Asian Journal of Chemistry; Vol. 34, No. 6 (2022), 1587-1591

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2022.23720

Simultaneous Estimation of Levodopa and Carbidopa in Mice Plasma by LC-MS/MS

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Received: 7 January 2022; *Accepted*: 17 April 2022; *Published online*: 18 May 2022; AJC-20827

A simple and sensitive liquid chromatographic method along with tandem mass detection has been developed for the determination of levodopa and carbidopa in mice plasma. Owing the hydrophilic nature of both the analytes, a hydrophilic interaction liquid chromatography (HILIC) setup was used for their separation by a Merck (Germany) HILIC column (4.6 × 250 mm, 5 µm; 200 Å). The mobile phase composed equal proportion of water and acetonitrile both containing 0.1% formic acid at a flow rate of 1.4 mL/min to achieve rapid separation of the compounds. The column was coupled with a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source using multi reaction monitoring (MRM) analysis. Since, recovery of both analytes in mice plasma posed significant challenge, a customized extraction procedure based on protein precipitation was adopted with best recovery. The optimized HILIC-MS/MS condition led to yield lower limit of quantification (LLOQ) of 9.9 ng/mL and 2.47 ng/mL for levodopa and carbidopa, respectively. The method was validated with suitable determination of correlation coefficient (R^2: 0.997), precision (1.6-17.2%), accuracy (84.7-120%). Successful application of this validated method was accomplished for analytes in biological samples.

Keywords: Levodopa, Carbidopa, Mice plasma, LC-MS/MS.

INTRODUCTION

Parkinson's disease is a progressive neurodegenerative disease that affect about 2% of the geriatric population [1]. The root cause of this disease is the degeneration of dopaminergic neurons resulting depletion of dopamine levels in the central nervous system [2]. As dopamine cannot readily cross the blood brain barrier, its metabolic precursor levodopa (3,4-dihydroxy phenyl alanine) in combined with a peripheral decarboxylase inhibitor carbidopa [(−)-l-2-(3,4-dihydroxybenzyl)-2-hydrazino-propionic acid] is used in combination to treat Parkinson's disease. This reduces daily dosage requirements of levodopa as well as its associated side effects [3].

Many chromatographic methods have been reported for the determination of levodopa and carbidopa in blood plasma to assist in the management of Parkinson's disease. However, these technique methods have their own limitations like cost factor, selectivity and sensibility, use of organic solvents, tedious sample preparation techniques, long analysis time, *etc*. Additionally, residual proteins in injected samples may shorter the lifetime and efficacy of the chromatographic columns [4]. For example, one of such study reported liquid-liquid extraction technique in rat plasma with perfluropentanoic acid having the less sensitive validated range of 50-10,000 ng/mL and 25-5,000 ng/mL for levodopa and carbidopa, respectively [5]. In another work, rat and monkey plasma with ethylene bridged hybrid (BEH) C18 column with ultra high performance liquid chromatography using two stabilizer as sodium metabisulfite and hydrazine dihydrochloride was used to stabilized the analyte [6], levodopa and carbidopa in human plasma with HPLC having electrospray ionization using the methyldopa as an internal standard [7].

The aim of the current study is to develop a simple, rapid and reliable method for levodopa and carbidopa in mice plasma having higher sensitivity with cost effective extraction technique along with single stabilizing agent getting maximum recovery by LC-MS/MS with adequate separation of peak with analyte interest and validated according to the guideline of the USFDA on bioanalytical method validation [8].

EXPERIMENTAL

Standards of levodopa, carbidopa and telmisartan were purchased from Sigma-Aldrich, India. Dimethylacetamide (DMA), formic acid, sodium metabisulphite, polyethylene glycol (PEG-200), Tween-80, methyl cellulose were obtained from Merck Limited, India. Dimethyl sulfoxide (DMSO) was procured from Rankem, Avantor. The LC-MS grade methanol, acetonitrile and water were purchased from J.T. Baker, Avantor Performance Materials India Limited. Syndropa CR tablets containing levodopa (200 mg) and carbidopa (50 mg) manufactured by Sun Pharma (Batch no. BSU09473) were purchased from a local pharmacy store.

Preparation of stock solutions and working solutions: Stock solutions (1 mg/mL) of levodopa, carbidopa and telmisartan standards were prepared by dissolving appropriate mass of the standards in acetonitrile. Working solutions was prepared from the marketed formulation having the ratio 4:1 of levodopa and carbidopa. The stock prepare by calculating the 1 mg/mL of levodopa in 1.5 mL Eppendorf tube and a few microliters of DMSO was added to dissolve the compound at room temperature. After substance dissolution, the final volume was made up to 1 mL with DMSO. According to this the preparation of working standard solution of levodopa is 1 mg/mL and carbidopa is 0.25 mg/mL.

Calibration standard and quality control samples: The processing volume of calibration standard and quality control samples were 20 µL. Firstly weighed the compound and then prepared the stock solution of 1 mg/mL. The serial dilutions were made with methanol:water (50:50) having 1% of sodium metabisulphite in water. Took 2 µL of aqueous calibration standard and properly vortex with the 18 µL of blank plasma and add 20 µL of 2%FA in water to the calibration standard and vortex properly. Then the calibration standard crash with the 200 µL of ACN containing internal standard and vortex properly and centrifuged it at 4000 rpm for 5 min. After taking out the sample from centrifuge, aliquot 180 µL and diluted it with 120 µL of water.

Animals: Male CD-1 IGS mice (20-25 g) were procured from the animal house of Aurigene Discovery and Technologies Limited, India. All the experiments were approved by the Institutional Animal Ethics Committee. Animals were caged separately in a quite untroubled environment for 7 days before the study, with free access to food (normal rat chows) and water. Prior to the experiment, animals were fasted for 12 h with free access to water. Then the drug were given IV and oral route by incorporating into 5% DMA + 20% peg-200 + Q.S. saline and 0.5% methy cellulose + 0.5% Tween-80 in water at a dose of 3 MPK and 10 MPK, respectively. After drug administration, blood samples were collected at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h and 24 h from jugular vein for estimating several biochemical parameters. The collected blood was centrifuged at 4000 rpm for 5 min at 4 ºC. After centrifugation, the separated plasma sample was transferred in a 1.5 mL microcentrifuge tubes and stored at -18 ºC until used.

Instrumental conditions: Experiments were performed by an AB-Sciex LC-MS/MS (5500, Q Trap) system coupled to a Nexeara X2 HPLC system (Shimadzu). Analyst (1.6.3) software was used for data acquisition. Chromatographic separation was carried out in a HILLIC (250 mm × 4.6 mm) 200Å column (Merck). The data was obtained by electro-spray ionization source (ESI^+) along with positive ion detection mode [9]. API 5500 triple quadrupole mass spectrometer (AB Sciex) accompanying with Turbo V Ionspray source working in the positive mode was carrying out for the detection of mass spectroscopy [10]. Analyst 1.4.2 software was used for the data acquisition. The mass spectrometer was work in the multiple-reaction monitoring (MRM) mode using the transitions from the protonated molecules at *m/z* 198 → 152 for levodopa, *m/z* 227 → 181 for carbidopa [5]. The details of the internal standards and MRM parameters used in the analysis are given in Table-1.

Chromatography conditions: Both levodopa and carbidopa being highly polar compounds were retained in the HILLIC column (250 mm × 4.6 mm) 200 Å (Merck) to prevent co-eluting interferences from complex biological matrix components. The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1% formic acid. Chromatographic run were performed for 5 min in the isocratic mode with solvent composition consisting of 50% A and 50%, at a flow rate of 1.4 µL min^{-1}. The injection volume was maintained at 15 µL and 1.4 µL min^{-1} running under isocratic mode.

Validation parameter: The developed method was validated according to the Bioanalytical Method Validation guidelines of USFDA [6]. The validation parameters were accuracy and precision, selectivity, sensitivity, linearity, limit of detection, limit of quantification, carry over matrix effect and stability [5].

RESULTS AND DISCUSSION

Both drugs levodopa and carbidopa were resolved in only 5 min using a mobile phase consisting of 1:1 proportion of water and acetonitrile, both containing 0.1% of formic acid in isocratic mode. Carbidopa was eluted first at retention time of 2.97 min followed by levodopa at 3.07 min and sodium metabisulphide was used as stabilizer to enhance stability of levodopa. The mass spectrometer was work in the multiple- reaction monitoring (MRM) mode using the transitions at *m/z* 198 → 152 for Levodopa, *m/z* 227 → 181 for carbidopa.

Specificity and selectivity: The developed LC-MS/MS method was found to be selective and specific as it was evalu-

TABLE-1
INTERNAL STANDARDS AND MRM PARAMETERS USED IN THE ANALYSIS

Analyte	Retention time (min)	Internal standard; Rt (min)	Declustering potential (V)	Collision energy (V)	MRM transition
Carbidopa	2.97	Telmisartan	80	15	*m/z* 227 → 181
Levodopa	3.07	Telmisartan	80	15	*m/z* 198 → 152

ated by six blank plasma injections and there was no quantifiable response or signal detected at the retention time of levodopa and carbidopa (Figs. 1 and 2). The selectivity of levodopa and carbidopa at the lower limit of detection are listed in Table-2. Standard deviation and percentage relative error (%RE) was found to be within the acceptance criteria.

Sensitivity: The sensitivity of the method was evaluated by analyzing at the lower level of quantifications of levodopa (9.90 ng/mL) and carbidopa (2.47 ng/mL). One matrix derived peak was observed in chromatogram but it was well separated by the method (Figs. 3 and 4). The nominal concentration and the %RE of the sample was within the acceptance range.

Linearity: It was determined by the regression analysis of standard plots correlated with different points of standard curve. The linearity range for levodopa is 9.90 to 9898.00 ng/mL and for carbidopa is 2.47 to 2474.50 ng/mL concentration.

TABLE-2
SELECTIVITY AT LLOQ

	Levodopa	Carbidopa
Nominal conc.	9.9	2.475
Calculated concentration (n = 6)	7.01	3.64
	14.21	3.41
	12.95	2.13
	10.17	3.28
	9.76	2.38
	10.68	2.97
Mean	10.203	2.493
SD	0.467	0.431
%RSD	4.571	17.299
%RE	3.060	0.727

Throughout the course of validation the average correlation coefficient (R^2) was ≥ 0.997.

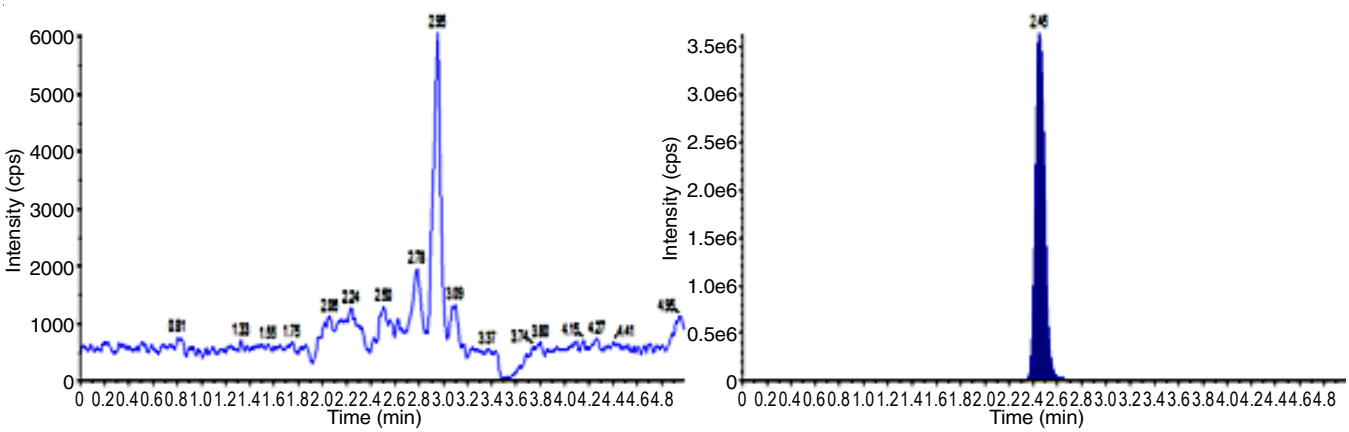

Fig. 1. Blank sample of levodopa

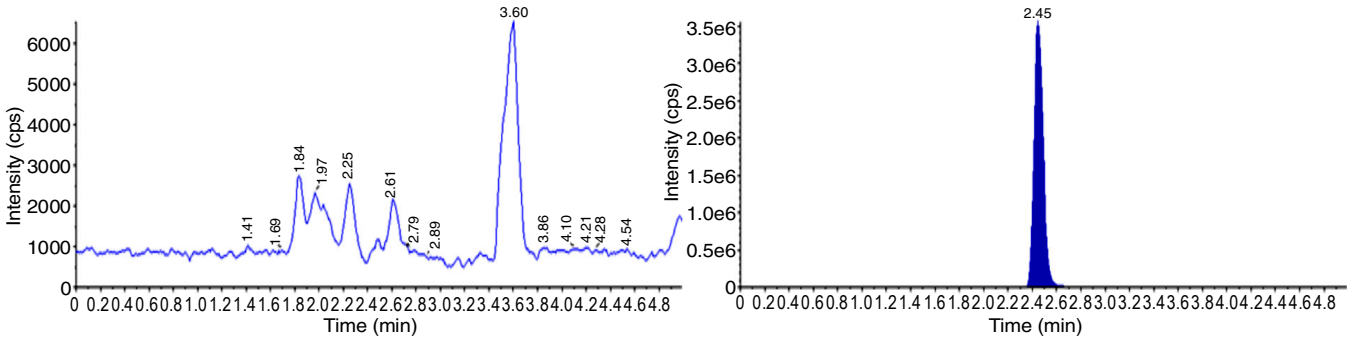

Fig. 2. Blank sample of carbidopa

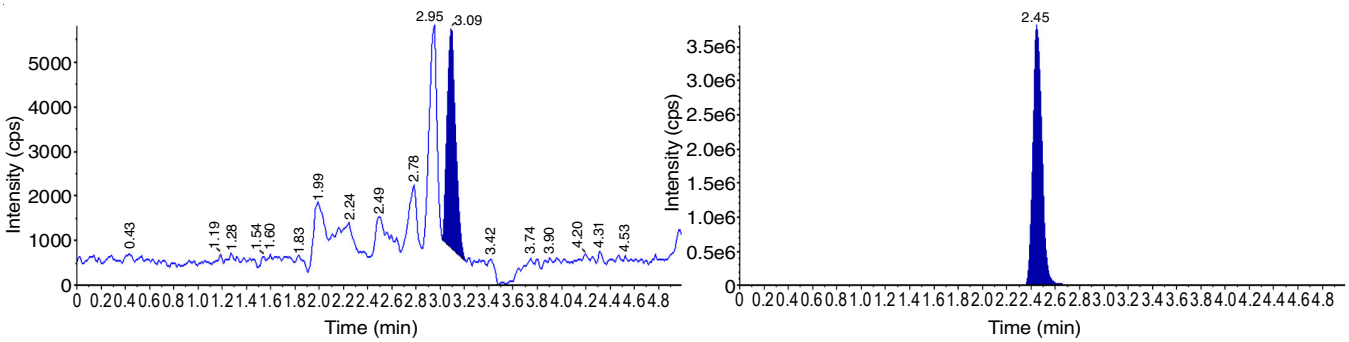

Fig. 3. LLOQ peak of the levodopa

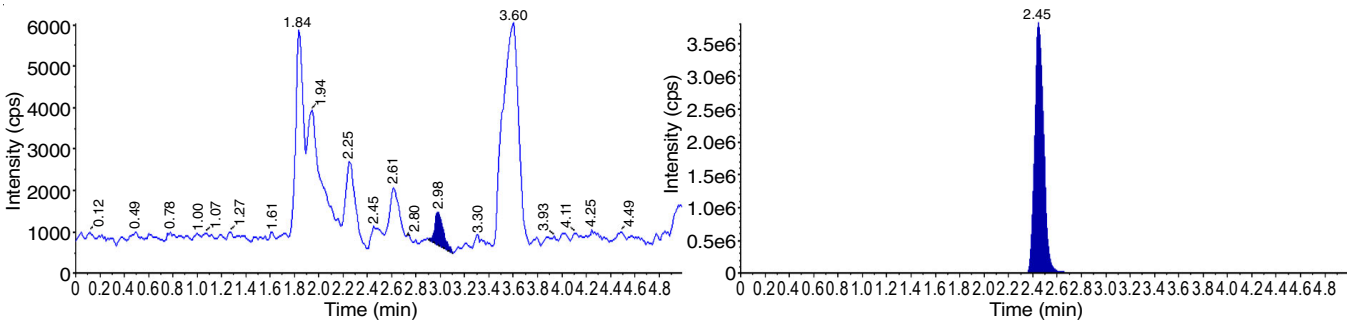

Fig. 4. LLOQ peak of carbidopa

Recovery: Extraction procedure done by the protein precipitation with 1:1 mixture of methanol and water. Inspite of both levodopa and carbidopa being highly unstable compounds, recovery of respectively 51.49% and 50.19% was obtained. 1% Sodium metabisulphide in water and it was carried in acidic medium by adding 2% formic acid in water for better stability.

Accuracy and precision: These were determined by analyzing the quality control (QC) samples at various concentrations (LLOQ, Low QC ,Mid QC and High QC), representing the entire range of the calibration curve. Six replicates at each QC levels was analyzed and standard deviation (SD), %RE (accuracy) and %RSD (precision) was calculated against the corresponding nominal concentration and listed in Tables 3 and 4 for levodopa and carbidopa, respectively.

Carryover and matrix effect: The detection of analyte may be affected by the presence of unmonitored and co-eluting compound from the matrix, which is commonly known as matrix effect. In present study, no carryover in both levodopa and carbidopa detected. This was performed by processing blank matrix (plasma) with protein precipitation extraction method. Aqueous quality control (QC) samples were separately prepared by adding 5 µL of aqueous QC (AQS: LQC, MQC and HQC) and 495 µL of RS. Blank samples were also reconstituted with aqueous QC samples (AQS: LQC, MQC & HQC to prepared post spiked samples. This was compared with those of the aqueous solutions (prepared using appropriate solvent in which analyte is freely soluble) and checked for suppression or enhancement in the ionization. The matrix factor for both levodopa and carbidopa was found to be 0.988 and 0.843, respectively.

Pharmacokinetics: The pharmacokinetics parameters were estimated using the WinNonlin 8.0 software [11]. After oral administration of 10 mg/kg, levodopa plasma concentration reached C_{max} of 2351.13 ng/mL at T_{max} 0.25 h, with $AUC_{(0-\infty)}$ of 2234 ng h/mL with 62% bioavailability. After IV administration of 3 mg/kg, levodopa exhibited medium clearance (46.627 mL/min/Kg) with moderate volume of distribution (1.34 L/kg) and terminal half-life of 0.39 h. After intravenous administration of 3 mg/kg, carbidopa exhibited high clearance (209.08 mL/min/Kg) with high volume of distribution (7.51 L/kg) and terminal half-life of 0.62 h. After the oral administration of 10 mg/kg, caridopa plasma concentration reached C_{max} of 64.39 ng/mL at T_{max} 0.25 h, with $AUC_{(0-\infty)}$ of 93 ng h/mL (Fig. 5).

Stability of quality control samples: The spiked quality control samples were stored at room temperature for 8 h to determine the bench top stability. The processed quality control samples were stored in the auto sampler, which was maintained at 15 ºC for 12 h to determine the autosampler stability. Freeze thaw stability of the spiked quality control samples were determined after three cycles stored at -80 ºC. Long term stability of the spiked quality control samples were determined after stored for 7 days at -80 ºC. Above 67% QC samples and more than 50% at each QC level passed the test. The percentage cumulative variance of levodopa and carbidopa (LQC and HQC) for bench top stability were 12.190, 1.026 and 6.686, 1.1484, for auto sampler stability 3.361, 1.389 and 9.341, 1.505. The

TABLE-3
ACCURACY AND PRECISION OF LEVODOPA

Sample	Nominal concentration (ng/mL)	Mean concentration (ng/mL) (n = 6)	SD	%RSD	%RE
LLOQ	9.9	10.203	0.467	4.517	3.060
LQC	27.23	24.307	0.767	3.155	-10.73
MQC	4841.2	4980.462	189.272	3.800	2.876
HQC	7448	7706.178	130.417	1.692	3.466

TABLE-4
ACCURACY AND PRECISION OF CARBIDOPA

Sample	Nominal concentration (ng/mL)	Mean (ng/mL) (n = 6)	SD	%RSD	%RE
LLOQ	2.475	2.493	0.431	17.299	0.727
LQC	6.808	7.262	0.367	5.051	6.668
MQC	1210.3	1236.042	43.190	3.494	2.126
HQC	1862	1958.823	36.522	1.862	5.19

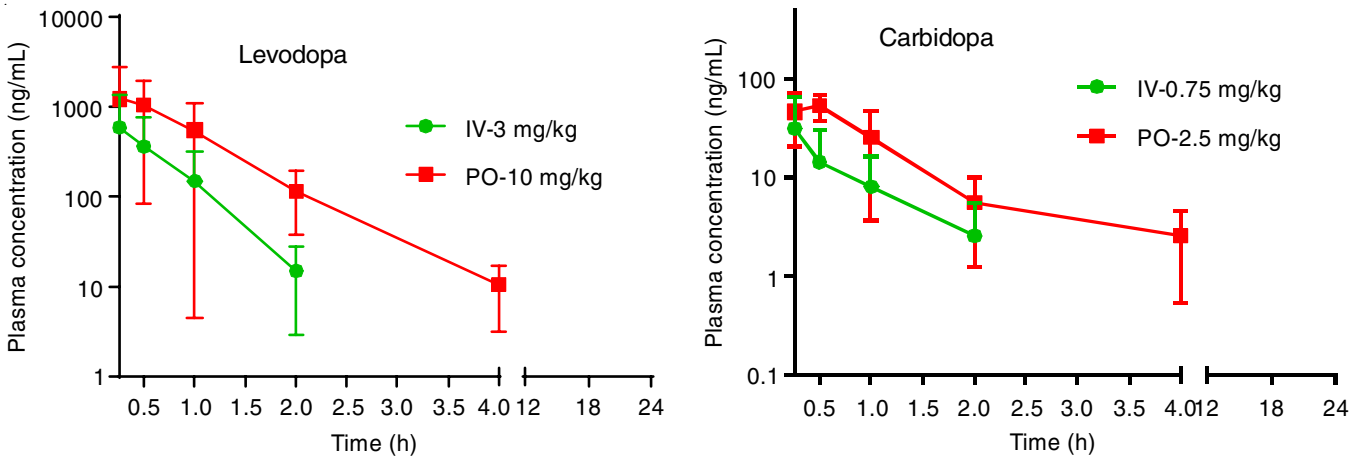

Fig. 5. PK profile of intravenous (IV) and oral (PO) levodopa and carbidopa administration

freeze thaw stability 5.030, 2.269 and 3.780, 2.126, while long term stability 9.112, 2.451 and 6.549, 2.408 for levodopa and carbidopa, respectively.

Conclusion

In this study, a selective and sensitive simultaneous bioanalytical method development and validation of levodopa and carbidopa in mice plasma was developed by LC-MS/MS. A bioanalytical sample preparation technique has been developed and achieved the higher sensitivity of analyte with cost effective protein precipitation extraction technique and getting maximum recovery in single stabilizing agent. The developed method was validated according to USFDA guidelines on Bioanalytical Method Validation for all the validation parameters.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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