dilution of the initial solution of methaqualone 1 mg/mL and pregabalin (from a stock solution of pregabalin 0.05 ng/μL), the test tube was vortexed and placed in an ultrasonic bath for 2 h at room temperature and 25 kHz. After centrifugation (7 min, 5000 rpm), the supernatant was collected and evaporated to dryness under nitrogen at 37°C. The residue was dissolved in 100 μL of LC–MS-MS Solvent A (95:5 H₂O/acetonitrile + 0.5% formic acid). For the analysis, 10 μL of the sample was injected into the ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UPLC–MS-MS) system.

Instrumentation

UPLC conditions

The chromatographic equipment consisted of an Acquity UPLC system (Waters, Zellik, Belgium). Elution was performed on an BEH C18 column (1.7 μm, 2.1 mm × 100 mm, Waters). A multi-step linear gradient of Solvent A (95:5 H₂O/acetonitrile + 0.5% formic acid) and Solvent B (acetonitrile + 0.5% formic acid) was applied. The initial mobile phase contained 99% Solvent A and 1% Solvent B. The system was run in a linear gradient from 0.10 to 4.20 min from 99 to 1% Solvent A. From 4.20 to 4.90 min, the gradient stayed at 1% Solvent A. From 4.90 to 5.00 min, the gradient decreased from 99% Solvent A down to 1% Solvent A to return into the initial conditions. The total run time was 5 min.
The injection volume was 10 μL (full loop). Column temperature was set to 40 °C, and sample temperature to 20 °C (13).

**MS–MS conditions**

The LC system was coupled with a Waters Xevo TQ MS mass spectrometer with an atmospheric pressure electrospray interface. Waters MassLynx version 4.1 SCN 644 was used for LC–MS system control and data analysis. Analyte identification was performed by monitoring the protonated molecule with the four most intense product ions. The parameters used for the mass spectrometer under the ESI+ mode were as follows: capillary voltage 3.00 kV, extractor voltage 3.00 V, source temperature 150 °C, desolvation temperature 650 °C, desolvation gas (nitrogen) flow 1000 L h⁻¹, cone gas flow 30 L h⁻¹, collision gas flow (argon) 0.15 mL min⁻¹, collision cell pressure 3.22 × 10⁻³ mbar, low mass (LM) 1 290 V, LM 2 280 V, high mass (HM) 1 14.90 V, HM 2 14.80 V, ion energy 0.3 V and ion energy 0.8 V. Parameters for the cone and collision energy are listed in Table I. Parameter optimization was achieved using MassLynx/Intellistart. Drug residues were monitored and quantified using multiple reaction monitoring. Two time windows were defined: pregabalin 1.1–1.6 min and methaqualone 2.2–2.8 min. Optimization of the mass spectrometer was performed by direct infusion of an aqueous solution containing the drug investigated (13).

**Method validation**

The following parameters have been validated: limit of detection (LOD) and lower limit of quantification (LLOQ), linearity, selectivity, intra- and interday precision and accuracy of a low and a high concentration of pregabalin, matrix effects and extraction yield. Application of the method to the analysis of a real case sample was also carried out.

**Linearity, LOD and LLOQ**

For the standard calibration curve, spiked hair samples with different concentrations of pregabalin were prepared: 50, 100, 200, 400, 800 and 1600 pg/mg. To verify the linearity of the curve, the correlation coefficient R² was calculated. The LOD was evaluated by calculation of the signal-to-noise ratio: S/N = 3. For the LLOQ, the S/N was set to 10.

**Selectivity**

For the selectivity, three different drug-free hair samples were tested to verify the absence of endogenous interference from different origins.

**Precision and accuracy**

For the intraday precision and accuracy, 10 samples of a low concentration (50 pg pregabalin/mg hair) and 10 samples of a high concentration (500 pg pregabalin/mg hair) were analyzed. For the interday precision and accuracy, six samples of these two concentrations were considered. A new calibration curve was established every day for the interday analyses. The precision was evaluated with a variation coefficient of <15%. The accuracy was determined as the percentage of deviation (bias) from the average of the intra- or interday analyses. The accuracy for the low concentration was acceptable with a deviation of 25% from the average, whereas for the high concentration a deviation from the average of only 15% was tolerated.

**Matrix effects**

Matrix effects were evaluated by preparing a hair sample with 200 μL of pregabalin and 20 μL of methaqualone, evaporated to dryness and collected in 100 μL of Solvent A. Injection into the LC–MS was done without further treatment. This analysis was compared with a standard sample of 500 pg/mg of pregabalin, with validation criteria of ±25% of the target value. The following equation was used to calculate the matrix effect (%): [(mean peak area of spiked standards in hair)/(mean peak area of standards spiked in neat mobile phase) × 100]. Values >100% show an ionization enhancement, whereas values <100% indicate an ionization suppression (14).

**Extraction yield**

To evaluate the extraction yield, two samples were prepared: for the first sample, methaqualone and pregabalin were added after extraction, and for the second sample, pregabalin was added before extraction and methaqualone after extraction. The concentration of pregabalin used for the analyses was 500 pg/mg. The validation criteria were ±40% of the target value. Using the following expression, the extraction yield (%) can be calculated: [(mean peak area for standards spiked before extraction)/(mean peak area for standards spiked after extraction) × 100]. The result of the extraction yield is not effected by matrix effects and this calculation can be considered the overall process efficiency (14).
Results and discussions

Linearity, LOD and LLOQ
The standard calibration curve was obtained with a correlation coefficient of 0.9979, an LOD of 0.76 pg/mg and an LLOQ of 2.5 pg/mg. The correlation coefficients of the intra- and interday analyses were in a range 0.9679–0.9981 (shown in Table II). The retention time of pregabalin was 1.24 min, and methaqualone did elute at 2.57 min.

Selectivity
The three different drug-free hair samples were tested to verify the absence of endogenous interference from different origins. No endogenous interference was detected.

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<td>Intra- and interday precision and accuracy of pregabalin in hair</td>
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<td>Average (pg/mg)</td>
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Precision and accuracy
All the results of the intra- and interday precision and accuracy are reported in Table II.

The precision of the intra- and interday analyses were in the range 2.8-12.3%.

For the low concentration (50 pg/mg), the accuracy range was between 9.2 and 21.3%. For the high concentration (500 pg/mg), the accuracy range was defined between 0.12 and 0.85%. Considering these results, the intra- and interday precision and accuracy were considered validated.

Matrix effects
Matrix effects were tested in duplicate on the same batch of hair, and a mean of 83.5% was calculated. The result indicates the presence of minor ionization suppression. With a difference of 16.5% between the spiked hair samples and the neat mobile phase, the result is located in the tolerated range ± 25%.

Extraction yield
The extraction yield was also tested in duplicate on the same batch of hair. The extraction yield was determined to be 75.5%.

Real case sample analysis
One real case of pregabalin determination has been performed using the described method. The patient was a 33-year-old woman with brown hair; she was treated with a daily dose of Lyrica 300 (300 mg pregabalin). We received 4.5 cm of the woman’s hair; the analysis has been carried out using 20 mg. A
concentration of 540 pg pregabalin/mg hair was found. The chromatogram of the real case sample is shown in Figure 2.

In conclusion, the method validation has been successfully completed using UPLC–MS-MS. The analysis method has been proved fast and simple with a run time for an analysis of only 5 min and can be applied to real cases.

References