Determination of Pregabalin in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry

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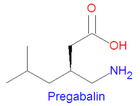
PURPOSE

- Pregabalin, (S)-3-(aminomethyl)-5-methylhexanoic acid, is an analogue of the neurotransmitter gamma amino butyric acid (GABA).



Pregabalin

- It is used for the treatment of peripheral and central neuropathic pain in adults and as an adjunctive therapy for refractory partial seizures.
- Pregabalin binds potently to the $\alpha_2\delta$ (alpha2delta) subunit of the voltage-dependent calcium channel in the central nervous system and its binding at this site reduces calcium influx at the nerve terminal, leading to the release of several neurotransmitters, such as glutamate and noradrenalin.
- The aim of this study is to develop a sensitive, specific, robust and rapid LC-MS/MS method:
  - Use simple extraction procedure;
  - With no interference from matrix;
  - With short run time.

METHOD DEVELOPMENT

- Positive electrospray ionization vs negative electrospray ionization: amine group, [MH^+]
- Mobile phase: 0.1% formic acid in water as mobile phase A Enhance the ionization of amine group and suppress the ionization of carboxy group
- Interference of endogenous compound with pregabalin at same retention time:
  Confirmed with 20 different lots of human blank plasma.
  Compared16 different types of columns
- Endogenous compound was separated with pregabalin using Synergi Max-RP (80A,4 µ, 50*2.0mm) column. See chromatograms.
- Reduce run time and maintain reproducibility: total run time 3.2 minutes with gradient elution

METHOD

Sample Extraction Procedure:

Aliquots of 50 µL of plasma sample with pregabalin-d_4 as internal standard (IS) added were extracted by protein precipitation with 500 µL of methanol. After vortexing and centrifuging for 5 minutes at 3500 rpm, 200 µL of the supernatant was mixed with 400 µL of purified water and 20 µl of the reconstitution solution was injected onto an LC/MS/MS system.

LC-MS/MS Conditions:

Shimadzu LC-20AC systems/Sciex API4000
Column: Synergi Max-RP column 50 X 2.0 mm 4 µm
Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: 100% methanol

HPLC Gradient:

Time (min)	Flow rate (mL/min)	%B
0.0	0.6	5
0.2	0.6	5
1.5	0.6	70
2.5	0.6	70
2.6	0.6	5
3.2	Stop	

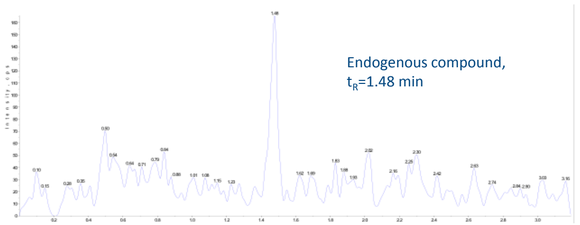
MS/MS Detection:

Interface: Turbo Ion Spray (ESI)
Positive
MRM channels:

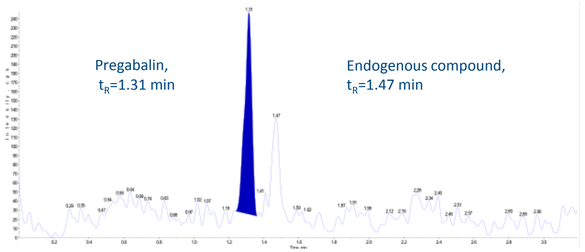
Analyte	Q1 → Q3
pregablin	160.2 → 142.2
pregabalin-d_4 (IS)	164.2 → 146.2

Chromatograms:

- Double blank of human plasma:



- Standard 1 (50 ng/mL) in human plasma:



RESULTS

- A short run time of 3.2 minutes for sample injection was achieved with an endogenous interference peak successfully separated with pregabalin by an optimized mobile phase gradient and the column selected. The assay range was linear over the concentration range of 50-50000 ng/mL with average coefficient determination (R^2) of three inter-runs greater than 0.99. The precision and accuracy for the intra-run and inter-run at four QC levels [low limit of quantitation (LLOQ), Low, Mid and High] are presented in table 1.
- The average extraction recovery from three concentration levels (150, 2500 and 3800 ng/mL) were 101.0 ±2.8%. The relative matrix effect (internal standard normalized effect) was 106.0 ±3.0% and 103.0 ± 7.0% at two concentration levels (150 and 3800 ng/mL) respectively as shown in table 2.
- No hemolysis effect was found for 2% hemolyzed plasma. The specificity, stability, and dilution integrity were also found within acceptance criteria. The stability data is shown in table 3.

Table 1. Intra-run and Inter-run Precision and Accuracy

Intra-run (n=6)	LLOQ	Low	Mid	High
Concentration Added (ng/mL)	50	150	2500	38000
Concentration Measured (ng/mL)	56	143	2430	34400
SD	3.54	7.37	146	694
C.V.(%)	6.3	5.2	6.0	2.0
Bias (%)	12.0	-4.7	-2.8	-9.5
Inter-run (n=18)	**LLOQ**	**Low**	**Mid**	**High**
Concentration Added (ng/mL)	50	150	2500	38000
Concentration Measured (ng/mL)	54.9	139	2380	35600
SD	5.43	9.36	119	1640
C.V.(%)	9.9	6.7	5.0	4.6
Bias (%)	9.8	-7.3	-4.8	-6.3

Table 2. Extraction Recovery and Matrix Effect Results

Extraction recovery	Low (150ng/mL)*	Mid (2500ng/mL)*	High (38000 ng/mL)*
Extracted %CV	3.6	3.1	4.4
Un-extracted %CV	7.9	5.0	4.9
% Recovery	101.5	103.6	98.0
Overall % recovery		101.0 ± 2.8	
IS normalized matrix effect	**Low (150ng/mL)****	**High (38000 ng/mL)****	
Average matrix factor	1.06	1.03	
%CV	2.8	6.8	

*n=3; **n=6

Table 3. Stability Data

Storage Condition	Low (150 ng/mL) * %bias	%CV	High (38000 ng/mL) * %bias	%CV
3 Freeze/thaw cycles (-20 °C)	-8	4.6	-5.8	1.4
3 Freeze/thaw cycles (-70 °C)	-1.3	4.0	-2.6	2.7
21 hours bench top stability at RT	-5.3	3.9	-5.8	2.9
161 days long term stability (-20 °C)	-5.3	4.3	-12.9	1.5
161 days long term stability (-70 °C)	-8	3.5	-11.8	0.7

*n=3

CONCLUSION

- A robust high throughput method for the determination of pregabalin in human plasma by LC-MS/MS using simple protein precipitation procedure and small sample volume was successfully developed and validated in accordance with FDA guidance for bio-analytical method validations.
- This method demonstrated robustness and high throughput with validation parameters (specificity, accuracy, precision, matrix effect, hemolysis effect, recovery and stability) met the FDA validation requirement, and was successfully applied to quantitation of the concentration of pregabalin in a human pharmacokinetic study.

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