DEVELOPMENT AND VALIDATION OF OPICAPONE IN HUMAN PLASMA USING TOLCAPONE AS INTERNAL STANDARD BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

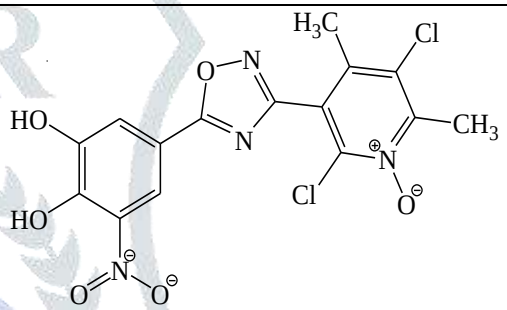
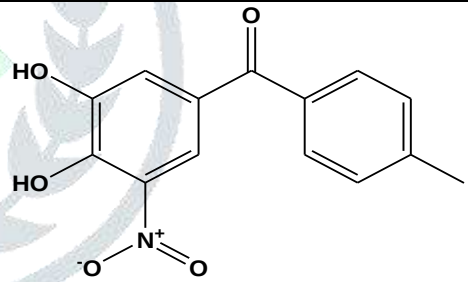
The present LC-MS/MS method for the estimation of Opicapone in human plasma by using Tolcapone as an internal standard was established and validated as per FDA guidelines The retention time of Opicapone and Tolcapone (Internal standard) was found to be 1.81 min and 2.1 min respectively. Linearity was established for Opicapone in the range of 40 ng/mL to 1800 ng/mL with correlation coefficient (r=0.999) and the overall percentage recovery was 94.63 % for Opicapone and 91.94 % for Tolcapone (Internal standard) respectively. The CV % values of accuracy and precision for Opicapone were found to be ≤ 15 %, which indicate accuracy and precision of the proposed method. The CV % values of accuracy and precision of Opicapone for stability studies were found to be ≤ 15 %, which indicate stability of the proposed method. The LC-MS/MS method for the estimation of Opicapone in human plasma by using Tolcapone as an internal standard 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 Opicapone.

Key words: Opicapone, Tolcapone, Linearity, Liquid Chromatography-Tandem Mass Spectrometry.

INTRODUCTION

A LC-MS/MS method was performed on a liquid chromatographic system consist of Shimadzu LC 10, an auto sampler of Shimadzu (SIL-HTc) coupled with an Applied Biosystems SCIEX a triple quadrupole mass spectrometer (API 4000) with electrospray ionization (ESI) used for analysis and Applied Biosystems/MDS SCIEX Analyst software (version 1.4.2) for processing and data collecting. Develosil ODS HG-5 RP C_{18} column (150 mm x 4.6 mm ID, 5 µm) is used as a stationary phase. An ultrasonic bath sonicator (Frontline FS 4, Mumbai, India), semi-micro analytical balance (India) and Whatman filter paper No. 41 is used in the study.

Table 1: IUPAC names and structures of Opicapone and Tolcapone (Internal standard)

Official Name	IUPAC Name	Structure
Opicapone	(4Z)-4-[3-(2,5-dichloro-4,6-dimethyl-1-oxidopyridin-1-ium-3-yl)-2H-1,2,4-oxadiazol-5-ylidene]-2-hydroxy-6-nitrocyclohexa-2,5-dien-1-one	
Tolcapone (Internal standard)	(3,4-dihydroxy-5-nitrophenyl)-(4-methylphenyl)methanone	

MATERIALS AND METHODS

Reagents used

Opicapone were procured from Yarrow chemicals, Mumbai, India. Tolcapone (Internal Standard) was procured from Mankind Pharma Limited, India. Acetonitrile of HPLC grade were procured from Rankem Ltd., India. Water of HPLC grade was obtained from Merck Specialties Private Limited, Mumbai, India. Ammonium phosphate and orthophosphoric acid of HPLC grade was procured from Merck Specialties Private Limited, Mumbai, India.

Preparation of mobile phase

An accurately weighed quantity of 1.32 g of Ammonium phosphate was taken into a 1000 mL beaker and diluted to 1000 mL with HPLC grade water and degassed in ultrasonic water bath and filtered through 0.45 µm nylon membrane filter using vacuum filtration gives required buffer concentration of 0.01 M Ammonium phosphate buffer and the pH was adjusted to 4 with orthophosphoric acid. 0.01 M Ammonium phosphate buffer with pH was adjusted to 4 with orthophosphoric acid were mixed with HPLC grade Acetonitrile in the proportion of 55:45, v/v and it was filtered through 0.45 µm nylon membrane filter and degassed by ultrasonication.

Preparation of standard and working solutions for Opicapone

The Opicapone stock solution was prepared by dissolving 10 mg of Opicapone in 1% ammonia solution in acetonitrile and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000µg/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 stock solution for Tolcapone (Internal standard)

A stock solution of Tolcapone (Internal standard) was prepared by dissolving 10 mg of Tolcapone in diluent (mixture of HPLC grade acetonitrile and water in a ratio (60:40, v/v) and made up the volume with the same in a 10 mL volumetric flask to produce a solution of 1000µg/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 40 ng/mL to 1800 ng/mL of Opicapone was prepared. Prepared quality control samples consisted of concentrations of 40 ng/mL (lower limit of quantification quality control sample), 120 ng/mL (lower quality control sample), 900 ng/mL (middle quality control sample) and 1560 ng/mL (higher quality control sample) for Opicapone. 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.

Preparation of plasma samples

For the preparation of plasma samples, human blood samples were collected into polypropylene tubes containing K_2-EDTA. Each tube was centrifuged for 15 min at 8500 rpm and the supernatant was collected in another tube. To the supernatant 1 mL of acetonitrile was added and kept for 10 min for the plasma proteins to precipitate and then the supernatant was collected for further use.

Procedure for Spiked Human Plasma

Liquid-liquid extraction was used to isolate Opicapone and Tolcapone IS from human plasma. For this, aliquots of 20 µL of internal standard and 100 µL of plasma sample was added into labelled polypropylene tubes and vortexed briefly. Followed by addition of 20 µL of diluent and vortexed. Then 20 µL of 5 % orthophosphoric acid buffer was added to it and vortexed. Followed by addition of 5 mL of ammonium phosphate and shaken for 30 min on reciprocating shaker at 500 rpm. Samples were centrifuged at 2000 rpm for 10 min at 5 °C. Then supernatant organic layer (5.0 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 which contains 0.01M ammonium phosphate buffer: acetonitrile (55:45; v/v) and 20 µL of sample were injected to HPLC with MS-MS detection.

Preparation of sample solution

After bulk spiking, aliquots of 100 µL for calibration curves and 100 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

RESULTS AND DISCUSSION

For the optimisation of LC-MS/MS method several parameters and mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Opicapone were obtained with Develosil ODS HG-5 RP C_{18} column (150 mm x 4.6 mm ID, 5 µm) and mobile phase containing a mixture of 0.01M Ammonium phosphate buffer with pH was adjusted to 4 with orthophosphoric acid and Acetonitrile in the proportion of (55:45, v/v) was delivered at a flow rate of 0.5 mL/min by positive ion mode (API 4000) with injection volume of 20 µL and a run time of 3 min. Detection is performed by atmospheric pressure electrospray ionization (ESI) tandem mass spectrometry in positive ion mode. The precursor to product ion transitions is *m/z* 414.2 to 261.1 for Opicapone and *m/z* 274.20 to 183.10 for Tolcapone (Internal standard) were used for quantization was shown in Figure. The retention time of Opicapone and Tolcapone (Internal standard) was found to be 1.81 min and 2.1 min respectively. A typical chromatogram of blank plasma, mobile phase, Opicapone and Tolcapone (Internal standard) is shown in Figure.1

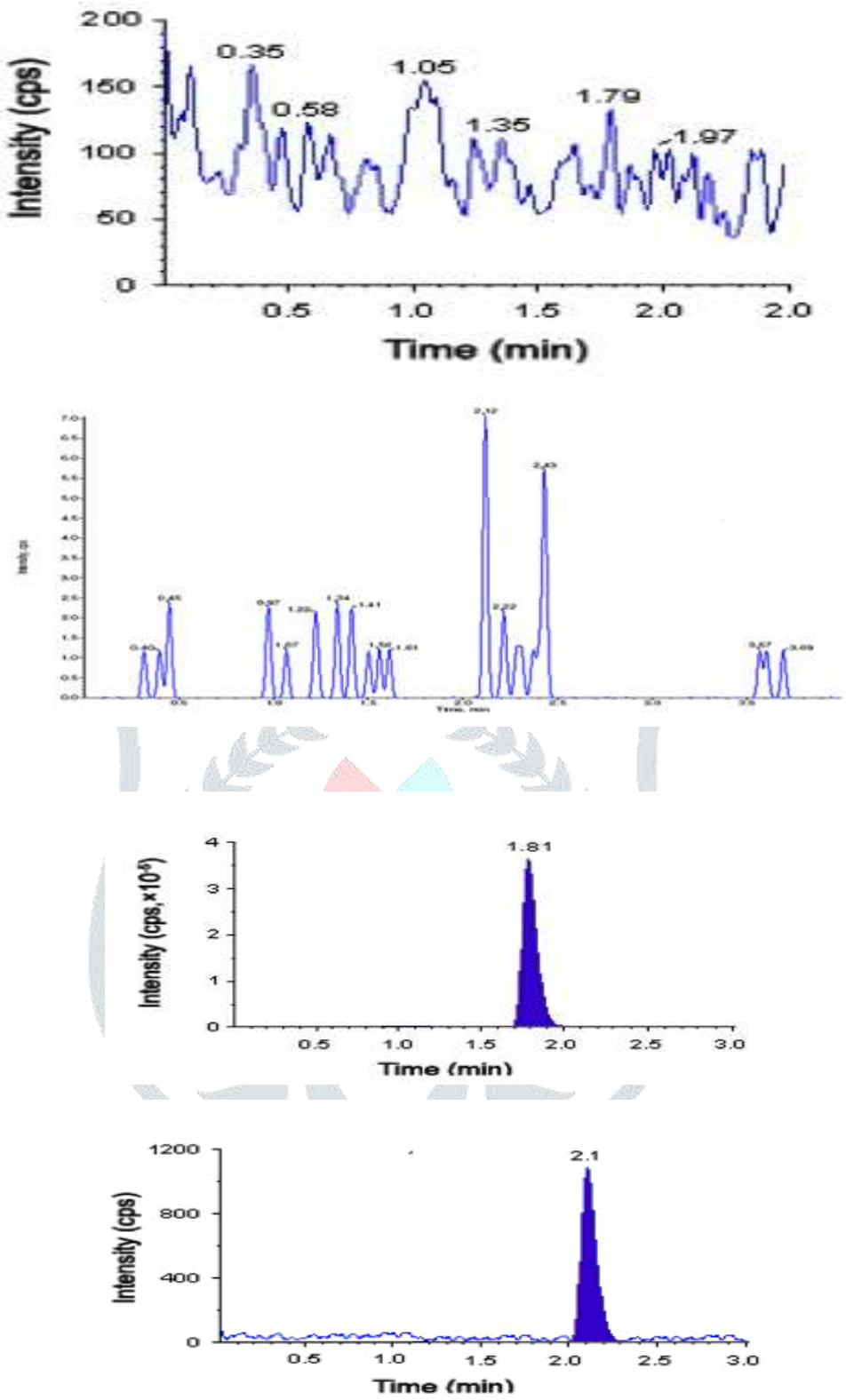


Fig. 1: Chromatogram of blank plasma, mobile phase, Opicapone and Tolcapone (Internal standard)

Method validation

The established LC-MS/MS method was validated for selectivity, specificity, sensitivity, linearity, accuracy, precision, recovery, stability and carry over test according to the principles of the FDA guidelines.

Screening of plasma lots and specificity

The selectivity of the present method was evaluated by screening six different lots of blank plasma. All of them were found to have no significant endogenous interferences at the retention times of the analyte and the internal standard. The same human EDTA plasma lots free of interfering substances were used to prepare the calibration curve standards and the quality control samples for the validation study.

Sensitivity

The lowest limit of reliable quantification (LLOQ) for Opicapone was set at the concentration of 40 ng/mL. The precision and accuracy for Opicapone at this concentration was estimated.

Linearity

The linearity of Opicapone was assessed at six concentration levels in the range of 40, 120, 420, 900, 1560 and 1800 ng/mL in plasma samples. Peak area ratios for each solution against its corresponding concentration were measured and the calibration curve was obtained.

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 Opicapone were compared against extracted samples of LQC, MQC and HQC of PA Batch-I (Precision and accuracy). The recovery comparison samples of internal standard were compared against the response of internal standard in MQC level.

$$\text{Extraction recovery (R \%)} = \frac{\text{Psbe}}{\text{Psae}} \text{ x } 100$$

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 Opicapone 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 Opicapone 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 Opicapone

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.

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

LC-MS/MS analysis

A binary mixture of 0.01 M Ammonium phosphate buffer with pH was adjusted to 4 with orthophosphoric acid and Acetonitrile in the proportion of (55:45, v/v) was proved to be the most suitable mobile phase 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* 414.2 to 261.1 for Opicapone and *m/z* 274.20 to 183.10 for Tolcapone (Internal standard). The retention time of Opicapone and Tolcapone (Internal standard) was found to be 1.81 min and 2.1 min respectively.

Linearity

The calibration curve was linear in the range of 40 ng/mL to 1800 ng/mL of the Opicapone as shown in Figure. 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) was 0.999 for Opicapone as shown in below Table and Figure.

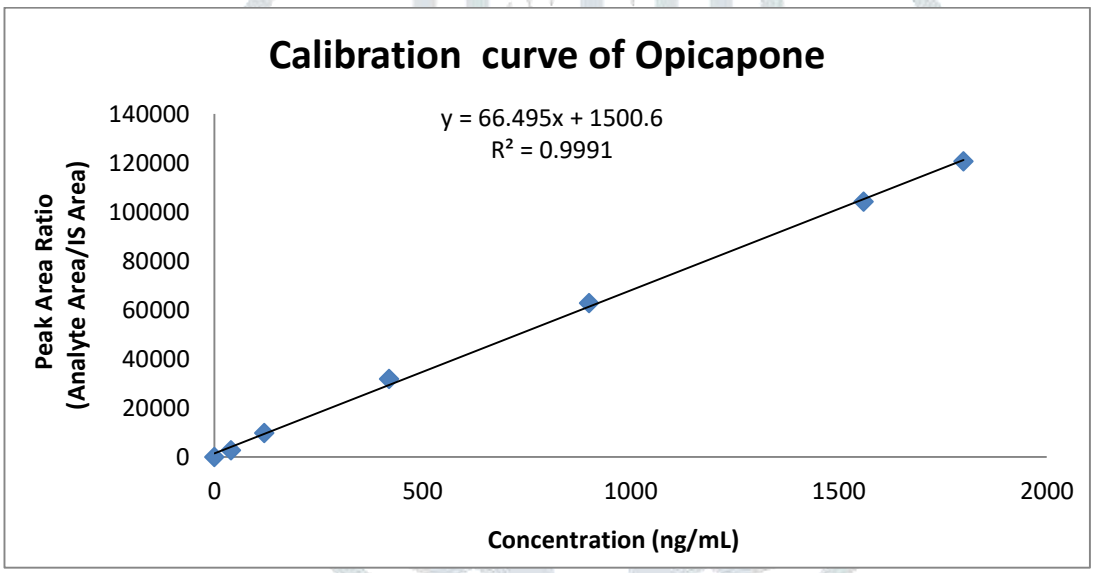


Fig. 2: Representative calibration curve for regression analysis of Opicapone

Table 2: Linearity of Opicapone

Concentration of Opicapone (ng/mL)	Peak Area Ratio (Analyte area/IS area)
40	2786
120	9817
420	31910
900	62872
1560	104218
1800	120738

Selectivity

There was no significant interference from endogenous components observed at the mass transitions of Opicapone and Tolcapone (internal standard).

Recovery of the Opicapone and Tolcapone (Internal standard)

Recovery for Opicapone was found to be in the range of 92.14% to 96.19% and the mean recovery for Opicapone was 94.63 %. While for Tolcapone (Internal standard) the mean recovery was 91.94 %.

Within-batch precision for LLOQ quality control ranged from 0.113 % to 0.199 % and for LQC, MQC and HQC ranged from 0.009 % to 0.183 %. Within-batch accuracy ranged for LLOQ quality control ranged from 100.05 % to 100.13 % and for LQC, MQC and HQC ranged from 99.92 % to 100.09 %. The results of within-batch precision and accuracy for Opicapone are represented in Table 3 and 4.

Table 3: Recovery of Opicapone 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	9873	10811	62713	65177	104216	108122
2	9802	10722	62691	65393	104291	108118
3	9821	10686	62889	65887	104415	110466
4	9864	10691	62718	65832	104217	109865
5	9877	10742	62727	65918	104589	107078
6	9892	10518	62682	65664	104214	107101
Mean	9855	10695	62737	65645	104324	108458
SD	35.28	97.80	76.53	300.32	151.53	1413.23
CV%	0.36	0.91	0.12	0.46	0.15	1.30
N	6	6	6	6	6	6
Recovery%	92.14		95.57		96.19	
Overall recovery%	94.63					

Table 4: Recovery of Tolcapone (Internal standard) 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	336965	NON EXTRACTED-MQC-1	368032
MQC-8	339043	NON EXTRACTED-MQC-2	367928
MQC-9	338573	NON EXTRACTED-MQC-3	368012
MQC-10	338457	NON EXTRACTED-MQC-4	368027
MQC-11	338521	NON EXTRACTED-MQC-5	367888
MQC-12	338320	NON EXTRACTED-MQC-6	368011
Mean	338313	**Mean**	367983
SD	704.2768	**SD**	60.02
CV%	0.21	**CV%**	0.02
N	6	**N**	6
Recovery%	91.94		

Intra-day precision and accuracy

Intra-day precision for LLOQ quality control was 0.202 % and for LQC, MQC and HQC ranged from 0.007 % to 0.045%. Intra-day accuracy for LLOQ quality control was 100.15 % and for LQC, MQC and HQC ranged from 100.01 % to 100.03 %. The results of intra-day precision and accuracy for Opicapone are represented in Table.

Between batch/inter-day precision and accuracy

Between batch precision for LLOQ quality control was 0.262 % and for LQC, MQC and HQC ranged from 0.024 % to 0.097 %. Between batch accuracy for LLOQ quality control was 100.03 % and for LQC, MQC and HQC ranged from 100.01 % to 100.05 %. The results of between batch/inter-day precision and accuracy for Opicapone are represented.

Table 5: Within-batch precision and accuracy for Opicapone

Quality control	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	40	120	900	1560
1	40.01	119.78	900.01	1554.12
2	40.08	120.03	900.23	1558.15
3	40.02	120.1	900.03	1560.03
4	40.13	120.08	900.04	1560.12
5	40.04	119.53	900.02	1560.01
6	40.03	119.94	900.1	1560.02
Mean	40.05	119.91	900.07	1558.74
SD	0.045	0.220	0.084	2.388
CV%	0.113	0.183	0.009	0.153
Nominal %	100.13	99.93	100.01	99.92
N	6	6	6	6
7	39.91	120.08	900.12	1560.08
8	40.01	120.01	900.03	1560.03
9	40.03	120.02	899.93	1561.03
10	39.98	119.97	899.34	1559.92
11	40.14	120.23	900.12	1560.81
12	40.08	120.34	900.04	1560.64
Mean	40.03	120.11	899.93	1560.42
SD	0.080	0.146	0.297	0.467
CV%	0.199	0.121	0.033	0.030
Nominal %	100.06	100.09	99.99	100.03
N	6	6	6	6
13	40.11	119.68	900.07	1561.08
14	40.02	120.21	899.67	1560.13
15	39.98	120.08	900.02	1560.01
16	39.96	120.02	900.1	1560.32
17	40.04	120.13	900.05	1561.11
18	40.01	119.96	901.19	1560.13
Mean	40.02	120.01	900.18	1560.46
SD	0.053	0.185	0.518	0.499
CV%	0.131	0.154	0.058	0.032
Nominal %	100.05	100.01	100.02	100.03
N	6	6	6	6

Table 6: Intra-day precision and accuracy for Opicapone

Quality control	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	40	120	900	1560
1	40.02	120.08	901.08	1560.11
2	39.98	119.95	900.12	1560.08
3	40.01	119.97	900.18	1560.37
4	40.03	120.12	899.98	1560.05
5	40.11	120.04	899.96	1560.08
6	40.03	120.03	900.16	1560.04
7	40.12	120.05	900.12	1560.11
8	40.04	120.11	899.97	1560.04
9	40.09	119.97	900.02	1560.03
10	40.07	120.07	900.01	1560.21
11	39.95	120.01	900.04	1559.89
12	40.26	120.04	900.01	1560.17
Mean	40.1	120.04	900.1	1560.1
SD	0.1	0.1	0.3	0.1
CV%	0.202	0.045	0.034	0.007
Nominal %	100.15	100.03	100.02	100.01
N	12	12	12	12

Table 7: Between batch/inter-day precision and accuracy for Opicapone

Quality control	Concentration (ng/mL)			
	LLOQ QC	LQC	MQC	HQC
	40	120	900	1560
1	40.01	120.15	900.01	1560.01
2	39.93	120.12	900.09	1560.08
3	40.13	120.01	900.01	1560.14
4	40.11	120.07	900.34	1559.41
5	39.89	120.02	900.07	1561.02
6	40.02	120.01	901.06	1561.03
7	39.93	120.24	899.92	1560.08
8	39.91	120.13	899.87	1560.04
9	39.92	120.15	900.02	1559.98
10	40.11	119.86	901.03	1559.88
11	39.96	119.73	900.16	1559.79
12	40.02	120.12	900.08	1560.08
13	39.91	120.14	899.94	1560.02
14	40.01	120.03	899.99	1560.03
15	39.89	120.08	900.22	1560.01
16	40.11	120.04	900.06	1560.06
17	40.24	120.13	900.31	1559.98
18	40.14	120.01	900.24	1560.25
Mean	40.01	120.06	900.19	1560.11
SD	0.10	0.12	0.34	0.38
CV%	0.262	0.097	0.037	0.024
Nominal %	100.03	100.05	100.02	100.01
N	18	18	18	18

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, bench top stability, auto sampler stability, freeze-thaw stability, re-injection stability and wet-extract stability. The results show that Opicapone is stable under the studied conditions, since in all cases the international acceptance criteria (variation values for area smaller than 15 %) were met.

Room temperature stock solution stability

The stability was found to be 100.2 % for Opicapone with the precision ranged from 0.11% to 0.14 %. The stability was found to be 100.1 % for Tolcapone (Internal standard) with the precision ranged from 0.11 % to 0.35 %.

Refrigerated stock solution stability (at 2-8 °C)

The stock solution was found to be stable for four days. The four days stock solution stability of Opicapone and Tolcapone (Internal standard) was found to be 100.1 % and 100.3 % respectively.

Room temperature spiking solution stability

The stability was found to be 100.2 % for Opicapone with the precision ranged from 0.07% to 0.071 %. The stability was found to be 100.4 % for Tolcapone (Internal standard) with the precision ranged from 0.03 % to 0.08 %.

Refrigerated spiking solution stability of Opicapone (at 2-8 °C)

The spiking solutions were found to be stable for three days. The three days spiking solution stability of Opicapone at LQC level was found to be 99.05 %.

Bench top stability

Opicapone was found to be stable upto 6 hours as per the acceptance criteria. The percent mean nominal ranged from 100.02 % to 100.07 % and the precision ranged from 0.014 % to 0.078 %.

Auto sampler stability

The results demonstrate that the processed samples were stable for 32 hours. The percent nominal at 32 hours ranged from 100.04 % to 100.06 % and precision ranged from 0.049 % to 0.050 %.

Freeze-thaw stability

Freeze-thaw stability of Opicapone is shown in Table. The percent nominal ranged from 99.98 % to 99.99 % and the precision ranged from 0.020 % to 0.121 % for four freeze-thaw cycles.

Re-injection stability

The results demonstrate that the reinjected samples were stable for 24 hours. The percent stability at 24 hours ranged from 99.98 % to 100.01 % and the precision ranged from 0.01 % to 0.37 % for 24 hours.

Wet-extract stability

Wet extract stability results are shown in Table. The results demonstrate that the processed samples were stable for 30 hours. The percent nominal at 30 hours ranged from 100.01 % to 100.02 % and the precision ranged from 0.042 % to 0.063 %.

Matrix effect

No significant matrix effect was observed in all the eight batches including haemolysis and lipemic plasma for Opicapone at low (LQC) and high (HQC) concentrations. The precision and accuracy for Opicapone at LQC concentration was found to be 0.05 % and 100.03 % respectively and at HQC concentration was found to be 0.01 % and 100.01 % respectively.

CONCLUSION

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. This method was intended for rapid and accurate estimation of Opicapone in human plasma. Good separation of the chromatographic peaks was observed and no interfering peaks are found.

Conflicts of Interest

The authors declare that they have no conflicts of Interest.

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