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Development and Validation of Entacapone in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry

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A novel liquid chromatography tandem mass spectrometry method is developed for the quantitative determination of entacapone in human plasma in positive ion mode and validated using tolcapone as internal standard according to linearity, selectivity, precision, recovery and various stability studies. Sample preparation was accomplished by liquid-liquid extraction technique. The eluted samples were chromatographed on ACE 3 C_{18} (150 × 4.6 mm, 5 µ) column (Agilent Technologies) using a mobile phase consisting of HPLC grade acetonitrile:10 mM ammonium phosphate (50:50, v/v) with injection volume of 15 µL and a run time of 3 min. The precursor to product ion transitions *m/z* 305.10 to 242.10 (entacapone) and *m/z* 272.20 to 212.10 (tolcapone) were used for quantization. The calibration graph of entacapone was linear with $r^2 > 0.99$ over a concentration range of 60.0 ng/mL to 2200.0 ng/mL. CV % of intra- and inter-day precisions were found satisfactory and well within the limits. The drug was found to be stable for the studied parameters and found to be interference free for matrix effect with appreciable recovery. The novelty of the method makes it highly valuable, rapid, selective and sensitive for quantification of entacapone in human plasma and can be used in therapeutic drug monitoring of this drug.

Keywords: Entacapone, High-performance liquid chromatography, Mass spectrometry, Human plasma.

INTRODUCTION

Entacapone is chemically known as 2-cyano-3-(5-dihydroxyamino-3,4-dioxo-1-cyclohexa-1,5-dienyl)-N,N-diethyl-prop-2-enamide and belongs to the class of antiparkinson agent[1]. Entacapone is a selective and reversible inhibitor of catechol-O-methyl transferase (COMT), with mainly peripheral actions. It is used in the treatment of Parkinson's disease as an adjunct to Levodopa/Carbidopa therapy[2]. The mechanism of action of entacapone is believed to be through its ability to inhibit COMT and alter the plasma pharmacokinetics of levodopa. When entacapone is given in conjunction with levodopa and an aromatic amino acid decarboxylase inhibitor alone leading to greater effects on the signs and symptoms of Parkinson's disease. Entacapone is rapidly absorbed, with a T_{max} of approximately 1 h. The absolute bioavailability following oral administrations is 35 %. Food does not affect the pharmacokinetics of entacapone due to its high plasma protein binding. The elimination of entacapone is biphasic, with an elimination half-life of 0.4-0.7 h based on the β-phase and 2.4 h based on the γ-phase. The γ-phase accounts for approximately 10 % of the total AUC. Entacapone is almost completely metabolized prior to excretion, with only a very small amount (0.2 % of dose) found unchanged in urine. After oral administration of a ^{14}C-labeled dose of entacapone, 10 % of labeled parent and metabolite is excreted in urine and 90 % in faces[3]. Several techniques have been reported in the literature for the quantitative estimation of entacapone in pharmaceutical[3] and biological fluids[4-9]. Few methods were developed by using LC-MS/MS[5,10-12]. Due to the increasing importance of speed and reliability of analysis in bioanalytical laboratories, a new method for determination of entacapone in human plasma with a short time of analysis (3 min) is described in this work. The LC-MS/MS technique was successfully employed to provide a satisfactory sensitivity and selectivity in a desirable time of chromatographic run.

EXPERIMENTAL

Entacapone (Fig. 1) was purchased from Yarrow chemicals, Mumbai, India and tolcapone, the internal standard (Fig. 2), from Mankind Pharma Limited (Calcutta, India). HPLC grade acetonitrile and methanol were obtained from J.T. Baker.

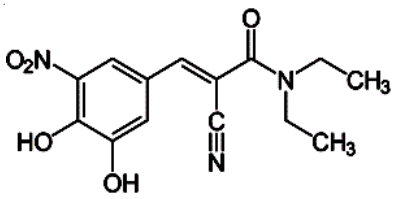

Fig. 1. Chemical structure of entacapone

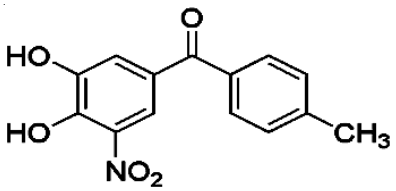

Fig. 2. Chemical structure of tolcapone

HPLC grade water was procured from Rankem Pharma. Ammonium Phosphate, acetic acid and ammonia solution (HPLC grade) were obtained from MERCK.

The liquid chromatographic system consist of LC Shimadzu LC10 from Shimadzu, an auto sampler of Shimadzu (SIL-HTc) coupled with an applied Bio systems SCIEX a triple quadrupole mass spectrometer (API 4000) with electro spray ionization (ESI) used for analysis. Date of acquisition and processing were controlled by Applied Biosystems/MDS SCIEX Analyst software (version 1.4.2) with ACE 3 C_{18} column (150 × 4.6 mm, 5 µ).

Bio-analytical conditions: The chromatographic analysis was performed by using a mobile phase of HPLC grade Acetonitrile: 10 mM Ammonium Phosphate buffer (50:50, v/v) with flow rate 0.5 mL/min by positive ion mode (API 4000). Detection is performed by atmospheric pressure electro spray ionization (ESI) tandem mass spectrometry in positive ion mode.

Mass spectrometry conditions

Acquisition duration:	3.0 min
Polarity:	Positive
Scan Time:	200 milli seconds (for each MRM)
Resolution:	Q1: Unit and Q3: Unit

Detection

	Q1 Mass	Q3 Mass
** Entacapone	305.10	242.10
** Tolcapone	272.20	212.10

Preparation of entacapone standard and working solutions: The entacapone stock solution was prepared by dissolving 10 mg of entacapone in 0.25 % ammonia solution in acetonitrile and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000000 ng/mL. This solution was kept in refrigerator at 2-8 °C. The stock solutions were diluted to suitable concentrations using diluent for spiking into plasma to obtain calibration curve standards, quality control samples for further use. All other dilutions were made in mobile phase.

Preparation of tolcapone stock solution (internal standard): A stock solution of internal standard (IS) was prepared by dissolving 10.00 mg of Tolcapone in diluent (mixture of HPLC grade acetonitrile and water in a ratio (50:50, v/v) and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000000 ng/mL. This solution was kept in refrigerator at 2-8 °C. Working IS solutions were prepared by suitably diluting the above mentioned stock solution a fresh before use.

Preparation of calibration curve standards and quality control (QC) samples: Calibration curve standard consisting of a set of eight non-zero concentrations ranging from 60 ng/mL to 2200 ng/mL of entacapone was prepared. Prepared quality control samples consisted of concentrations of 60 ng/mL (lower limit of quantification quality control sample), 180 ng/mL (lower quality control sample), 1100 ng/mL (middle quality control sample) and 1760 ng/mL (higher quality control sample) for entacapone. These samples were stored at –70 °C ± 10 °C until use. Twelve sets of LQC and HQC samples were stored at -20 °C ± 5 °C to check stability.

Sample preparation procedure: After bulk spiking, aliquots of 200 µL for calibration curves and 200 µL for quality controls of spiked plasma samples were pipetted out into a pre-labelled polypropylene micro centrifuge tubes and then all the bulk spiked samples were stored to deep freezer at –70 °C ± 10 °C, except twelve replicates each of LQC and HQC, which were stored in -20 °C ± 5 °C for generation of stability data. The thawed samples were vortexed to ensure complete mixing of the contents. 100 µL of the plasma sample was pipetted into stoppered test tubes, 20 µL of internal standard spiking solution were added to it and vortexed, except in blank plasma samples where 20 µL diluent was added to it and vortexed. Then 20 µL of 5 % phosphoric acid buffer was added to it and vortexed. Followed by addition of 3 mL of ammonium phophate and shaken for 15 min on reciprocating shaker at 250 rpm. Samples were centrifuged at 6000 rpm for 4 min at 5 °C. Then supernatant organic layer (3.0 mL) was transferred to pre-labelled glass dry test tubes and evaporated to dryness in turboVap at 40 °C. The samples were reconstituted in 1000 µL of mobile phase and 15 µL sample was injected to HPLC with MS-MS detection.

Method Validation: The method was validated for selectivity, linearity, accuracy, precision, recovery, stability and carry over test according to the principles of the FDA industry guidance.

Sensitivity: The lowest limit of reliable quantification (LLOQ) for entacapone was set at the concentration of the LLOQ *i.e.* 60 ng/mL. The precision and accuracy for entacapone at this concentration was estimated.

Linearity: The linearity of calibration curve for entacapone was assessed at eight concentration levels in the range of 60 ng/mL to 2200 ng/mL in plasma samples. Peak area ratios for each solution against its corresponding concentration were measured and the calibration curve was obtained from the least squares linear regression presented with their correlation coefficient.

Extraction recovery: Twenty four blank matrix samples were processed and six sets of each blanks samples were reconstituted with the aqueous quality control dilutions at low, middle and high concentration without internal standard, which represents 100 % extraction of analyte(s) (non-extracted samples). Six blanks were reconstituted with the internal

standard solution, which represents 100 % extraction of internal standard. (Non-extracted sample). The non-extracted samples were injected. The recovery comparison samples of entacapone were compared against extracted samples of LQC, MQC and HQC of PA BATCH-I (Precission and accuracy). The recovery comparison samples of internal standard were compared against the response of internal standard in MQC level.

$$R\ (\%) = (Psbe/Psae) \times 100$$

where: R is extraction recovery, Psbe is the mean value of the peak area responses obtained from plasma samples spiked with analyte before extraction and Psae is the mean value of the peak area responses obtained from plasma samples spiked with analyte after extraction.

Accuracy and precision: Intra assay precision and accuracy were determined by analyzing six replicates at four different quality control levels in two runs on the same day. Inter-assay precision and accuracy were determined by analyzing six replicates at four different quality control levels on five different runs. The acceptance criteria included accuracy within ≤ 15 % deviation (SD) from the nominal values, except LLOQ quality control, where it should be ≤ 20 % and a precision of ≤15 % relative standard deviation (RSD), except for LLOQ quality control, where it should be ≤ 20 %.

Stability: Stability of entacapone in plasma was performed using six replicates of two quality control samples at low and high levels. Samples were prepared by spiking drug-free plasma with appropriate volumes of entacapone standard solutions. The stability was evaluated with different studies such as room temperature stock solution stability, refrigerated stock solution stability, room temperature spiking solution stability, refrigerated spiking solution stability, freeze-thaw, short term stability, bench top stability *etc*. Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2-8 °C) was performed by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (6 h), processed sample stability (auto sampler stability for 32 h), freeze thaw stability (four cycles), reinjection stability (24 h), wet extract stability (30 h) and plasma samples stability at –20 °C were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (≤ 15 % SD) and precision (≤ 15 % RSD).

Matrix effect test of entacapone: Two sets of extracted blank plasma samples each containing six tubes (plasma taken from six different lots) are taken. One set of tubes are reconstituted with equivalent aqueous concentration of LQC and the other set of tubes are reconstituted with equivalent aqueous concentration of HQC. These samples are known as post spiked samples. These samples are analyzed along with equivalent aqueous LQC and HQC samples. The matrix effect is evaluated by determining the % response ratio using the formula.

$$\text{Response ratio}\,(\%) = \frac{\text{Mean area ratio of post spiked samples}}{\text{Mean area ratio of equivalent aqueous samples}} \times 100$$

RESULTS AND DISCUSSION

LC-MS/MS analysis: A binary mixture of acetonitrile and 10 mM ammonium phosphate in a ratio of 50:50, v/v was proved to be the most suitable of all the combinations since the chromatographic peaks obtained were well defined and resolved and free from tailing. A mobile phase flow rate of 0.5 mL/min. with a splitness of 25/75 was found to be suitable in the study range of 0.3 -1.0 mL/min. Detection of the ions were performed by multiple reaction monitoring (MRM) of the transitions *m/z* 305.10 and 242.10 for entacapone and *m/z* 272.20 and 212.10 for the internal standard and the mass spectrum of the drug molecule is given (Fig. 3).

A model chromatogram showing the separation of entacapone is presented in Figs. 4 and 5. Under the above optimized conditions retention times of 2.32 and 2.78 min were obtained for entacapone and tolcapone respectively.

Linearity: The calibration line was linear in the range of 60 ng/mL to 2200 ng/mL of the drug as shown in Fig. 6. A straight-line fit made through the data points by least square regression analysis showed a constant proportionality with minimal data scattering. The correlation coefficient (r^2) ranged from 0.9902 to 0.9994 for entacapone.

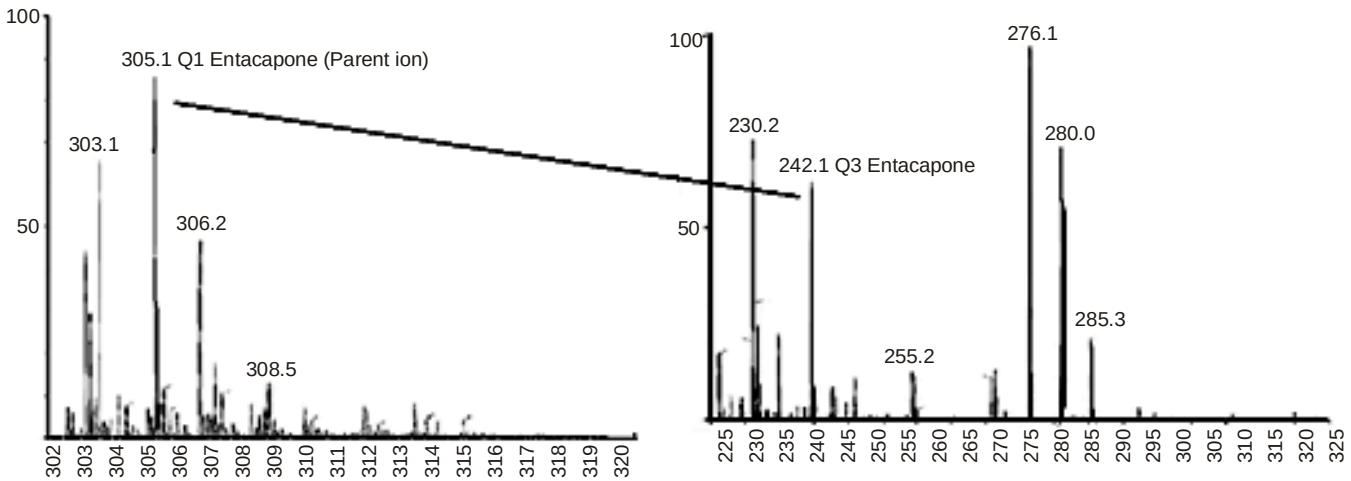

Fig. 3. Mass spectra of entacapone for parent and product masses

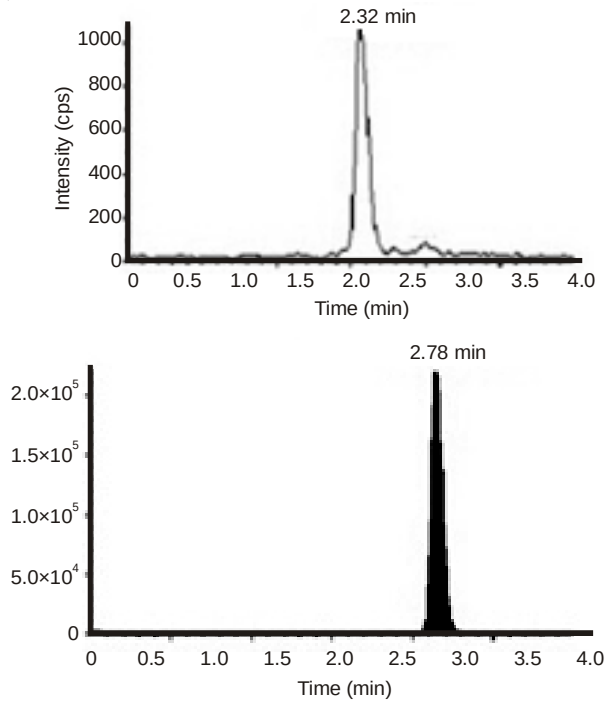

Fig. 4. Representative chromatogram of an aqueous standard and internal standard mixture

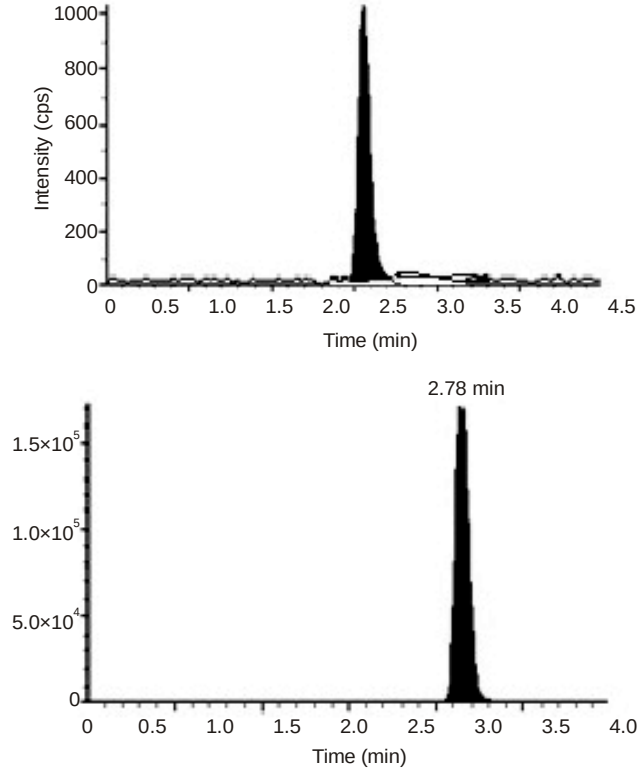

Fig. 5. Representative chromatogram of LLOQ sample of entacapone with internal standard

Selectivity: There was no significant interference from endogenous components observed at the mass transitions of entacapone and internal standard.

Recovery of the drug and internal standard: Recovery for entacapone was found to be in the range of 76.5 to 98.28 % (mean recovery: 90.22 %). While for tolcapone was 91.83 %. The results are shown in Tables 1 and 2.

Within-batch precision and accuracy: Within-batch precision for LLOQ quality control ranged from 0.71 % to 0.93 % and for LQC, MQC and HQC ranged from 0.07 % to 0.284 %. Within-batch accuracy ranged for LLOQ quality control ranged from 99.56 % to 100.4 % and for LQC, MQC and HQC ranged from 99.84 % to 100.07 %.

Intra-day precision and accuracy: Intra-day precision for LLOQ quality control was 0.66 % and for LQC, MQC and HQC ranged from 0.03 to 0.44 %. Intra-day accuracy for

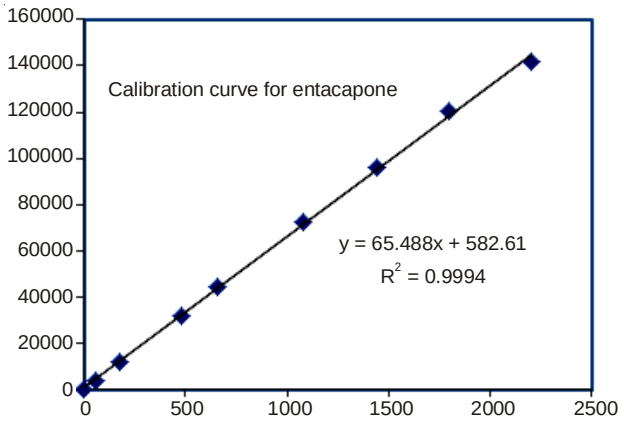

Fig. 6. Representative calibration curve for regression analysis of entacapone

TABLE-1
RECOVERY OF ENTACAPONE FROM HUMAN PLASMA

	LQC response		MQC response		HQC response	
	Extracted quality control	Non extracted quality control	Extracted quality control	Non extracted quality control	Extracted quality control	Non extracted quality control
Sample ID	LQC (07-12)	LQC (1-6)	MQC (07-12)	MQC (1-6)	HQC (07-12)	HQC (1-6)
1	12052	16052	72121	75152	120023	122058
2	12060	16124	72125	75452	120012	122023
3	12055	15958	72023	75685	120032	122054
4	12121	16023	71998	75124	120065	122089
5	12265	15025	72158	75002	120045	122124
6	12352	16123	72542	75123	120054	122456
Mean	16131.7	15884.17	72161.17	75256.33	120038.5	122134
SD	1275.25	417.7942	195.8553	253.0619	18.38047	157.2052
CV (%)	0.97	2.63	0.27	0.33	0.01	0.12
N	6	6	6	6	6	6
Recovery (%)	76.5		95.88		98.28	
Overall recovery	90.22					

TABLE-2
RECOVERY OF TOLCAPONE FROM HUMAN PLASMA

Extracted quality control ID	IS response in extracted samples (area)	Non-extracted quality control ID	IS response in non-extracted samples (area)
MQC-7	326652	MQC-1	354578
MQC-8	325546	MQC-2	356898
MQC-9	324565	MQC-3	357845
MQC-10	325689	MQC-4	356412
MQC-11	328598	MQC-5	351245
MQC-12	324578	MQC-6	352648
Mean	325938	Mean	354937.7
SD	1479.258	SD	2581.703
CV (%)	0.45	CV %	0.72
N	6	N	6
Recovery (%)		91.83	

LLOQ quality control was 100.29 % and for LQC, MQC and HQC ranged from 99.91 to 99.98 %.

Between batch/inter-day precision and accuracy: Between batch precision for LLOQ quality control was 1.129 % and for LQC, MQC and HQC ranged from 0.037 to 0.43 %. Between batch accuracy for LLOQ quality control was 100.06 % and for LQC, MQC and HQC ranged from 99.81 to 99.99 %.

The results of within batch, intraday and between batch precision and accuracy results are represented in Tables 3-5 respectively.

Stability: The processing and storage conditions of clinical samples need to maintain the integrity of a drug or at least keep the variation of pre-analysis as minimal as possible. For this reason, stability studies play an important role in a bio-analytical method development. In this study, the stability was assessed by considering different studies such as room temperature stock solution stability, refrigerated stock solution stability, room temperature spiking solution stability, refrigerated spiking solution stability, freeze-thaw, short term stability, bench top stability *etc*. The results presented in Table-6 shows that entacapone is stable under the studied conditions, since in all cases the international acceptance criteria (variation values for area smaller than 15 %) were met.

Matrix effect: No significant matrix effect was observed in all the eight batches including haemolysis and lipemic plasma for entacapone at low (LQC) and high (HQC) concentrations. The precision and accuracy for entacapone at LQC concentration was found to be 0.31 % and 100.21 % respectively and at HQC concentration was found to be 0.016 % and 100.01 %, respectively and shown in Table-7.

Conclusion

An alternative HPLC/ESI/MS/MS method for quantification of entacapone in human plasma has been successfully developed and validated. A simple and inexpensive liquid-liquid extraction procedure and an isocratic chromatography condition using a reversed-phase column provided an assay well suited for real time analysis. The method exhibited excellent performance in terms of selectivity, linearity, accuracy, precision, recovery, stability and matrix effect test. In addition, the reported method has a short analysis run time, an advantage over previously reported methods. Therefore, this method is suitable for therapeutic drug monitoring of entacapone.

TABLE-3
CALCULATED CONCENTRATION OF QUALITY CONTROL SAMPLES FOR ENTACAPONE (WITHIN-BATCH)

Quality control	LLOQ quality control	LQC	MQC	HQC
	Concentration (ng/mL)			
	60.0	180.0	1100.0	1760
1	60.010	180.01	1100.21	1760.08
2	61.210	180.02	1099.02	1759.98
3	60.020	180.21	1099.99	1759.90
4	60.235	179.84	1100.01	1759.99
5	60.200	179.89	1100.12	1761.05
6	60.120	180.02	1100.00	1759.97
Mean	60.29917	179.9983	1099.892	1760.162
SD	0.433513	0.128471	0.407543	0.437196
CV (%)	0.71	0.07	0.037	0.024
Nominal (%)	100.4	99.99	99.9	100.004
N	6	6	6	6
7	59.90	180.21	1100.11	1759.95
8	59.80	180.00	1099.99	1759.92
9	59.95	180.11	1100.20	1759.9
10	60.01	180.00	1100.00	1760.06
11	58.75	179.01	1100.99	1759.10
12	60.02	178.99	1098.00	1759.99
Mean	59.73833	179.72	1099.882	1759.82
SD	0.484601	0.511054	0.988682	0.351705
CV (%)	0.81	0.284	0.089	0.02
Nominal (%)	99.56	99.84	99.98	99.98
N	6	6	6	6
13	60.25	179.99	1100.11	1760.05
14	59.98	180.01	1099.99	1760.09
15	59.98	180.02	1099.99	1759.93
16	59.25	179.88	1100.32	1759.90
17	61.02	180.01	1100.56	1760.11
18	60.02	179.88	1104.03	1761.02
Mean	60.08333	179.965	1100.833	1760.183
SD	0.564363	0.130907	1.542214	0.413665
CV (%)	0.93	0.07	0.14	0.023
Nominal (%)	100.13	99.98	100.07	100.01
N	6	6	6	6

TABLE-4
INTRA-DAY PRECISION AND ACCURACY FOR ENTACAPONE

Quality control	LLOQ quality control	LQC	MQC	HQC
	Concentration (ng/mL)			
	60.0	180.0	1100.0	1760.0
1	59.89	179.93	1099.08	1759.92
2	60.09	179.09	1099.99	1759.09
3	59.99	180.08	1100.09	1759.10
4	59.90	181.09	1100.04	1759.99
5	59.94	179.08	1100.04	1759.05
6	61.02	179.92	1099.04	1760.01
7	61.00	178.99	1099.00	1761.00
8	59.92	180.04	1099.06	1760.08
9	60.09	181.02	1100.01	1759.03
10	60.10	179.01	1100.09	1760.09
11	59.99	180.06	1100.99	1760.01
12	59.89	179.93	1099.08	1759.92
Mean	60.17545	179.8464	1099.766	1759.761
SD	0.400031	0.717727	0.606844	0.593441
CV (%)	0.66	0.44	0.05	0.03
Nominal (%)	100.29	99.91	99.97	99.98
N	12	12	12	12

TABLE-6
STABILITY RESULTS OF ENTACAPONE AND TOLCAPONE

Analyte	Entacapone		Tolcapone	Acceptance criteria	
Stability method	Nominal (%)	Precision		Nominal (%)	Precision
Room temperature stock solution stability (0 & 6 h)	99.28 % (6 h)		100.05 % (6h)	Comparison response: 90-110 %	
Refrigerated stock solution stability (4 days)	99.31 %		97.68 %		
Room temperature spiking solution stability (6 h)	98.43 % (6 h)		95.25 % (6 h)	Comparison response: 90-110 %	
Refrigerated spiking solution stability (3 days)					
LQC	99.43 %		–	Comparison response: 90-110 %	
Bench-top stability (6 h)	99.98-100.02 %	0.025-0.319 %	–	85-115 %	≤ 15 %
Auto sampler stability (32 h)	99.99-100.02 %	0.036-0.221 %	–	85-115 %	≤ 15 %
Freeze thaw stability (IV cycle)	99.99-100.0 %	0.036-0.29 %			
Reinjection stability (24 h)	99.84-99.98 %	0.021-0.4 %	–	85-115 %	≤ 15 %
Wet extract stability (30 h)	99.96-100.0 %	0.026-0.24 %			

TABLE-7
MATRIX EFFECT OF ENTACAPONE

Plasma (Batch No.)	M-163 ME QC#	LQC 180.0 (ng/mL)			Mean	M-163 ME QC#	HQC 1760.0 (ng/mL)			Mean
P040310-253	1, 2, 3	180.044	181.08	179.99	180.3713	1, 2, 3	1759.58	1761.99	1759.05	1760.207
P050510-287	1, 2, 3	179.950	179.49	181.92	180.4533	1, 2, 3	1760.99	1759.00	1759.96	1759.983
P050510-288	1, 2, 3	181.540	181.02	179.97	180.8433	1, 2, 3	1760.43	1759.87	1759.38	1759.893
P240610-309	1, 2, 3	179.910	179.55	180.05	179.8367	1, 2, 3	1761.98	1759.09	1760.29	1760.453
P240610-313	1, 2, 3	181.010	180.97	180.43	180.8033	1, 2, 3	1759.94	1760.66	1761.94	1760.847
P050510-290	1, 2, 3	180.970	179.02	179.94	179.9767	1, 2, 3	1759.02	1761.94	1759.25	1760.070
P070310-254 (Lipemic)	1, 2, 3	179.080	180.76	179.00	179.6133	1, 2, 3	1758.98	1759.91	1761.89	1760.260
P070310-255 (Hemolytic)	1, 2, 3	181.790	180.01	181.89	181.2300	1, 2, 3	1759.90	1760.93	1759.97	1760.267
Mean					180.391					1760.248
SD					0.556277					0.299621
CV (%)					0.31					0.016
Nominal (%)					100.21					100.01
N					8					8

TABLE-5
BETWEEN BATCH/INTER DAY PRECISION AND ACCURACY FOR ENTACAPONE

Quality control	LLOQ quality control	LQC	MQC	HQC
	Concentration (ng/mL)			
	60	180	1100	1760
1	59.01	179.09	1099.98	1759.09
2	59.09	179	1099	1759
3	59.99	179.05	1099.99	1760.01
4	60.09	179.89	1100	1761
5	60.1	181	1100.08	1761.01
6	60.08	180.09	1100.05	1760.09
7	59.89	180.05	1100.09	1760.03
8	61.09	181.08	1100.1	1759.99
9	60.05	179.04	1099.89	1759.08
10	60.98	179.02	1099.11	1759.03
11	59.04	179	1099.04	1759
12	59.09	179.02	1099.98	1759.09
13	60.03	179.04	1099.93	1760.03
14	61.05	179.99	1099.97	1759.01
15	61	181.07	1100.05	1760.03
16	60	179.01	1100.99	1760.09
17	60.09	180	1100.02	1760.08
18	59.01	179.09	1099.98	1759.09
Mean	60.03941	179.6729	1099.898	1759.745
SD	0.678697	0.766595	0.458029	0.658037
CV (%)	1.129	0.43	0.041	0.037
Nominal (%)	100.06	99.81	99.99	99.98
N	18	18	18	18

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