CODEN (USA): IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

Available online at: http://www.iajps.com *Research Article*

SELECTIVE AND RAPID LC-MS/MS METHOD FOR THE SIMULTANEOUS QUANTITATION OF LEVODOPA AND CARBIDOPA IN HUMAN PLASMA USING ALUMINA SPE CARTRIDGES.

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Abstract:

A simple, sensitive and rapid high-performance liquid chromatography-tandem mass spectrometry method was developed and validated for the simultaneous quantification of the levodopa (LEV) and carbidopa (CAR) in human plasma. Following solid phase extraction, the electron rich elements in the protein, which were triggered during analysis of small molecules, were eliminated to avoid matrix effect, using alumina-A cartridges. The analytes (levodopa and carbidopa) and internal standard (methyldopa) were analysed using mobile phase of constant eluting strength on phenominex kinetex biphenyl, 50 mm x 3 mm, 2.6 µm column and were analyzed by an LC-MS/MS in the multiple reaction monitoring mode using the respective $[M+H]^+$ ions, m/z 198→ 152 for levodopa, m/z 227→ 181 for carbidopa and m/z 212→ 166 for the internal standard. The assay exhibited a linear dynamic range of 5 to 2000 ng/mL for levodopa and 2 to 500 ng/mL of carbidopa. The lower limit of quantification was 5 ng/mL for levodopa and 2 ng/mL for carbidopa with a relative standard deviation of less than 1.0%. This LC-MS/MS method was validated with intra-batch and inter-batch precision and accuracy. Results for precision and accuracy for LEV and CAR are in range of 3.4 – 5.6 %, 4.1 to 6.4 % and 99.6 – 101.3 %, 94.5 – 102.2 % respectively. This validated method is simple and repeatable to use in bioequivalence/pharmacokinetic studies.

Key Words: *Levodopa, Carbidopa, liquid chromatography-tandem mass spectrometry, human plasma.*

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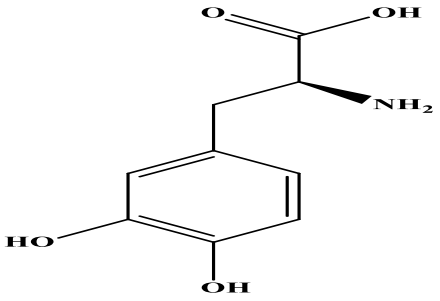
Please cite this article in press as Sreekanth Kakarla et al, ***Selective and Rapid LC-MS/MS Method for the Simultaneous Quantitation of Levodopa and Carbidopa in Human Plasma Using Alumina SPE Cartridges****, Indo Am. J. P. Sci, 2016; 3(8).*

INTRODUCTION:

Parkinson's disease is a common age-related neuro-degenerative disorder characterized by cardinal motor symptoms that include bradykinesia with resting tremor, rigidity and gait disturbance [1]. In recent years, new drugs have become available for the treatment of Parkinson's disease. However, since the introduction of dopamine (DOPA) supplementation, levodopa (LEV) has been considered the gold standard treatment for motor symptoms [2–4]. Current Parkinson's disease therapy is largely based on a dopamine replacement strategy, and the oldest, most efficacious and best tolerated drug for dopaminergic substitution therapy is levodopa [5-6]. Levodopa administration was improved by the addition of the dopa decarboxylase inhibitors, such as carbidopa, which reduces the peripheral degradation of levodopa to dopamine. [5] The concomitant administration of levodopa and carbidopa enhances the clinical benefit of levodopa by decreasing its peripheral metabolism, thus providing more drug for delivery to the brain and conversion into dopamine.



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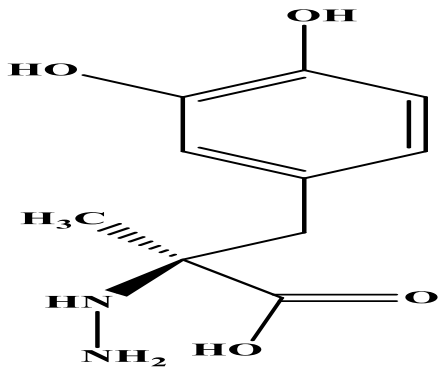

b)

Fig 1: Chemical structures of (a) levodopa and (b) carbidopa.

By reducing daily levodopa dosage requirements, the addition of carbidopa also reduces the side effects associated with levodopa administration. [7-8]. Prolonged use of LEV leads to fluctuations and motor complications such as the "wearing-off" phenomenon characterized by moments without benefits. [9] Thus, the determination of levodopa and carbidopa in biological fluids has an essential role in the diagnostics and control of Parkinson's disease.

Several analytical methods have been described in the literature for the determination of LEV and CAR in biological matrices using high-performance liquid chromatography (HPLC) and various detection techniques such as electrochemical detection [10-18], tandem mass spectrometry (MS/MS) [19-23], and fluorescence [24-25]. All the methods mentioned have less sensitivity and more runtime (≥8 min). Cesar et al. developed LC-MS/MS method for simultaneous quantitation of levodopa and carbidopa in human plasma [26] with a run time of 6 min and also used perchloric acid for sample extraction technique. However, strong acids are unsuitable for ESI-MS/MS owing to the deterioration of sensitivity, as noted by Li et al. [27]. Acids can hydrolyze proteins. In fact, this is the major reason the human stomach secrets hydrochloric acid after a meal. The acid content provides hydrogen ions (H^+) which will protonate any element possessing what are called lone electron pairs. Oxygen and nitrogen possesses such lone electron pairs, which are ideal targets for hydrogen ions provided by any acid; strong acids provide copious quantities of hydronium ions. These ions protonate as it is called the electron rich elements such as oxygen and nitrogen of the proteins. Under very strong acid

treatment, disruption of the bonding in protein can result, matrix effect during ionization in mass spectrometer [28- 30]. Vilhena et al. developed a LC-MS/MS method for simultaneous quantitation of levodopa and carbidopa in human plasma using HILIC column [31]. This method having disadvantage of 10 min run time and also having less stability period in plasma as 1 hours at room temperature and 4 hours in auto sampler which is not sufficient enough for bio-analysis. None of the methods were compatible for LC-MS/MS pharmacokinetic studies.

Thus, it was decided to develop and validate [32-36] a more sensitive, selective and compatible LC-MS/MS method in plasma with less run time and also to establish more stability period which would be useful for therapeutic drug monitoring and pharmacokinetic studies.

EXPERIMENTAL:

Reagents and samples

The reference standards (levodopa and carbidopa, Fig. 1(a) and 1(b)) and internal standard (ISTD; methyldopa) were obtained from Clearsynth Labs. (Mumbai, India) Gradient grade LiChrosolv methanol, acetonitrile (ACN), analytical grade formic acid (GR Grade) was purchased from Merck (Worli, Mumbai, India). Sodium metabisulphite (GR Grade) was obtained from Thermo Fisher

Scientific India Private Limited (Sion East, Mumbai, India). Ultrapure type-1 water from Milli-Q system (Millipore, Bedford, MA, USA) was used for preparing solutions. 4 ml RIA vials and 25 ml volumetric flasks were obtained from Tarsons products Pvt. Ltd., New Delhi, India. For sample preparation, a solid phase extraction speed disk equipped with 50mg/1CC Alltech Alumina-A cartridges (Orochem Technologies Inc. (Naperville, Illinois, USA) was used.

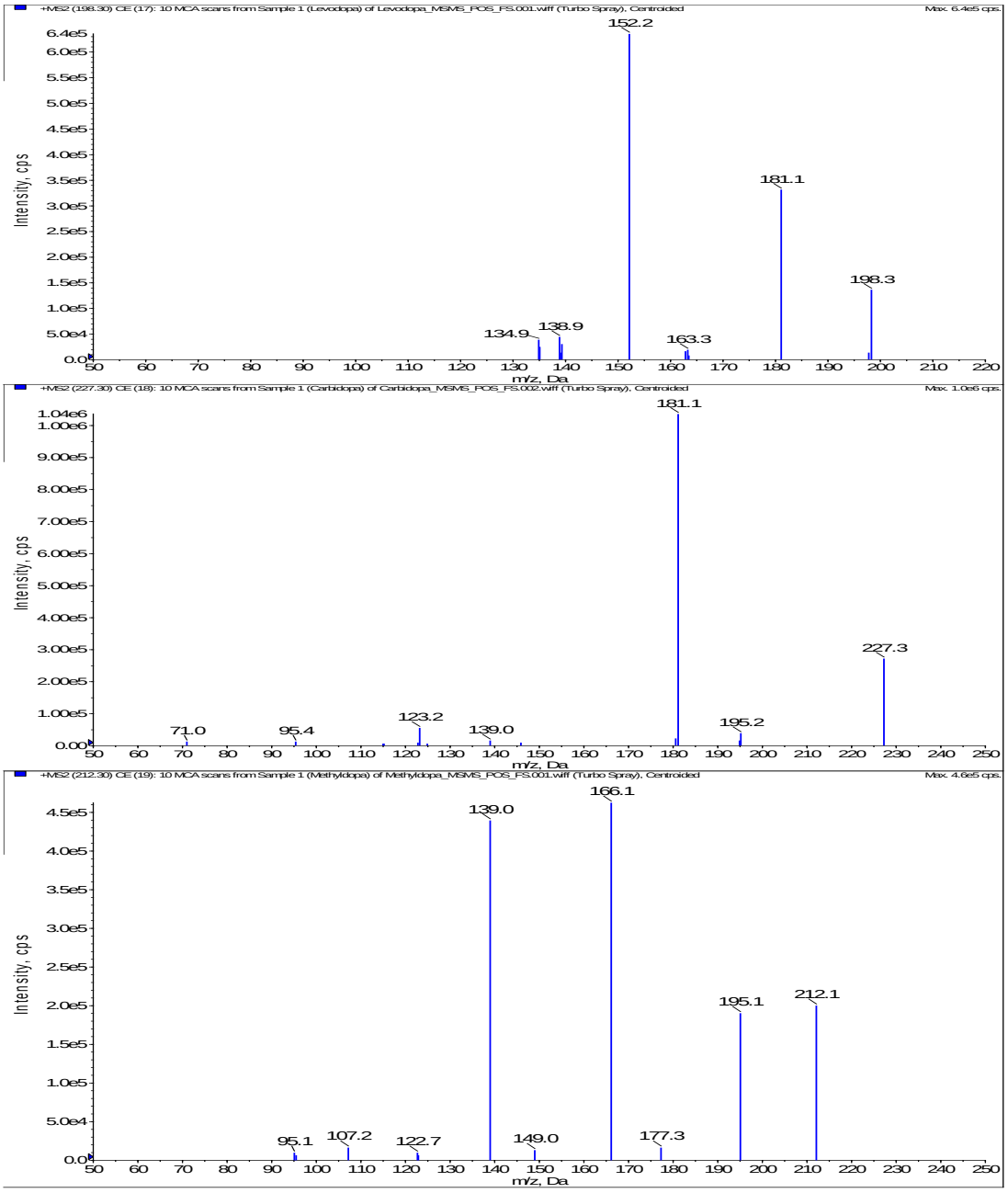

Fig 2: Product ion spectra of (upper) levodopa, (middle) carbidopa and (lower) methyldopa.

LC-MS/MS instrument and conditions

The HPLC SIL HTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with an LC-AD VP binary pump, a DGU20A5 degasser, and a SIL-HTC auto sampler with a CTO-10AS VP thermostat column oven maintained at 35°C temperature. A Kinetex biphenyl 50 mm x 3 mm, 2.6 µm was used as stationary phase. Both the analytes and internal standard were eluted by isocratic elution mode consisting of a mixture of 0.1% formic acid and acetonitrile (80/20, v/v). The flow rate was 0.3 mL/min. The mobile phase was split using a flow splitter and 60% of the flow was sent in to the MS system.

Mass spectrometric detection in a multiple reaction mode (MRM) was performed using an API 4000 triple quadrupole instrument (MDS-SCIEX, Concord, Ontario, Canada). A turbo ion spray interface operating in positive ionization with the turbo-gas temperature at 450°C, an ion spray needle voltage at 5500 V, entrance potential at 10 V and collision cell exit potential at 10 V were used for both analytes and ISTD. Declustering potential & collision energy was set at 40 V & 17 V for levodopa and 40 V & 19 V for carbidopa and methyldopa respectively. The precursor/product ion pairs in the MRM mode monitored were *m/z* 198/ 152 for levodopa, 227/181 for carbidopa and *m/z* 212/166 for ISTD. The collision gas and curtain gas were set at 6 and 40 (arbitrary units) respectively. Data acquisition was performed with analyst 1.4.2 software (MDS-SCIEX, Concord, Ontario, Canada). The product ion spectra of levodopa, carbidopa and methyldopa are shown in Fig. 2.

Preparation of stock and working solutions

Four hundred microgram per milli liter of stock solution of LEV, CAR and MET (ISTD) were prepared separately by dissolving 2.0 mg of respective drugs using 5 mL of 0.1% formic acid. The final concentration of the stock solutions were calculated by considering the amount of stock weighed and % purity on as is basis. The prepared stock solutions were stored in refrigerator at 2-8 °C with protection from light.

The working solutions of LEV, CAR and MET were prepared from the stock solution using 0.1% formic acid as a diluent.

Internal standard working solution at 5 µg/mL concentration was prepared in 0.1% formic acid. The prepared working solutions were stored at room temperature with protection from light and fresh dilutions were made on day-to-day basis during the analysis. All the volumetric measurements were made using calibrated micropipettes.

Calibration and QC samples preparation

Calibration standards and quality control (QC) samples were prepared by spiking stabilized blank plasma (2%) with freshly prepared working solutions. The stabilized blank plasma was prepared by 0.4 mL of 20% sodium metabisulfite in 0.2% formic acid was added to each 10 mL of plasma. Spiking volume was kept low to avoid unintended changes in sample matrix. Blank plasma lots obtained from healthy, non-smoking volunteers were individually screened and pooled before use. Calibration standards (STD 1 to STD 8) for LEV at concentrations of 5, 10, 50, 200, 500, 1000, 1600 and 2000 ng/mL, for CAR at concentrations of 2, 5, 10, 50, 100, 300, 400 and 500 ng/mL were prepared. Quality control samples were prepared for LEV at 5 ng/mL (LLOQ QC), 15 ng/mL (LQC), 990 ng/mL (MQC), 1570 ng/mL (HQC) and 4000 ng/mL (DQC), for CAR at 2 ng/mL (LLOQ QC), 6 ng/mL (LQC), 250 ng/mL (MQC), 390 ng/mL (HQC) and 1000 ng/mL (DQC). LLOQ QC samples were prepared only during validation batch runs. 0.300 mL each of the plasma spiked standards and quality control samples were distributed in to single use polypropylene tubes and stored at -70 ± 15 °C, until analysis.

Sample preparation

The stored plasma samples were retrieved from freezer thawed un-assisted at room temperature and are subjected to the following sample preparation procedure. A 200 µL aliquot of each sample was dispensed into 5 mL polypropylene tubes and added 50 µL of ISTD solution (MET, 5 µg/mL), followed by 100 µL of Milli-Q water. The resulting samples were vortex mixed and then subjected to the SPE procedure using Alumina-A 50 mg/1 CC cartridges (Orochem, USA) on a positive pressure SPE unit. Before the samples were loaded, the SPE columns were sequentially conditioned with 1 mL of methanol and 1 mL of Milli-Q water. After samples loading, washing of the columns was sequentially made with 1 mL of Milli-Q water for 2 times. The elution was performed with 0.6 mL of 0.1% formic acid. Then the eluent was transferred to auto sampler vials and a 10-µL sample volume was injected for LC-MS/MS analysis.

RESULTS AND DISCUSSION:

Method development

Method development was initiated with scanning of the compounds for parent and product ions to perform multiple reaction monitoring. 10 ng/mL solutions of LEV, CAR and MET were separately prepared in their respective diluents and infused using a syringe pump at a rate of 10 µL/min. Both analytes and ISTD were tuned in positive ionization mode using electrospray ionization technique and were found to be good, further mass spectral and chromatographic conditions were optimized. The mass spectral scanning was performed over the range of 100 to 1000 amu for

analytes and ISTD. After selecting the parent and stable product ion, compound and gas parameters were optimized in flow injection analysis. In the mass spectrometer, zero air was used as source gas and pure nitrogen was used as collision gas. The [M+H]+ peaks were observed at m/z of 198 for LEV, 227 for CAR and at 212 for MET. With the application of appropriate collision energy, abundant product ions were found at m/z of 152 for LEV, 181 for CAR and at 166 for MET. Optimized all the compound parameters and applied to LEV, CAR and CIT for better sensitivity. Source temperature at 450 °C has produced high signal intensities for both analyte and ISTD compared to 400 °C or higher. Moreover it required a high temperature due to the % of the buffer content is more (80%) in mobile phase. Ion spray voltage at 5500v was found appropriate and a 20% change in its value does not affect the signal intensities.

The LC conditions were optimized so that the retention time was kept at 4.0 min to assure high throughput. The analyte and ISTD were set under isocratic mode. Initial chromatographic conditions were applied as per Cesar et.al. LEV, CAR and ISTD were injected using a mobile phase consisting of a mixture of 0.1% formic acid, acetonitrile (90:10 v/v). ACE C18, 5 µm (50 mm × 4.6 mm, i.d.) was used as a stationary phase. Both analyte and ISTD were eluted around 5 min and leads more run time. In order to reduce the run time, the column was changed to Luna HILIC 150 × 2.0 mm, 3 µm; Phenomenex. On this column, observed more run time (≥ 10 min) and bad peak shapes due to its more affinity towards the stationary phase. In order to achieve the short run time, the column was changed to kinetex biphenyl 50 × 3.0 mm, 2.6 µm; Phenomenex, as it is more selective and stable even for 100% aqueous mobile phase and it allows excellent reverse phase retention and aromatic selectivity. The same mobile phase conditions were applied to check the peak retention. It was observed that all the compounds were retained well and also observed good peak shape for all the drugs. However the retention time was observed little higher. Thus, the organic content in the mobile phase was increased to 20% to decrease the retention time of all analytes. The chromatographic run was completed within 4 min and also helped to enhance the sensitivity. Column temperature is also tested at three temperature levels: 30, 35 and 40 ºC. The retention time decreased slightly and no change in peak shape was observed with increasing temperature. Therefore, the temperature was maintained at 35 ºC. All the optimized chromatographic conditions yielded the target sensitivity and short run time. The final chromatographic conditions were set as follows, a mobile phase consisting of a mixture of 0.1% formic acid : acetonitrile (80:20, v/v) using a 0.3mL flow rate, kinetex biphenyl 50 X 3 mm, 2.6µm column and a run time of 4 min was used.

In the literature, the most commonly used sample extraction technique is precipitation and the common precipitating agent used for catecholamines is perchloric acid. However, strong acids are unsuitable for ESI-MS/MS owing to the deterioration of sensitivity, as noted by Li et al. [27]. Later, several procedures for sample preparation were tested, aiming at obtaining the best recovery of analytes and IS. The procedures included protein precipitation (PPT; methanol or acetonitrile containing or not formic acid) and solid-phase extraction (Oasis Hydrophilic-Lipophilic-Balanced (HLB) sorbent, Waters Corporation, Milford, Ireland). The protein precipitation method showed inappropriate recovery, ion suppression and insufficient sample cleaning for LC–MS/MS analysis. Moreover, number of co-eluent peaks were observed and also the response was decreased to 3 times as the sample was undergone more dilution. The SPE technique using HLB cartridges was provided very low recovery due to matrix effect. Liquid–liquid extraction was not tested because catecholamines are sensitive to temperature and degrade over time [37, 38]. Thus, the time and/or temperature necessary to evaporate the extraction solvent would compromise the assay.

In order to get good chromatography with high recovery, the sample preparation was changed to normal phase mechanism with Solid-Phase extraction. Alumina acidic (1 ml capacity with 50 mg sorbent) cartridges were used to selectively extract stabilized plasma samples. Lewis acid properties of alumina allow the sorbent to be more retentive towards electron-rich compounds. Where the proteins have electron rich elements and retained on cartridges helped to avoid co eluting peaks. The recovery for all the analytes was achieved and also matrix effect was eliminated.

As the method requires less time, facilitating more rapid sample preparation and making the analysis simple and easily handled. This method showed adequate recovery, reproducibility, minimal ion suppression and cleaner samples. Final SPE conditions are described in above section sample extraction procedure.

Method validation

Method validation has been carried out for the levodopa, carbidopa in compliance with the US-FDA and ANVISA resolution (Brazil) guidelines. Results were evaluated for precision (CV ≤ 15%; LLOQ and LLOQ QC: CV ≤ 20%) and accuracy (back calculated concentrations within 85 - 115%; LLOQ and LLOQ QC: 80 - 120%). All stability experiments in plasma were carried out by comparing with freshly extracted calibrators and QC samples. The stability experiments in plasma were considered stable if the deviation from nominal value (± 15.0%) and precision (CV ≤ 15%) were within the acceptable limits.

Selectivity and matrix factor

The selectivity of the method was determined to test the potential interferences of endogenous compounds co-eluting with the analytes and ISTD. A Total of 10 human blank plasma samples were tested: 8 normal, 1 lipemic and 1 heamolyzed plasma lots. They were found to be free of interferences at the retention time of LEV, CAR and ISTD. Representative chromatograms of levodopa, carbidopa and methyldopa in blank plasma are shown in Fig. 3.

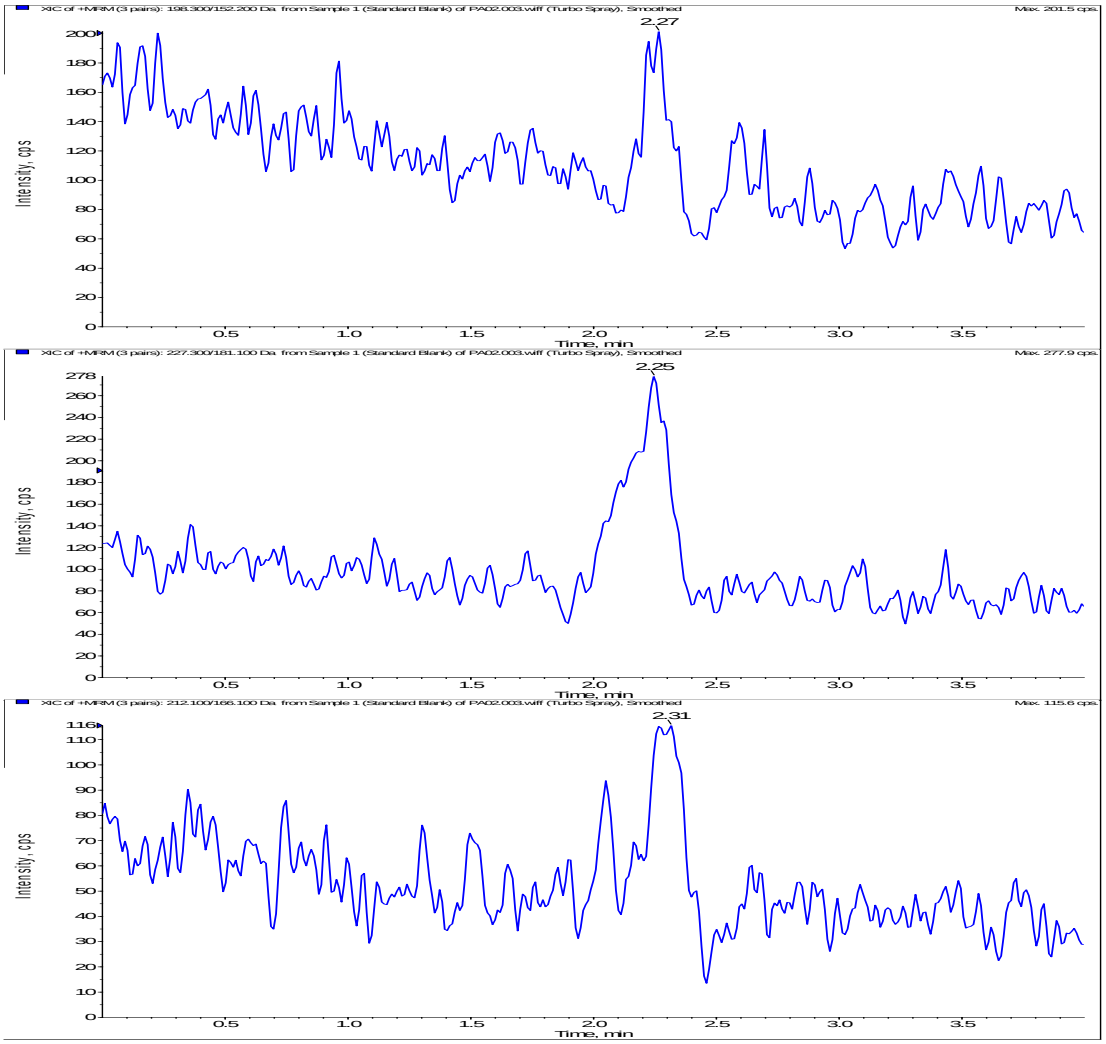

Fig 3: Representative chromatograms of (upper) levodopa, (middle) carbidopa and (lower) methyldopa in blank plasma.

The matrix effect on LEV and CAR was evaluated in 6 different volunteer plasma lots (4 normal, 1 lipemic and 1 hemolyzed) containing K_2EDTA as an anticoagulant. From each plasma lot, 6 replicates (three replicates each for LQC and HQC) of blank plasma were extracted. After collecting the final eluent from the cartridges of the extracted blank plasma samples in ria vial tubes, working solutions of LQC and HQC were added to obtain post extraction spiking samples. The response of ratio of LEV and CAR in the post extracted spiking samples was compared with that of the aqueous samples (considering it as 100%). Precision (%CV) of the ISTD normalized matrix factor for LEV in K_2EDTA was 5.1 and 6.2 and response ratio (%) was 98.0 and 96.6% for LQC and HQC respectively for CAR it was 3.2 and 6.9 and response ratio (%) was 106.3 and 101.2% for LQC and HQC respectively (calculated from 6 different plasma lots). This indicates that there is no ion suppression or enhancement.

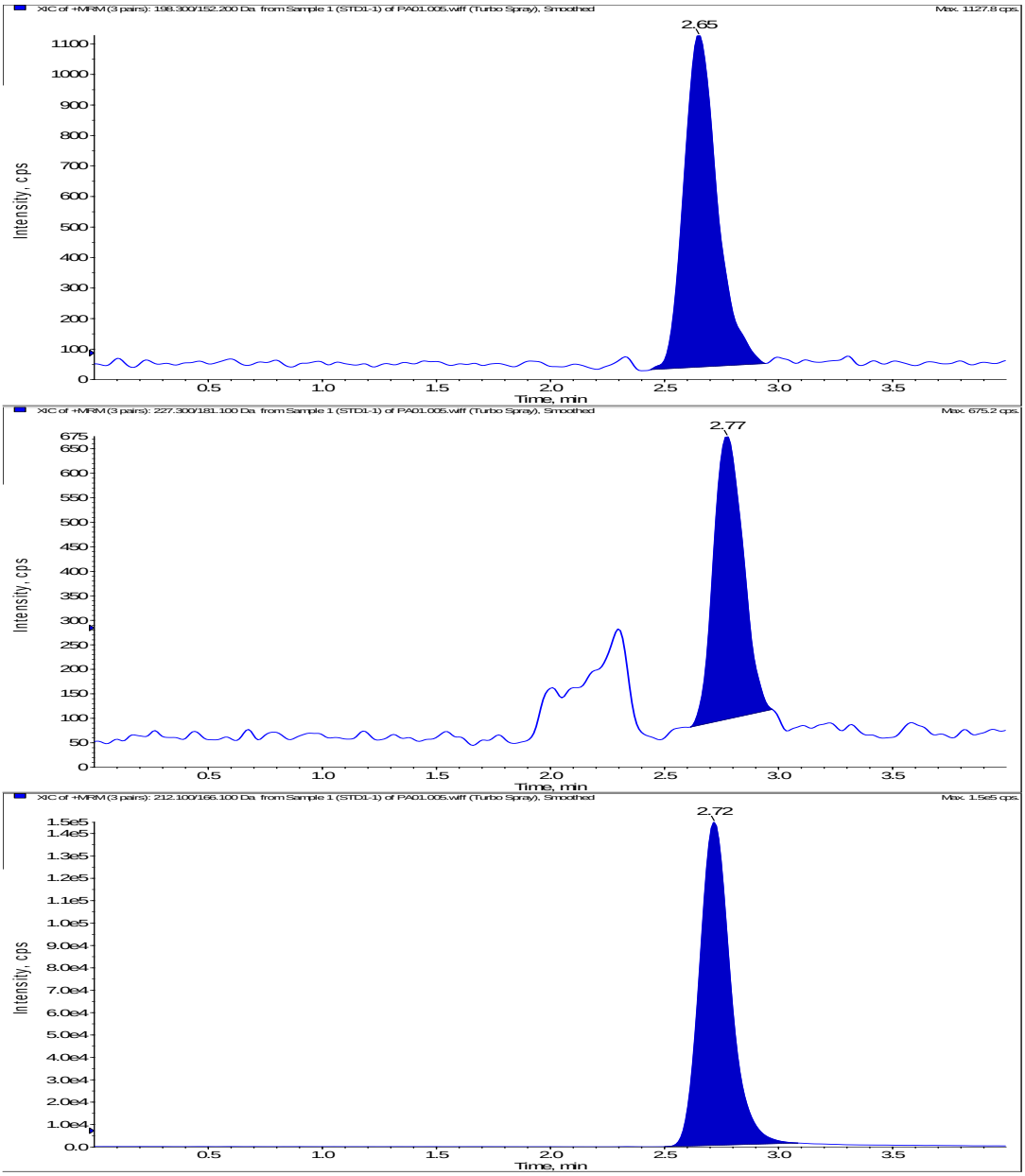

Fig 4: Representative chromatograms of (upper) levodopa, (middle) carbidopa and (lower) methyldopa in LLOQ sample.

Lower Limit of quantification and Linearity

The LLOQ was determined at 5 ng/mL for LEV and 2 ng/mL for CAR concentration for 6 analyte spiked plasma samples. The Average signal to noise ratio was found to be 190 for LEV and 85 for CAR respectively. The average % accuracy for LEV & CAR was found to be 100.5 % & 102.2 %, with a %CV of ≤ 5.8 & 7.8 respectively. The signal response in the blank plasma extracted samples at the retention time of the analyte and ISTD was not more than the acceptable limits of analytical protocol: not more than 20% for analyte and 5 % for ISTD. Representative chromatograms of levodopa, carbidopa and methyldopa in LLOQ sample are shown in Fig. 4.

Linearity was determined at eight calibration points for 4 calibration curves in range of 5 to 2000 ng/mL for LEV and 2 to 500 ng/mL for CAR. A linear regression analysis with weighing ($1/x^2$) was used to determine slopes, intercepts, and correlation coefficients. The average precision (% CV) and accuracy of the calibration curves was in the range of 0.4 to 6.6% and 93.4 to 105.3% respectively for LEV. The average precision (% CV) and accuracy of the calibration curves was in the range of 1.2 to 6.7% and 94.7 to 106.0% respectively for CAR. The coefficient of determination (r) was greater than 0.9994 for all the curves.

Recovery, precision and accuracy

The extraction recovery of the analyte and ISTD from human plasma was determined by analyzing the quality control samples. The recovery at three concentrations levels (LQC: 15 ng/mL, MQC: 898 ng/mL and HQC: 1571ng/mL for LEV and LQC: 6 ng/mL, MQC: 250 ng/mL and HQC: 393 ng/mL for CAR) was determined by comparing the peak areas of aqueous QC samples with that of the extracted QC samples. The recovery at LOQ, MQC and HQC was found to be 72.5 %, 66.1% and 67.7% for LEV and 64.7%, 59.7% and 61.7 for CAR respectively. The overall average recovery of levodopa, carbidopa and ISTD was found to be 68.8, 62.0 and 72.8% respectively.

Precision and accuracy (P&A) for this method were evaluated by calculating intra- (within a batch) and inter-batch (total 4 batches of P&A conducted in two different days) variations at five QC sample concentrations (5, 15, 989, 1571 and 4000 ng/mL for LEV and 2, 6, 250, 393 and 1000 ng/mL for CAR); six replicates at each concentration. As shown in Table 1, the intra- and inter-day precision (% CV) was less than 6.5 % and the accuracy was in the range of 98.5 % to 102.1 % for LEV and the intra- and inter-day precision (% CV) was less than 6.5 % and the accuracy was in the range of 89.7 % to 102.2 % for CAR respectively. These results indicate the adequate reliability and reproducibility of the method within the analytical curve range. The developed method was further tested for the ruggedness by using a different analyst, a different column and different equipment. Results (not shown) were within the specified acceptance criteria described.

Table 1: Precision and accuracy of the method for determining levodopa and carbidopa concentrations in human plasma samples

Analyte	Sample	Concentration added (ng/mL)	Within-batch (n=1)			Between-batch (n=4)		
			Concentration found [a] (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)	Concentration found [b] (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)
Levodopa	LLOQ QC	5	5.1 ± 0.33	6.5	101.1	5.1 ± 0.28	5.6	100.9
	LQC	15	14.7 ± 0.63	4.3	99.0	15.0 ± 0.51	3.4	101.3
	MQC	989	977.6 ± 25.34	2.6	98.8	1002.4 ±37.45	3.7	101.3
	HQC DQC	1571 4000	1547.8 ± 37.37 4083.1 ± 203.76	2.4 5.0	98.5 102.1	1584.9 ±57.57 3982.6 ± 157.48	3.6 4.0	100.9 99.6
Carbidopa	LLOQ QC	2	2.0 ± 0.09	4.7	98.9	2.0 ± 0.13	6.4	97.6
	LQC	6	5.3 ± 0.10	1.9	89.7	5.5 ± 0.29	5.2	94.5
	MQC	250	243.9 ± 9.23	3.8	97.6	255.4 ± 10.47	4.1	102.2
	HQC DQC	393 1000	381.9 ± 6.28 967.3 ± 13.37	1.6 1.4	97.2 96.7	401.2 ± 17.94 1002.7 ± 51.08	4.5 5.1	102.1 100.3

[a] *Mean of 6 replicates at each concentration.*
[b] *Mean of 24 replicates at each concentration.*

Table 2: Stability data of levodopa and carbidopa in human plasma under various storage conditions (n=6)

stability conditions	Analyte	QC level	Nominal concentartion (ng/mL)	Calculated concentration (mean ± S.D.) (ng/mL)	Stability (%)	Precision (%)
Bench-top for 12.25 h at ~10 °C	Levodopa	LQC	15	14.3 ± 0.60	96.2	4.2
		HQC	1571	1452.2 ± 17.95	92.4	1.2
	Carbidopa	LQC	6	5.3 ± 0.17	91.1	3.1
		HQC	393	374.0 ± 4.78	95.2	1.3
Four freeze-thaw cycles	Levodopa	LQC	15	14.5 ± 0.43	98.0	3.1
		HQC	1571	1513.3 ± 20.87	96.5	1.4
	Carbidopa	LQC	6	5.6 ± 0.14	97.9	2.5
		HQC	393	378.4 ± 13.64	97.0	3.6
Auto sampler (at 10°C) for 23.25 h	Levodopa	LQC	15	14.5 ± 0.26	98.0	1.8
		HQC	1571	1480.0 ± 16.51	94.2	1.1
	Carbidopa	LQC	6	5.6 ± 0.14	94.9	2.5
		HQC	393	378.4 ± 8.91	96.3	2.4
Long term (at 70°C) for 60 days stability conditions	Levodopa	LQC	15	14.4 ± 0.31	96.8	2.1
		HQC	1571	1496.7 ± 34.97	95.2	2.3
	Carbidopa	LQC	6	5.2 ± 0.13	89.4	2.5
		HQC	393	380.8 ± 3.87	96.9	1.0

Dilution Integrity

Pool plasma containing a concentration approximately twice the high CC standard (STD 8); 4000 ng/mL of the levodopa and 1000 ng/mL of the carbidopa; was prepared to assess dilution integrity. This plasma sample was diluted 1/5 times before extraction with blank human K_2EDTA plasma. After dilution, diluted samples were analyzed as a part of precision and accuracy batch. Within the batch, precision and accuracy (n = 6) for dilution integrity of the levodopa was 5.0 % and 102.1 % respectively. Within the batch, precision and accuracy (n = 6) for dilution integrity of the carbidopa was 1.4 % and 96.7 % respectively

Stability in plasma and extracted samples

Stability of the levodopa and carbidopa was assessed in human plasma and in the extracted samples. Bench top stability, free and thaw cycle's stability, in-injector stability and long term stability at -70 °C were tested as per analytical protocol. For each stability experiment, LQC and HQC stability samples (n = 6) stored at −70 °C were retrieved from the deep freezer, extracted and analyzed. Stability was determined by comparing with a freshly spiked, extracted and analyzed calibration curve. The analyte was considered to be stable in the tested conditions if the precision and accuracy was within ≤15% and ±15% (85-115%) respectively of their nominal concentrations. Summary of the results are given in Table 2. From the table, it can be seen that the levodopa and carbidopa are stable under the tested conditions.

Concomitant Medication

During clinical trials, physicians may use some medications to treat unexpected or expected adverse events like fever, body pain, vomiting, etc. Therefore; selectivity, precision and accuracy of the method shall be tested in presence of concomitant drugs to check their interference. As per analytical protocol, interference of paracetamol, ibuprofen, aceclofenac, ranitidine and ondansetron drugs on the levodopa and carbidopa analysis was tested. Each drug's working solution was spiked to three replicates of LQC and HQC samples (total 36 samples) and to a blank plasma sample at a concentration of approximately C_{max} of the respective drugs. These samples were further extracted and analyzed along with blank plasma samples (six replicates) and a calibration curve. The % interference at the retention time of the analyte and ISTD was evaluated using the area obtained, if any, from the blank plasma sample against that of the LLOQ. No significant interferences at the analyte and ISTD retention times were observed due to concomitant medication. The precision and accuracy were within the acceptable limits.

Stability in whole human Blood

Stability of the both analytes in whole human blood was evaluated at temperature at ~10 °C. Stability samples at LQC and HQC concentration levels were prepared by spiking the respective working solutions to a 2 mL whole human blood and placed on the working bench. After a period of 2.2 hours, comparison samples were also prepared similar to the stability samples. For separating the plasma,

comparison and stability samples were centrifuged at 3000 rpm at 4ºC for 20 min. Each plasma sample after separating from the whole human blood was aliquoted into three 3 RIA vial tubes, extracted and analyzed. The percent stability at LQC and HQC levels was calculated as follows:

$$\% \text{ Stability} = \frac{\text{Mean response ratio of stability samples}}{\text{Mean response ratio of comparison samples}} \times 100$$

LEV and CAR were stable at ~10 °C temperature up to 2.2 hrs in whole human blood and the %stability at low & high QC levels was found to be 96.0 & 91.3 and 96.2 & 95.5 respectively. The % CV (n =3) was found to be in the range of 1.4 to 6.7 for LEV and 4.8 to 8.3 for CAR.

CONCLUSION:

A highly selective, sensitive and fast LC-MS/MS method was developed for the quantification of levodopa and carbidopa in human K_2EDTA plasma in the concentration range of 5 – 2000 ng/mL and 5 – 1000 ng/mL respectively. This is the first simultaneous method developed for levodopa and carbidopa using alumina-A SPE cartridges. These cartridges were selected as it allows the sorbent to be more retentive towards electron-rich elements., The proteins or endogenous matrix consisting lot of electron rich elements help to retain on cartridge in order to avoid matrix effect and also unwanted co-eluting peaks, to achieve shorter runtime. Precision and accuracy experiments show that the developed method is repeatable, reproducible and robust. Stability evaluations of the levodopa and carbidopa in human K_2EDTA plasma demonstrated that there is insignificant degradation in the specified storage conditions and time periods.

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