



A Simple Validated Stability Indicating RP-HPLC Method for the Determination of Three Antiparkinsonism Compounds in Oral Contraceptive Tablet Formulations

B. Jala chandra Reddy, N. C. Sarada*

Chemistry Division, School of Advanced Sciences, VIT University, Vellore, Tamil Nadu, India.

Abstract : The present study describe the simple, novel stability-indicating assay method was developed and validated for determination of Levodopa, Carbidopa and Entacapone along with their degradation products in pharmaceutical formulation (Tablet) by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). During the ICH prescribed stress study, Levodopa, Carbidopa and Entacapone were found susceptible to degrade under oxidative (Peroxide) and hydrolytic (acid and base) conditions. The separation was achieved with Hypersil BDS (250 x 4.6 mm, 5 μ m) column using 0.1% orthophosphoric acid as a buffer and acetonitrile containing 35% of 0.1% orthophosphoric acid (95:5 v/v%) as mobile phase, at a flow rate of 1.2 mL min⁻¹ column temperature kept at 30°C and photodiode array detector at 282 nm. The average retention times for Levodopa, Carbidopa and Entacapone, were 2.4, 4.6 and 8.0 min, respectively. The optimal condition, method was validated according to the ICH and USP guidelines. The method were linear in the concentration range for Levodopa, Entacapone were 50-300 μ g mL⁻¹ for Carbidopa 12.5-75 μ g mL⁻¹ ($r^2 > 0.999$) and all three compounds recoveries were above 99%. There were no chromatographic or spectral interferences from excipients and proposed method suitable for the routine quality control of analysis.

Keywords : Levodopa, Carbidopa, Entacapone, Validation and Pharmaceutical formulations.

Introduction

Levodopa and Carbidopa and Entacapone is an Antiparkinsonism drugs, it is a single tablet combination used once in a day for the treatment for Parkinson's disease in adults. Parkinson's disease (PD) symptoms are leads to depletion of dopamine, when Levodopa (L) administrates it will be converted decarboxylation in to dopamine. It is controlling cardinal signals caused by PD, as well as the tremor, hyperkinesias and rigidity. Carbidopa (C) inhibits decarboxylation of peripheral levodopa, more availability of levodopa in brain to cross the blood brain barrier. Entacapone (E) a selective and reversible inhibitor of catechol-O-methyl transferase. The clinical response of the individual Levodopa is stable up to several hours. Administrated through entacapone with conjunction of carbidopa and levodopa, increasing the plasma levels of Levodopa in the brain. New combination treatment containing Levodopa, Carbidopa and Entacapone (LCE) in different strengths on market is Stalevo® [1-4].

Levodopa an aromatic amino acid, chemically as (-)-L-a-amino- β -(3,4-dihydroxybenzene) propanoic acid. Molecular formula and mass is C₉H₁₁NO₄ and 197.2. levodopa was dopamine biosynthesis clinically, its

improve muscle control and imbalance. Carbidopa an inhibitor of aromatic amino acid decarboxylation, chemically as (-)-L-α-hydrazino-α-methyl-β-(3,4-dihydroxybenzene) propanoic acid monohydrate. Molecular formula and mass is $C_{10}H_{14}N_2O_4 \cdot H_2O$ and 244.3. Carbidopa inhibits the peripheral metabolism of Levodopa, improve bioavailability of Levodopa, and crosses the blood - brain barrier for central nervous system effect.

Entacapone was chemically as (E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethyl -2-propenamide and inhibitor of catechol O- methyltransferase. Molecular formula and mass is $C_{14}H_{15}N_3O_5$ and 305.3. It is used in the combination of levodopa and carbidopa to treat symptoms of Parkinson 'disease.

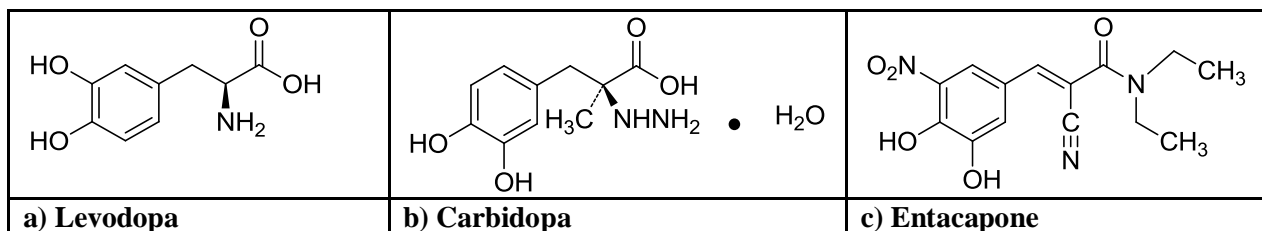


Figure 1: Chemical Structures of a) Levodopa, (b) Carbidopa and (c) Entacapone.

The present literature survey revealed that some of the analytical methods are accessible using to determination of LCE, such as Spectrophotometric [5], Spectrofluorometric [6], TLC [7], HPTLC [8], ATR-FTIR [9], NMR [10] LC-MS [11] and capillary zone electrophoresis [12] methods for Levodopa and Carbidopa pharmaceutical formulation. Assay of Levodopa and Carbidopa by HPLC [13-17] and UPLC [20] dissolution profile Levodopa and Carbidopa and Entacapone [18-19]. The stability indicating method was validated as per the ICH, USP [21-26] and references guideline recommendation [27-37].

The scientific novelty of present work is to establish a validated stability indicating RP-HPLC method for assay of three anti-parkinsonism compounds in oral contraceptive tablet formulations and presence of its degradation products and assessment of the stability of its dosage forms and purity of the bulk drug. Stability study of three anti-parkinsonism compounds shows the piece of information which will help in formulation and characterization of degradation compounds, it will open a new scope for the toxicity study of degraded components. The proposed method is simple, accurate, rapid, reproducible and economical. The method is less time consuming and expensive compared to other published methods. The effective separation of all the degradants from the drug and runtime were 11.0 minutes.

Experimental

Chemicals and reagents

Pharmaceutical grade reference standards of Levodopa 99.94%, Carbidopa 92.50% and Entacapone of 99.96% purity according to the certificate of analysis of manufacturer were provided as gift samples from Dr. Reddy's pharmaceutical industry, Hyderabad, Telangana, India. Ultrapure water was obtained from Milli-Q plus water purified system, Millipore, Bedford, MA, USA for making the solutions. HPLC grade acetonitrile and methanol, orthophosphoric acid (OPA), chemicals of analytical grade were procured from local market (Merck, India).

Instrumentation

Analysis of the sample was performed on an HPLC system (Waters 2695 Alliance, USA) equipped with inbuilt auto-sampler, quaternary gradient pump, on-line degasser, column oven, photodiode array detector and Empower software-2 (all from Waters, USA). The chromatographic separation was performed on Hypersil BDS, 250 x 4.6 mm, 5 μm analytical column, 0.45 μm nylon membrane filters, (Millipore, Bangalore India), An ultrasonic bath (spincotech Pvt Ltd Mumbai, India). A precision water bath furnished with MV controller (Thermostatic Classic Scientific India Ltd, Mumbai, India) was used for hydrolytic study. A humidity chamber (Labline Sun Scientific Ltd. New Delhi, India) for stability studies and a photo stability chamber (95 Th-400G, Thermo lab, Mumbai, India) for the photolytic study were used. Thermal stability study was carried out in a hot air oven (Kumar Scientific Works, Pune, India). A pH meter (Lab India) was used for pH adjustment of solutions.

Chromatographic separation

A Hypersil BDS, 250 x 4.6 mm, 5 μ m analytical column (30°C; column temperature) was used with a mobile phase comprised 0.1% orthophosphoric acid buffer and acetonitrile containing 35% of 0.1% orthophosphoric acid (95 : 5 v/v%) pumped at a flow rate of 1.2 ml/min for the HPLC studies on all reaction solutions individually. The detection was a photodiode array detector set at 282 nm. The sample injection volume was 10 μ L.

Preparation of analytical solutions

Preparation of 0.1% Ortho-Phosphoric Acid buffer:

One ml from the concentrated orthophosphoric acid (OPA) was diluted to 1000ml with HPLC grade water and sonicated to degas for 20 min and filter through 0.45 μ m membrane filter and mix well.

Preparation of mobile phase:

The mobile phase was prepared by taking a mixture of solution A and solution B in a ratio of 95A: 5B (v/v). Where, Solution A: 0.1% OPA Solution B: 0.1% OPA and acetonitrile (35:65). The mobile phase filtered through 0.45 μ m membrane filter, mix well and degassed.

Preparation of Diluent:

A mixture of 0.1% OPA and acetonitrile (80:20 v/v %) was used as a diluent.

Standard Stock Solution Preparation:

In order to prepare the stock solution, 200 mg of Levodopa, and 50 mg of Carbidopa and 200 mg of Entacapone were accurately weighed and transferred into 3 separate standard 100ml volumetric flasks. To that 70ml of diluent were added and sonicated to completely dissolve and then volume was made up to mark with the same diluent. From the above solutions, 5.0 ml was taken into a 50ml volumetric flask and made up to mark with the diluent final concentrations are 200, 50 and 200 μ g mL⁻¹ respectively.

Sample Solution Preparation:

A number of 20 tablets were accurately weighed ground in to a fine powdered using a mortar and pestle. A portion weight equivalent to about one tablet powder was transferred into a 100 mL volumetric flask added approximately 70mL of diluent sonicated for 15 min with intermediate shaking and made up the remaining volume with diluent. A portion of the above solution was filtered through 0.45 μ m nylon membrane filters and 5.0 ml of filtered solution was transferred into a 50 ml volumetric flask and volume was made with the diluent final concentration 200, 50 and 200 μ g/ml.

Specificity and stress degradation studies

Degradation study:

The degradation samples were prepared by transferring tabletpowder equivalent to 200 mg of Levodopa and 50 mg of Carbidopa and 200 mg of Entacapone into a 250 ml round bottomed flask and drug content were employed for acidic, alkaline and oxidant and also for thermal and photolytic conditions. After the forced degradation samples treatments were completed, the stress content solutions were allowed to room temperature. Resultant solutions were further diluted to obtain final concentration.

Forced degradation Studies

Acid Degradation Studies:

To the one ml of stock solutions of LCE and 1.0 ml of 1N HCl was added and refluxed for 3.0 hours at 70°C. The reactant mixture was cooled and neutralized by 1N NaOH solution.

Alkali Degradation Studies:

To the one ml of stock solutions of LCE and 1.0 ml of 1N NaOH was added and refluxed at 3.0 hours at 70°C. The reactant mixture was cooled and neutralized by 1N HCl solution.

Oxidation:

To the one ml of stock solutions of LCE and 1.0 ml of 30% hydrogen peroxide (H₂O₂) was added and refluxed for 3.0 hours at room temperature. The reactant mixture was cooled to room temperature and neutralized by addition of 30% sodium thiosulfate.

DryHeat Degradation:

The one ml of stock solutions of LCE was placed in oven at 70°C for 3 hours, After the stipulated time, the solution was cooled to room temperature.

UV lightstudies:

The stability of the sample (LCE) was studied by exposing the standard stock solution to UV Light by keeping the beaker in UV Chamber for 200Watt hours/m² for 7days.

Neutral Degradation Studies:

Stress testing under neutral conditions was also studied by refluxing the drug in water for 3.0 hours at a temperature of 70 °C.

Method Validation

At the optimal condition, the proposed HPLC method has been validated with respect to the following parameters outlined by [ICH Q2 (R1) and USP]:

Linearity and range:

Suitable dilutions from the working standard solutions were prepared to yield a series of solutions in the concentration range of 50-300µg/ml, 12.5-75 µg/ml and 50-300µg/ml for Levodopa, Carbidopa and Entacapone respectively. The resultant solutions were chronologically injected in triplicate into the HPLC column. A calibration curve was constructed thereby plotting the corresponding peak areas against concentration to obtain regression equation and correlation coefficient, indicate the linearity method.

Precision:

The intra-day precision was determined using three quality control samples (50, 100 and 150 µg/mL) each six injections on the same day and % RSD were calculated. Furthermore, these experiments were repeated on three consecutive days to assess inter-day precision.

Accuracy:

Accuracy was determined by analyzing a known concentration of the drug, via, 50%, 100%, and 150% spiked with stressed sample in triplicate and then determining the percent recovery.

Detection and quantitation limits:

The limit of detection (LOD) and limit of quantification (LOQ) represent the lowest concentration of the analyte yield a signal-to-noise ratio of 3 and 10, respectively.

Robustness:

The robustness study was carried out to assess the influence of minor variations in the optimal chromatographic factors. A deliberate variation in the separation parameters i.e.: flow rate (± 0.1 ml/min), percent acetonitrile in mobile phase ($\pm 1\%$), column temperature ($\pm 5^\circ\text{C}$).

Result and Discussion

Development and optimization of the stability-indicating method

The stability-indicating chromatographic method was optimized to separate Levodopa, Carbidopa and Entacapone from their major degradant products under different stress conditions. Several buffer systems at several pH values were trailed in various ratios with methanol, and acetonitrile as a mobile phase. Separation with good resolutions was studied different types of columns (C8 and C18). Decisively after several experimental trials, Hypersil BDS (250 x 4.6 mm, 5mm) column was found to contribute best separation than others with 0.1% orthophosphoric acid buffer and acetonitrile containing 35% of 0.1% orthophosphoric acid (95 : 5 v/v%) as a mobile phase. Among different flow rate values, better selectivity and good peak parameters were observed at 1.2 ml/min. The temperature of column oven was set at 30°C and detection photodiode array detector set at 282 nm, optimal condition are shown in Figure 2.

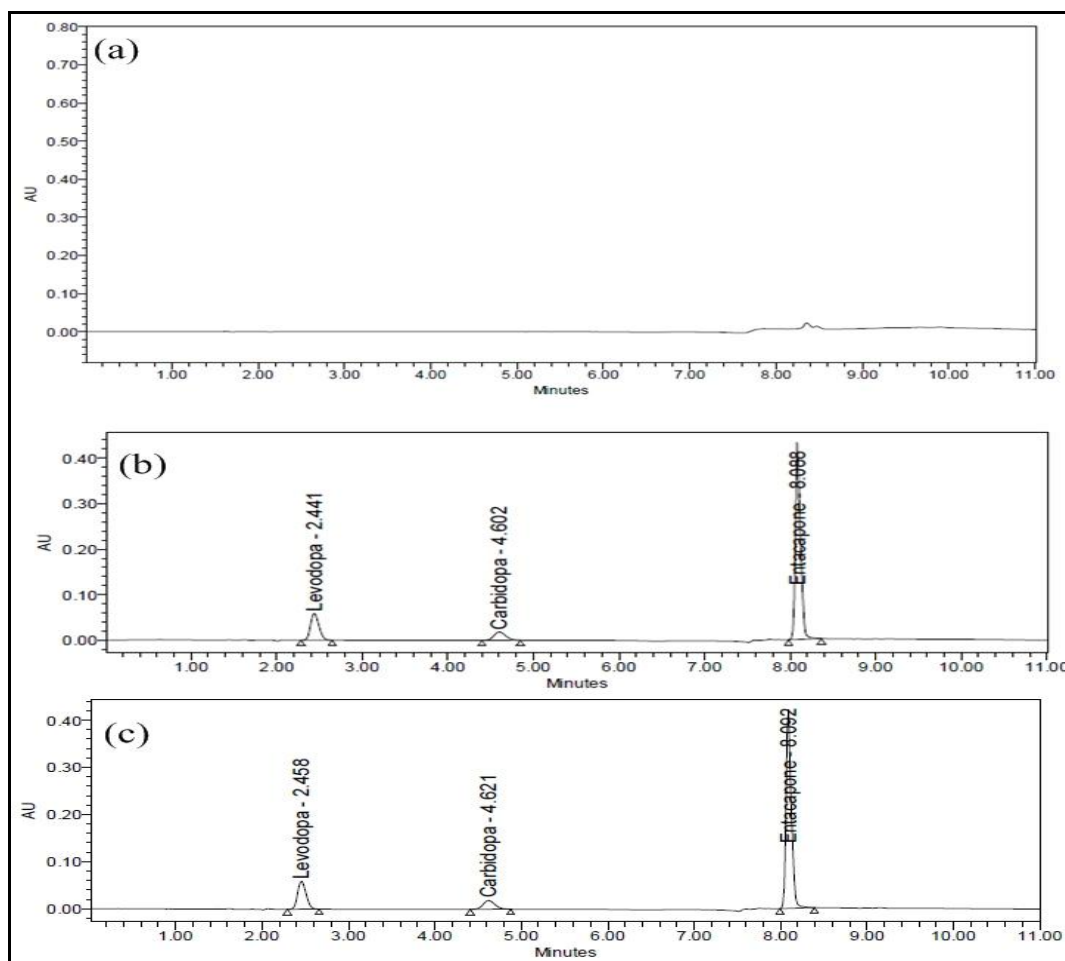


Figure 2: Chromatograms of Levodopa, Carbidopa and Entacapone a) Blank chromatogram b) Standard c) Formulation.

Validation of the stability-indicating method

From the system suitability test results, it has been confirmed that the system was to be suitable as it complies with the limits of peak parameters. Resolution was >2 and peak asymmetry was <1.4 for all the parameters confirms that good selectivity of the method. The peak areas for drug samples were precisely linear in the concentration range between $50 - 300 \mu\text{g mL}^{-1}$, $12.5-75 \mu\text{g mL}^{-1}$ and $50-300 \mu\text{g mL}^{-1}$ for Levodopa, Carbidopa and Entacapone respectively. The data were analyzed with least squares regression indicating good linearity for Levodopa, Carbidopa and Entacapone with the correlation coefficient r^2 0.999, results was in (Table 1).

Table 1: Linearity parameters for the simultaneous estimation of Levodopa, Carbidopa and Entacapone

Parameter	Levodopa	Carbidopa	Entacapone
Linearity ($\mu\text{g mL}^{-1}$)	50-300	12.5-75	50-300
Slope	2019.3 \pm 1.52	3486 \pm 2.64	8841 \pm 4.72
Intercept	565.3 \pm 3.22	595.5 \pm 6.55	664.5 \pm 2.38
Correlation coefficient(r)	0.9998 \pm 0.0001	0.9998 \pm 0.0003	0.9997 \pm 0.0005

All the values are reported as mean \pm S.D. of three calibration curves, six concentrations in the linearity range were evenly distributed.

The obtained LOD values for Levodopa, Carbidopa and Entacapone are 0.52, 0.06 and 0.08 $\mu\text{g mL}^{-1}$ respectively and LOQ values are 1.57, 0.19 and 0.27 $\mu\text{g mL}^{-1}$ respectively. The results demonstrate that the method is adequately sensitive. Precision study is represented in (Table 2) for intermediate precision and repeatability experiments. The calculated %R.S.D values for the intra-day precision study were 0.57, 0.43 and 0.29 for Levodopa, Carbidopa and Entacapone respectively, proving that the method was suitably precise. The mean percentage recovery from the accuracy study was calculated for fortified and unfortified solutions results are in (Table 2). Excellent recoveries were obtained for Levodopa, Carbidopa and Entacapone are 100.17, 100.05 and 100.44% respectively. From the solution stability study at different time intervals, it was concluded that test preparation was found to stable up to 48 h at 2-8 $^{\circ}\text{C}$. The method was found to be sufficiently robust under the tested conditions, specificity of the method was determined by checking the interference of placebo with an analyte and peak purity of Levodopa and Carbidopa and Entacapone during the forced degradation study was carried results are in (Table 4).

Table 2: Method validation results for Levodopa, Carbidopa and Entacapone

Parameter	Levodopa	Carbidopa	Entacapone
System suitability test			
RT (min)	2.447	4.630	8.090
Theoretical plates	2947	5647	8318
Asymmetry(A_s)	1.16	1.11	1.18
Resolution(R_s)	2.5	7.6	18.3
Repeatability $^a, t_R$ (%RSD)	0.6	1.3	0.5
Repeatability $^a, A$ (%RSD)	0.4	1.5	0.8
Validation			
Precision a,b (%RSD)	0.57	0.43	0.29
Accuracy a,b (%RSD)	0.68	0.73	0.63
Accuracy (%recovery)	100.17	100.05	100.44
selectivity	No interference	No interference	No interference
LOD ($\mu\text{g mL}^{-1}$)	0.52	0.06	0.08
LOQ ($\mu\text{g mL}^{-1}$)	1.57	0.19	0.27

^a Intra-day precision (repeatability), inter-day precision and accuracy were tested by analyzing three replicate Qc Standard Samples At 50,100 And 150 % Levels For L and C and E under the guidelines of ICH. ^b Inter day precision and accuracy were determined with three replicates on three consecutive days.

The peak purity of the Levodopa and Carbidopa and Entacapone was found satisfactory 0.999 under different stress conditions therefore no interferences of any peak of degradation product with drug peak. The robustness responses to the variations were compared with the proposed method results was demonstrated in (Table 3).

Table 3: Method validation data for the Robustness study

Robust condition	%Assay			USP Theoretical Plates			Asymmetry		
	L	C	E	L	C	E	L	C	E
Flow 1.1 ml/min	100.61	99.92	100.84	2502	4020	8366	1.16	1.16	1.43
Flow 1.3 ml/min	101.66	100.8	100.54	2480	4790	9452	1.13	1.15	1.41
ACN: Buffer high organic	100.54	99.33	99.19	2265	4134	8145	1.13	1.17	1.44
ACN: Buffer low organic	100.15	100.68	99.74	2598	4020	8366	1.22	1.20	1.44
Temperature High	99.68	99.93	99.27	2534	4120	9334	1.13	1.16	1.45
Temperature low	100.53	100.32	100.13	2148	4136	9651	1.14	1.23	1.44

Degradation behaviour of Levodopa, Carbidopa, and Entacapone

Stress testing of Levodopa, Carbidopa, and Entacapone under several conditions with the aid of RP-HPLC suggested the following degradation behaviour:

Acid hydrolysis:

In acidic condition, it was found that around 5.82% of Levodopa, 6.29% of Carbidopa and 5.34% of Entacapone was degraded. The data obtained were enumerated in Table 4 and chromatogram of diluent Figure 3a and acid degradation was represented in Figure 3b.

Base hydrolysis:

In alkaline condition, it was found that around 4.6% of Levodopa, 5.42% of Carbidopa and 6.64% of Entacapone was degraded a major degradation peak at RT of 2.440 min was detected. The data obtained were enumerated in Table 4 and chromatogram was represented in Figure 3c.

Oxidative:

Oxidative condition, three major degradation products are 7.0% of Levodopa, 7.11% of Carbidopa and 7.06 % of Entacapone peaks at RT of 3.789 min, 8.653 min and 9.142 min was detected. The data obtained were enumerated in Table 4 and chromatogram was represented in Figure 3d.

Table 4. Data obtained from the degradation study

Stress conditions	RT of main peaks	Asymmetry	Purity Angle	Purity Threshold	%Degraded	% Remained
Acid (1N HCl, 3.0 hours, 70°C)						
Levodopa	2.457	1.1	0.074	0.262	5.82	94.18
Carbidopa	4.691	1.2	0.147	0.355	6.29	93.71
Entacapone	8.087	1.3	0.481	0.645	5.34	94.66
Neutral (H₂O, 70°C, 3 hr)						
Levodopa	2.442	1.1	0.081	0.250	1.56	98.44
Carbidopa	4.607	1.1	0.137	0.340	1.73	98.27
Entacapone	8.078	1.3	0.411	1.245	1.77	98.23
Base (1N NaOH, 3.0 hr, 70°C)						
Levodopa	2.440	1.1	0.069	0.254	4.60	95.40
Carbidopa	4.643	1.1	0.149	0.348	5.42	94.58
Entacapone	8.084	1.3	0.358	0.246	6.64	93.36
Oxidation (30% H₂O₂ at room temperature for 30 min)						

Levodopa	2.448	1.1	0.080	0.255	7.00	93.00
Carbidopa	4.649	1.2	0.158	0.352	7.11	92.89
Entacapone	8.084	1.3	0.110	0.245	7.06	92.94
Photolytic (UV, 200 Watt hours/m², 7days)						
Levodopa	2.439	1.1	0.072	0.250	2.86	97.14
Carbidopa	4.613	1.1	1.127	0.323	2.73	97.27
Entacapone	8.083	1.3	0.661	1.252	2.29	97.71
Thermal (Dry heat, 70°C, 3 hr)						
Levodopa	2.436	1.1	0.071	0.252	3.02	96.98
Carbidopa	4.617	1.1	0.182	0.377	3.11	96.89
Entacapone	8.094	1.2	0.370	1.245	3.47	96.53

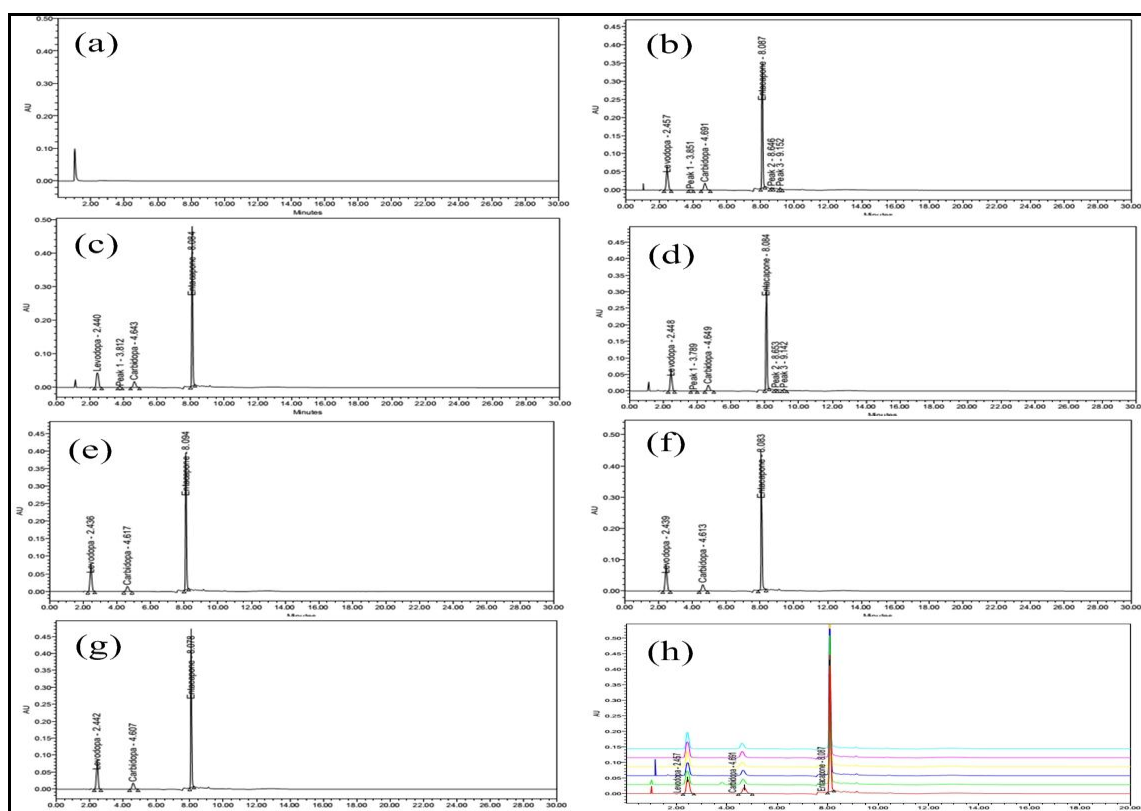


Figure 3: Forced degradation chromatograms a) Diluent b) Acid degradation c) Base degradation d) oxidative degradation e) Thermal degradation f) neutral degradation g) UV degradation h) Overlay chromatograms for degradation studies.

Thermal, light (UV) and neutral (water) hydrolysis:

Levodopa, Carbidopa, and Entacapone were stable to thermal, light and neutral hydrolysis stress conditions and no decomposition was found. The results obtained were enumerated in Table 4 and chromatogram was presented in Figure 3 e, f and g .The overlaid chromatograms for the all degradation study were presented in Figure 3h.

Conclusion

The proposed method was economical, simple, accurate and robust stability-indicating method was developed for the determination of LCE. Stress study demonstrates that LCE is highly sensitive to oxidative, acid and alkali hydrolysis, stable in thermal, light and neutral stress conditions. The developed method proved

to be selective, sensitive, as per ICH guidelines. The separate all degradant from its main compounds were within 11 minutes. The proposed RP-HPLC method was found to be more selective than published HPLC methods, which showed the less retention time for separation of all degradation products. The proposed method several samples can be run as simultaneously using the small quantity of mobile phase within a less time. The method applicable for routine analysis to the determination of possible degradants in bulk pharmaceutical formulations.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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