

## ESTIMATION OF GABAPENTIN IN HUMAN PLASMA USING LC-MS/MS METHOD

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## ABSTRACT

**Objective:** The objective of this research was to develop and validate a simple, sensitive and specific Liquid Chromatography–Tandem Mass Spectrometry (LC–MS/MS) quantification of Gabapentin in human plasma. The analytical method consists of liquid-liquid extraction of plasma sample followed by the determination of Gabapentin by a LC–MS/MS.

**Method:** The analyte was separated on a Zorbax Eclipse XDB -C18 (150 x 4.6 mm, 5  $\mu$ ) column with an isocratic mobile phase of Methanol: Water (50: 50 v/v, pH of 3.0) at a flow rate of 0.8 mL/min. Protonated ions formed by a turbo ionspray in a positive mode was used to detect analyte and internal standard (IS). The MS/MS detection was made by monitoring the fragmentation of  $m/z$  172.20 $\rightarrow$ 154.20 for Gabapentin and  $m/z$  176.40 $\rightarrow$ 158.10 for internal standard on a mass spectrometer.

**Results:** The method was validated over the concentration range of 51.356 ng/mL to 8217.008 ng/mL for Gabapentin in human plasma with correlation coefficient greater than 0.98.

**Conclusion:** The developed method was successfully applied for analyzing Gabapentin in plasma samples for a bioequivalence study with healthy volunteers.

**Keywords:** Gabapentin; Validation; Analysis; Bioavailability; LC-MS/MS.

## INTRODUCTION

Gabapentin (1-(aminomethyl) cyclohexaneacetic acid) (Fig. 1), is a new antiepileptic drug which is a structural analogue of neurotransmitter  $\gamma$ -aminobutyric acid (GABA). Gabapentin, unlike GABA, has a cyclohexane molecule system and is able to penetrate through blood–brain barrier. Gabapentin is used for the treatment of partial onset seizures with or without secondary generalized tonic-clonic convulsions in clinical practice. Gabapentin is indicated as adjunctive therapy in the treatment of partial seizures with and without secondary generalization in adults with epilepsy and for the management of postherpetic neuralgia. After oral administration, Gabapentin is well absorbed and reaches maximal plasma concentrations within 2–3 h. The elimination half-life of the drug is 5–7 h after a single oral dose of 200–400 mg. Gabapentin is not metabolized and mainly excreted by kidney. The drug does not bind plasma proteins. Pharmacokinetics of Gabapentin is not affected by foods and other drugs. Gabapentin can be actively transported across the brain–blood barrier and the gut via the L-system amino acid transporter, which recognizes L-isoleucine, L-leucine, L-phenylalanine and L-valine [1, 2].

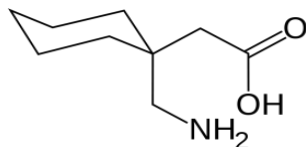


Fig. 1: Chemical Structure of Gabapentin

Several analytical methods have been reported for the determination of Gabapentin such as HPLC [1, 3-9], Capillary electrophoresis [10], Gas chromatography [11], GC-MS/MS [12-14] and mass spectrometry [15-19]. The purpose of the present study was to develop and validate an LC-MS-MS method as per USFDA Bioanalytical Method Validation Guideline [20], with simple sample preparation technique to determine Gabapentin concentration in human plasma and to apply it to a bioequivalence study of 400 mg Gabapentin capsule in 48 healthy volunteers. This assay method demonstrated acceptable sensitivity (LLOQ: 50 ng/mL), precision,

accuracy, selectivity, recovery and stability, and less absolute and relative matrix effect. In addition, most of the reported method for Gabapentin utilizes non deuterated/non stable isotope as internal standard. For control of extraction of analyte and internal, HPLC injection and ionization variability, it is recommended to use a deuterated/ stable isotope of analyte. The present study utilizes deuterated Gabapentin-d4 as an internal standard which has advantage over the other reported method.

## MATERIAL AND METHODS

## Instrumentation

Agilent 1200 Series HPLC System and AB Sciex MS/MS API-4000.

## Reagents / Materials

Methanol (HPLC Grade), Acetonitrile (HPLC Grade), Water (HPLC Grade), Formic Acid (AR Grade), Methylene chloride (HPLC Grade), Gabapentin USP Working Standard and Gabapentin-D4 internal standard.

## Stock Solutions

Gabapentin stock solutions and Gabapentin-D4 stock solutions were prepared in methanol

## Biological Matrix

Human plasma containing Sodium Heparin as anticoagulant was used as a biological matrix during method validation. Selectivity and sensitivity tests were performed before bulk spiking.

## Calibration Curve (CC) Standards and Quality Control (QC)

## Sample Concentrations

The Calibration Curve standards (CC) were prepared at ranges from 51.356 ng/mL to 8217.008 ng/mL concentrations for Gabapentin. The quality control samples for Gabapentin were prepared at concentrations of 51.400 ng/mL (LLOQC), 157.224 ng/mL (LQC), 1007.843 ng/mL (MQC), 3876.319 ng/mL (MQC1) and 6528.538 ng/mL (HQC).

**Buffer (Formic Acid (1.0% v/v)):** Mixed 1.0 mL of Formic Acid in 100 mL HPLC grade water. It was mixed well and sonicated in an ultrasonic bath for 5 minutes. The buffer was prepared as and when required.

#### Mobile Phase

A mixture of Methanol: Water (50: 50 v/v) was prepared. The pH of the above solution is adjusted to 3.00 with Formic Acid and mixed. The mobile phase was sonicated in an ultrasonicator for 5 to 10 minutes. The mobile phase was degassed in an ultrasonicator for 5 to 10 minutes.. The mobile phase was prepared as and when required.

#### Diluent

A mixture of Methanol: water (80: 20) was prepared. The mixture was mixed well and degassed in an ultrasonicator for 5 minutes. The diluent was prepared as and when required.

#### Bioanalytical Conditions

A summary of the chromatographic conditions is given in Table 1:

**Table 1: Summary of Bioanalytical Chromatographic conditions**

Parameters	Conditions
System	LC-MS/MS API 4000
Detector	Mass Spectrometry
Software	Analyst software 1.4.2
Column	Zorbax Eclipse XDB -C18 150 x 4.6 mm, 5 $\mu$
Mobile Phase	Methanol : Water (50 : 50 v/v) pH 3.0
Flow Rate	0.8 mL / min. Gabapentin - 172.20/154.20
m/z	Gabapentin-D4 - 176.40/158.10
Injection Volume	5 $\mu$ L
Column Oven Temperature	35° C
Diluent	Mobile Phase
Auto Sampler Temp	10° C

#### Sample Preparation

The thawed plasma samples were vortexed to ensure complete mixing of the contents. 250  $\mu$ L of the sample was pipetted into RIA vials. 20  $\mu$ L of 400  $\mu$ g/mL Gabapentin-D4 dilution was added to it as an internal standard (IS) except in blank sample wherein 20  $\mu$ L of HPLC grade water was added, and vortexed for 30 seconds followed by addition of 10  $\mu$ L of 1% Formic Acid and vortexed again for 30 seconds. Then 500 $\mu$ L of Acetonitrile was added and vortexed for 1 minute. Samples were centrifuge for 5 minute at 2000 rpm 10°C. Withdraw 500 $\mu$ L of supernatant into 15 mL glass centrifuge tube. To it add 1000 $\mu$ L of water and vortexed for 30 seconds. Again add 2 mL of methylene chloride and vortexed for 1.0 minute. Centrifuge for 10 minute at 2500 rpm 10°C. Withdraw 500 $\mu$ L of supernatant layer into HPLC vials and injected in to HPLC system.

#### Data Processing

The chromatograms were acquired and the data was processed by peak area ratio method using Analyst 1.4.2 software. The concentration of the unknown was calculated from the following equation using regression analysis of spiked calibration standard with the reciprocal of the drug concentration ratio as a weighting factor ( $1/x^* x$ ).

$$y = mx + c$$

Where,

y = peak area ratio of Gabapentin to internal standard

m = slope of the calibration curve

x = concentration ratio of Gabapentin ng/mL

c = y-axis intercept of the calibration curve

#### RESULTS AND DISCUSSION

During validation selectivity, sensitivity and recovery exercise was carried out. Precision and accuracy exercise was carried out by processing four precision and accuracy batches. Results of various stabilities, anticoagulant effect, partial volume, dilution integrity, reinjection reproducibility and ruggedness were carried out.

#### Selectivity

No significant interference from endogenous components was observed at the retention times of Gabapentin and internal standard in all the batches screened.

#### Sensitivity

The lowest limit of reliable quantification for Gabapentin was set at the concentration of the LLOQ, 51.400 ng/mL. The precision and accuracy for Gabapentin at this concentration was found to be 5.08% and 106.82%, respectively for plasma containing sodium heparin as anticoagulant.

#### Anticoagulant Effect

Six sets of spiked LLOQ QC samples spiked in plasma containing K2EDTA as an anticoagulant were processed and analysed along with processed calibration curve standards freshly spiked in plasma containing Sodium Heparin as an anticoagulant. Results demonstrate absence of anticoagulant effect. The within batch precision and accuracy 4.44% and 114.09% respectively.

#### Matrix Effect

Extracted three blank samples from each of six batches of matrix. Prepared LQC, MQC, MQC1 & HQC spiking dilution and spiked in the above extracted blank samples. Prepared aqueous sample at LQC, MQC, MQC1 & HQC and injected six replicates of the same and spiked samples. The matrix effect ratio is between 0.98 to 1.05 at all three QC levels. Precision of area response of replicate injection of aqueous samples is 1.39% to 2.19% and precision of area response of post extracted samples is 0.75% to 3.87%. So there is no matrix effect.

#### Carryover Test

For carryover test two samples of upper limit of quantification (ULOQ) and 4 samples of blank plasma were processed. These samples were injected in the following sequence.

- a) 2 blank samples b) 2 ULOQ samples c) 2 blank samples

The step (b) and (c) were repeated 2 times. The results demonstrate that there was no interference from the previous injection.

#### Linearity

A regression equation with a weighting factor  $1/x^*x$  of ratio of drug to IS concentration was judged to produce the best fit for the concentration response relationship for Gabapentin in human plasma. Correlation coefficients (r) were greater than 0.98 in the concentration range of 51.356 ng/mL to 8217.008 ng/mL for Gabapentin.

#### Precision and Accuracy

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQ, LQC, MQC, MQC1 and HQC samples respectively during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the low, middle and high quality control samples to their respective nominal values, expressed in percentage.

#### Within-Batch Precision and Accuracy

For LLOQ within-batch precision ranged from 3.08% to 11.71% and the within batch accuracy ranged from 99.16% to 105.55% for Gabapentin. For LQC, MQC MQC1 & HQC within-batch precision ranged from 1.11% to 7.53% and the within batch accuracy ranged from 96.98% to 108.90% for Gabapentin.

### Intra-day Precision and Accuracy

For LLOQC, the intra-day precision ranged from 4.39% to 7.39% and the intra-day accuracy ranged from 99.16% to 105.55% for Gabapentin. For LQC, MQC, MQC1 & HQC, the intra-day precision ranged from 1.64% to 7.53% and the intra-day accuracy ranged from 96.98% to 106.33% for Gabapentin.

### Between Batch / Inter-run Precision and Accuracy

For LLOQC, the inter-day precision was 6.45% and the inter-day accuracy was 100.29% for Gabapentin. For LQC, MQC, MQC1 & HQC, the inter-day precision ranged from 2.74% to 3.44% and the inter-day accuracy ranged from 98.35% to 105.60% for Gabapentin.

### Stabilities

#### Standard Stock Solution Stability

#### Room Temperature Stock Solution Stability 6 Hrs

Room temperature stock solution stability was carried out at 6 hours by injecting six replicates of stock dilutions of both stability standard stock solution and comparison (fresh) standard stock solution of Gabapentin and Gabapentin-D4. The response of stability sample was corrected by multiplying with correction factor. The 6.0 Hours stock solution stability of Gabapentin and Gabapentin-D4 was found to be 101.53% and 101.25%, respectively. The stock solution stability of Gabapentin and Gabapentin-D4 is 6 hours at room temperature.

#### Refrigerated Stock Solution Stability 8 days at 2 to 8°C

Refrigerated stock solution stability was carried out by injecting six replicates of stock dilutions of both stability standard stock solution and comparison (fresh) standard stock solution of Gabapentin and Gabapentin-D4. The response of stability sample was corrected by multiplying with correction factor. The eight days stock stability of Gabapentin was found to be 99.23% and seven days stock stability of Gabapentin-D4 was found to 100.44 %. The refrigerated stock solution stability of Gabapentin and Gabapentin-D4 is 8 days.

#### Bench Top Stability in Human Plasma 6 Hrs

Bench top stability, using six sets each of LQC, MQC, MQC1 and HQC, was determined at six hours. The quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for calculation of precision and accuracy. Gabapentin was found to be stable up to 6 hours as per the acceptance criteria. The percent nominal ranged from 96.15% to 100.87% and the precision ranged from 2.06% to 4.74%.

#### Auto sampler Stability 13 Hrs

In assessing the auto sampler stability, six sets of QC samples (LQC, MQC, MQC1 and HQC) were processed and placed in the auto sampler. These samples were injected after a period of 13 hours and were quantified against freshly spiked calibration curve standards of concentration range equivalent to that used for calculation of precision and accuracy. The results demonstrate that the processed samples were stable for 13 hours. The percent nominal at 13 hours for Gabapentin ranged from 95.76% to 102.62%. And precision at 18.0 hours for Gabapentin ranged from 2.03% to 4.79%.

#### Freeze-Thaw Stability 3rd Cycle

The stability in human plasma was determined for three freeze-thaw cycles. Six replicates of LQC, MQC, MQC1 and HQC were analysed on third freeze-thaw cycles. The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for the calculation of precision and accuracy. The percent nominal ranged from 99.06% to 106.12% for three freeze-thaw cycles and precision ranged from 2.12% to 6.84% for three freeze thaw cycles.

### At -20°C Stability 5 days

The stability of Gabapentin in case of temporary storage of plasma samples at -20°C deep freezer for five days was carried out by quantifying six sets each of LQC, MQC, MQC1 and HQC against the freshly spiked calibration curve standards of concentration range equivalent to those used for the calculation of precision and accuracy. Gabapentin was found to be stable up to 5 days at -20°C deep freezer as per the acceptance criteria. The mean stability ranged from 97.59% to 102.88% and the precision ranged from 1.61% to 8.51%, for 5 days stability samples.

### Below -70°C long term stability in human plasma 99days

The stability of Gabapentin in plasma samples below -70°C deep freezer for 99days was carried out by quantifying six sets each of LQC, MQC and HQC against the freshly spiked calibration curve standards of concentration range equivalent to those used for the calculation of precision and accuracy. Gabapentin was found to be stable up to 99 days below -70°C deep freezer as per the acceptance criteria. The % nominal ranged from 96.46% to 100.88% and the precision ranged from 1.86% to 3.06%, for 99days stability samples.

### Reinjection Reproducibility

Reinjection reproducibility was carried out by reinjecting six sets of LQC, MQC, MQC1 and HQC of PA-batch 4 quality control samples after 10.0 hours and was back calculated concentration against initial calibration curve standards injected at zero hours. After 10.0 hours precision and accuracy ranged from 1.09% to 4.36% and 99.93% to 106.73%, respectively.

### Recovery

Prepared six sets of recovery comparison samples by spiking 50 µL of spiking dilution of quality control samples (LQC, MQC, MQC1 and HQC) Gabapentin in 950 µL Eluent of extracted blank. For recovery comparison sample of IS, 20 µL of internal standard dilution 400.000 µg/mL was spiked in 950 µL Eluent of extracted blank. The recovery comparison samples of Gabapentin were compared against extracted samples of LQC, MQC, MQC1 and HQC of PA-batch 3, and recovery comparison samples of Gabapentin-D4 were compared against extracted samples of MQC and MQC1 of PA-batch 3, which were within the acceptance criteria. The mean overall recovery of Gabapentin was 74.61% with a precision of 4.67% & mean recovery of internal standard (Gabapentin-D4) was 77.49%.

### Dilution Integrity

Dilution integrity samples were prepared by spiking approximately 1.8 times highest standard concentration (14689.210 ng/mL). Six sets of dilution integrity samples were processed by diluting them twice and four times. These quality control samples were analysed along with a processed calibration curve standards. The quality control sample concentrations were calculated using appropriate dilution factor. Results demonstrate acceptable dilution integrity for two times and four times dilution. The precision and accuracy, for a dilution factor of 2 was 4.49% and 103.70%, respectively. Similarly the precision and accuracy, for a dilution factor of 4 was 1.89% and 109.05% respectively. Dilution integrity for two times and four times dilution is acceptable as per acceptance criteria.

### Partial Volume Analysis

Six sets of spiked MQC, MQC1 and HQC were processed using 25% and 50% of the processing volume. These quality control samples were analysed along with processed calibration curve standards. The concentrations were calculated using appropriate multiplication factor. Results demonstrated partial volume analysis acceptable for 50% and for 25% partial volume for Gabapentin, since the QC samples were within the bioanalytical batch acceptance criteria. The precision and accuracy for a processing volume of 50% ranged from 2.24% to

4.48% and 104.69% to 110.62% respectively. The precision and accuracy for a processing volume of 25% ranged from 3.37% to 6.09% and 101.21% to 105.25%, respectively.

#### Ruggedness

Ruggedness was carried out on another analyst. Ruggedness exercise was carried out by processing one precision and accuracy batch. A regression equation with a weighting factor  $1/x^2$  of ratio of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Gabapentin in human plasma. Correlation coefficients were greater than 0.99 in the concentration range of 51.356 ng/mL to 8217.008 ng/mL. Within-batch precision ranged from 1.64% to 5.70% and the within batch accuracy ranged from 94.97% and 104.44% for Gabapentin

#### CONCLUSION

The LC-MS/MS validated method has proved to be very simple, sensitive and reliable and successfully applied for the pharmacokinetic study in human plasma. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. The major advantage of this method is the use of deuterated Gabapentin-d4 as an internal standard. The run time is within 2 min and only 0.250 mL of plasma was required for each determination of Gabapentin, and thus the stress to volunteers or patients in clinical studies was greatly reduced. This method is very suitable and convenient for pharmacokinetics and bioavailability study of the drug Gabapentin.

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