Stability-indicating method of entacapone-related substances using UPLC in finished dosage form

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A rapid, specific, sensitive and robust method for entacapone-related substances in carbidopa, entacapone and levodopa film-coated tablet was developed using ACQUITY UPLC with BEH C18 column. Mobile phase (A), i.e. 0.1% orthophosphoric acid and mobile phase (B), i.e. acetonitrile with water in the ratio 75 : 25 (v/v) with gradient method were employed. The method was evaluated for identification of process impurities and unknown impurities. It has been validated according to ICH (Q2) R1 guidelines. The values of LOD and LOQ for impurity and entacapone were found to be 0.01% and 0.03% respectively. Degradation studies were carried out in peroxide, acid, alkali conditions and contribution to mass balance was established. It was stable under heat, light-exposed and humid conditions.

Keywords: Entacapone-related substances, dosage form, force degradation study, liquid chromatography.

THE mechanism and action of entacapone are due to its ability to inhibit catechol-O-methyltransferase (COMT) and alter the plasma pharmacokinetics of levodopa. When entacapone is given in conjunction with levodopa and an aromatic amino acid decarboxylase inhibitor, such as carbidopa, plasma levels of levodopa are greater and more sustained than after administration of levodopa and an aromatic amino acid decarboxylase inhibitor alone. At a given frequency of levodopa administration, the more sustained plasma levels of levodopa result in more constant dopaminergic stimulation in the brain, leading to greater effects on the signs and symptoms of Parkinson's disease. The higher levodopa levels also lead to increased adverse effects, sometimes requiring a decrease in drug dose[1].

Many liquid chromatography methods for the estimation and determination of drug in solid dosage form and active pharmaceutical ingredients using HPLC, LC/MS techniques are available in the literature. Compared with all analytical methods, stability indicating method is less time consuming and cost effective. The method was developed for entacapone-related substances in the combination product using ultra performance liquid chromatography. It is a highly sensitive, accurate and robust stability indicating method. This is a combination product of carbidopa, levodopa and entacapone film-coated tablet. According to BCS study, carbidopa and levodopa molecules are class-I drugs[2] and entacapone is a class-IV drug[3]. Based on the solubility study data, a separate method was developed for entacapone-related substances in solid dosage form. This method can be used for entacapone-related substances in single or combination product.

Orthophosphoric acid (88%), acetonitrile (HPLC-grade), water (Milli-Q-grade), hydrochloric acid (37%), sodium hydroxide and hydrogen peroxide were used in this study. Entacapone, carbidopa and levodopa all active pharmaceutical ingredients (APIs), entacapone reference standard and impurity of *Z*-isomer, film-coated tablets were provided by AET Laboratories Private Limited, India. Standard sample is weighed and sample and impurity of *Z*-isomer were dissolved in a diluent with the help of sonicator. Known impurity, unknown impurities and degradation impurities were identified using ultra performance liquid chromatography (UPLC) technique (Waters India Limited, model: ACQUITY UPLC system with PDA detector, Empower software-2). Good resolution was achieved between impurity of *Z*-isomer and entacapone with the help of BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 75 mm.

The method reported here was developed for entacapone-related substances UPLC with BEH C18 column (18% of carbon loading, end capped, spherical particle shape, surface area: 185 m^2/g, low silanol activity, pH range 1 to 12), 130 Å, 1.7 µm, 2.1 mm × 75 mm, flow rate 0.22 ml/min, wavelength 300 nm, injection volume 2 µl, sample cooler 15°C, run time 15 min. Table 1 shows the gradient programme.

Mobile phase (A) was prepared using 0.1% conc. orthophosphoric acid solution[4] and mobile phase (B) was prepared using acetonitrile and water in the ratio 75 : 25 (v/v). Diluent was prepared using acetonitrile and water in the ratio 50 : 50 (v/v).

Entacapone standard was prepared in 0.002 mg/ml concentration with diluent. Sample was prepared in 1.0 mg/ml concentration with diluent. Entacapone and *Z*-isomer impurities were prepared in 0.002 mg/ml concentration with diluent.

The main objective of this study is to develop a stability-indicating method of entacapone-related substances

Table 1. Gradient programme

Time	Flow rate (ml)	Mobile phase (A)	Mobile phase (B)
0	0.22	80	20
5	0.22	40	60
10	0.22	40	60
12	0.22	80	20
15	0.22	80	20

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Table 2. Entacapone, carbidopa and levodopa – basic information

Drug	Entacapone	Carbidopa	Levodopa
Molecular weight	305.29	244.24	197.19
Molecular formula	$C_{14}H_{15}N_3O_5$	$C_{10}H_{14}N_2O_4$	$C_9H_{11}NO_4$
pKa value	4.5	2.32	2.32, 8.72, 9.74, 13.40
Polarity nature	Mid-polar	High-polar	High-polar

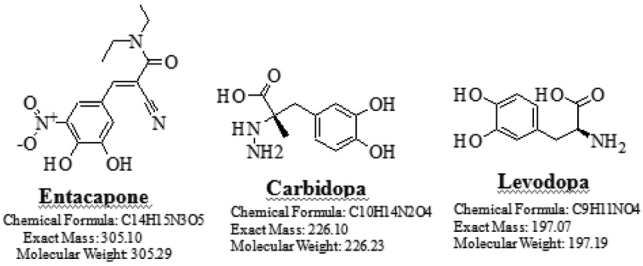

Figure 1. Chemical structure of entacapone, carbidopa and levodopa.

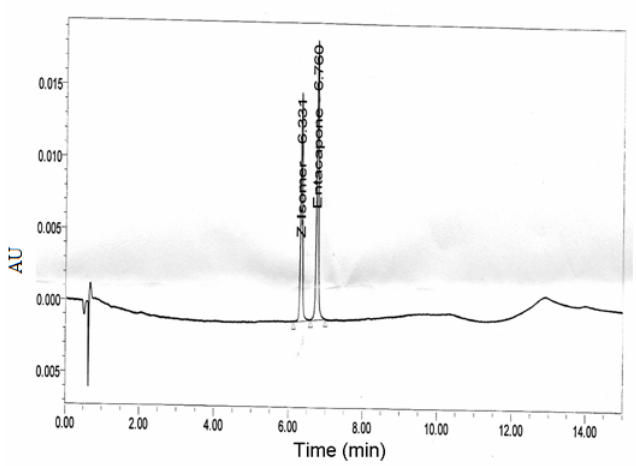

Figure 2. Chromatogram of entacapone and *Z*-isomer.

in the combination product. Table 2 and Figure 1 provide the details of physico-chemical properties of the drug, pKa value, solubility, molecular formula, molecular weight, etc.[5–13]. We have selected the reverse phase technique and BEH stationary phase C18 column as it contains pore size 130 Å, particle size 1.7 µm, internal diameter 2.1 mm and length 75 mm, 18% of carbon loading, end capped, spherical particle shape, surface area 185 m^2/g, low silanol activity and pH range 1–12. Solubility and pKa value aid in the selection of mobile phase pH and diluent optimization. Entacapone is a mid-polar molecule, soluble in organic solution. Carbidopa and levodopa are soluble in acidic medium (e.g. 0.1 N hydrochloric acid). All three compounds are not soluble in the same diluent solution. Based on the solubility information, entacapone-related substances method was developed separately in the combination product. According to the literature, some of the trials were made. In the first trial, the mobile phase (A) contained 0.1% orthophosphoric acid (pH 2.00) and the mobile phase (B) contained 100% acetonitrile for separation between *Z*-isomer and entacapone using BEH C18 column. The observed resolution between impurities of *Z*-isomer and entacapone was low. In the second trial, for better resolution, the mobile phase (B) contained acetonitrile and water in the ratio (90 : 10) v/v. The observed resolution between impurity of *Z*-isomer and entacapone did not meet the USP criteria. In the final trial, mobile phase (A) contained 0.1% orthophosphoric acid (pH 2.00) and mobile phase (B) contained acetonitrile and water in the ratio 75 : 25 v/v and linear gradient was given for better resolution. The observed resolution was 3.2 and no interference was observed at impurity of Z-isomer and entacapone retention time in the method. Carbidopa and levodopa along with impurities were eluted for <1 min owing to their highly polar nature[14–19]. The entacapone and *Z*-isomer compounds were eluted in a narrow region, because both molecules had similar polarity. For good resolution between entacapone and *Z*-isomer impurity, a linear gradient was given; polarity ratios formed between two compounds and good separation was observed. Carbidopa and levodopa were not responsive at 300 nm. Entacapone was degraded in acid-, base-hydrolysis and peroxide degradation studies.

Analytical method validation parameters, known and unknown impurity specifications were used based on the ICH Q2 (R1) guidelines[20,21].

Specificity parameter was performed using UPLC with PDA detector. Prepared blank, placebo, standard, test as such and test spiked sample solutions were injected in the ACQUITY UPLC system. We observed all chromatograms along with degradation samples. No interference was observed at entacapone and *Z*-isomer retention time. Figures 2 and 3 show purity of entacapone and *Z*-isomer in the degradation chromatograms and spiked chromatograms.

Force degradation study was as follows:

(1) Preparation of 1 N hydrochloric acid – 8.5 ml concentrated hydrochloric acid was taken in 1000 ml volumetric flask and diluted with Milli-Q water.

(2) Preparation of 1 N sodium hydroxide solution – 4.0 g of sodium hydroxide pellets was transferred to 1000 ml beaker; 500 ml of Milli-Q water was added and then the pellets were dissolved and diluted with Milli-Q water.

(3) Preparation of 3% hydrogen peroxide solution – 10 ml of 30% hydrogen peroxide solution was taken in 100 ml volumetric flask, diluted with Milli-Q water.

Table 3. Force degradation study results

Condition	Entacapone drug recovered (A)	Entacapone drug decomposed (B)	Mass balance (A + B)
Control sample	100.2	0.00	NA
Acid degradation	93.2	5.21	98.4
Base degradation	96.8	2.62	99.4
Peroxide degradation	92.8	5.92	98.7
Water degradation	99.2	0.28	99.5
Thermal degradation	99.8	0.28	100.1
Humidity degradation	100.1	0.30	100.4
Photolytic degradation	99.6	0.29	99.9

Table 4. Method precision results

Preparation	Initial as such sample (*Z*-isomer impurity)	*Z*-Isomer of entacapone impurity spiked in the sample (0.2% specification level)	Actual percentage of *Z*-isomer present in the sample	
Sample 1	0.06	0.27	0.21	0.21
Sample 2	0.06	0.27	0.21	0.21
Sample 3	0.06	0.26	0.20	0.20
Sample 4	0.06	0.26	0.20	0.20
Sample 5	0.06	0.26	0.20	0.20
Sample 6	0.06	0.26	0.20	0.20
Sample average value	0.20	NA		
Sample STDEV	0.005	NA		
Sample % RSD	2.4	NA		
Intermediate precision results				
Sample 7	0.06	0.26	NA	0.20
Sample 8	0.06	0.26	NA	0.20
Sample 9	0.06	0.27	NA	0.21
Sample 10	0.06	0.26	NA	0.20
Sample 11	0.06	0.27	NA	0.21
Sample 12	0.06	0.26	NA	0.20
Sample average value				0.20
Sample STDEV				0.005
Sample % RSD				2.32

NA, not applicable.

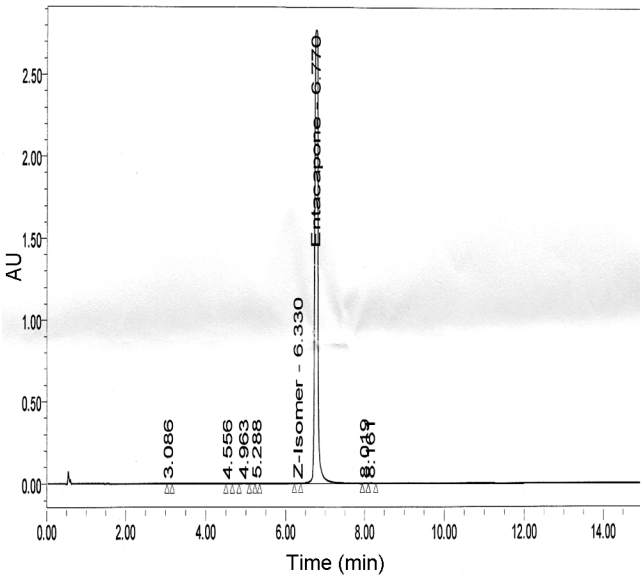

Figure 3. Chromatogram of spiked sample (0.2% specification level).

(4) Thermal degradation – sample was kept in 105°C for 7 days.

(5) Photolytic degradation – sample was kept in the photo stability chamber for 5 days.

(6) Humidity degradation – sample was kept in the 85% RH humidity desiccators.

All these degradation samples were prepared for entacapone drug degradation in carbidopa, levodopa and entacapone film coated tablets. Table 3 shows the results of the force degradation study.

The precision of an analytical procedure shows the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Six samples were prepared and injected in the UPLC system; results obtained were within the acceptance criteria. Intermediate precision analysis was performed with the second analyst; six samples were prepared in the same homogeneous sample, injected in the different UPLC system using

Table 5. Accuracy results – true concentration

Drug	Accuracy levels	STD stock preparation		Dilution		Potency (%)	Concentration (mg/ml)	
Entacapone	50	5	25	1	200	99.5	100	0.0010
	100	5	25	2	200	99.5	100	0.0020
	150	5	25	3	200	99.5	100	0.0030
Entacapone *Z*-isomer impurity	50	5	25	1	200	99.5	100	0.0010
	100	5	25	2	200	99.5	100	0.0020
	150	5	25	3	200	99.5	100	0.0030

Table 6. Accuracy results – experimental concentration

Drug	Accuracy level (%)	Area		Standard preparation				%P	CF	mg/ml
		Sample	STD							
Entacapone	50	35,831	69,733	20.16	100	2	200	99.5	1.000	0.00103
	100	71,805	69,733	20.16	100	2	200	99.5	1.000	0.00207
	150	108,909	69,733	20.16	100	2	200	99.5	1.000	0.00313
Entacapone *Z*-isomer impurity	50	38,531	69,733	20.16	100	2	200	99.5	0.932	0.00103
	100	78,298	69,733	20.16	100	2	200	99.5	0.932	0.00210
	150	118,493	69,733	20.16	100	2	200	99.5	0.932	0.00318

Table 7. Accuracy results – recovery

Drug	Accuracy level (%)	True concentration	Experimental concentration	% Recovery (true concentration/ experimental concentration) × 100
Entacapone	50	0.0010	0.00103	103.6
	100	0.0020	0.00207	103.8
	150	0.0030	0.00313	105.0
Entacapone *Z*-isomer impurity	50	0.0010	0.00103	103.9
	100	0.0020	0.00210	105.0
	150	0.0030	0.00318	105.9

Table 8. Linearity and correction factor

	Entacapone		*Z*-isomer impurity	
Linearity level (%)	Concentration (mg/ml)	Obtained area	Concentration (mg/ml)	Obtained area
50	0.00100	34,843	0.0010	38,173
75	0.00149	53,233	0.0015	58,409
100	0.00199	71,343	0.0020	78,320
125	0.00249	88,084	0.0025	95,978
150	0.00299	109,196	0.0030	118,413
Correlation coefficient	0.9994		0.9995	
Slope	36,895,879.4		39,609,800	
RRF	1		1.073	
Correction factor	1		0.932	

different columns. Results obtained were within the acceptance criteria. Table 4 shows the results.

Accuracy should be established across the specified range of the analytical procedure. Recovery study was performed at different level of concentrations according to ICH guidelines. Results obtained were within the acceptance criteria. Results are given in Tables 5–7.

The linearity parameter was used within a given range to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity solutions were prepared at different levels – 50%, 75%, 100%, 125% and 150% and correction factor was simultaneously employed. Acceptance criteria: correlation coefficient (>0.99). Results are provided in Figure 4 and Table 8.

Table 9. Filter compatibility results

Filter study for as such sample	Area	Difference	% Difference
Entacapone *Z*-isomer impurity in as such sample			
Centrifuged sample	32,440	NA	NA
Millipore PVDF 0.45 µm	33,250	–810	–0.02
Nupore-PVDF 0.45 µm	33,223	–783	–0.02
Whatman PVDF 0.45 µm	34,101	–1661	–0.05
Entacapone *Z*-isomer impurity in spiked sample			
Centrifuged sample	116,555	NA	NA
Millipore PVDF 0.45 µm	116,238	317	0.00
Nupore-PVDF 0.45 µm	117,443	–888	–0.01
Whatman PVDF 0.45 µm	118,095	–1540	–0.01

Table 10. Organic composition variation in mobile phase B

System suitability parameters	Actual composition (acetonitrile : water; 75 : 25)	Lower volume (acetonitrile : water; 65 : 25)	Higher volume (acetonitrile : water; 85 : 25)
Entacapone retention time	6.78	7.59	5.81
Z-isomer retention time	6.33	7.14	5.46
USP place count	53,598	58,256	49,586
USP resolution between two adjacent peaks	3.2	3.6	2.8
USP tailing factor	1.09	1.1	1.07
% RSD of entacapone (five standard injections)	0.42	0.49	0.38

Table 11. Flow rate variation

System suitability parameters	Actual flow rate (0.22 ml)	Flow rate	
		0.20 ml	0.24 ml
Entacapone retention time	6.78	7.51	5.85
Z-Isomer retention time	6.33	7.08	5.42
USP place count	53,598	53,985	52,896
USP resolution between two adjacent peaks	3.2	3.6	2.9
USP tailing factor	1.09	1.08	1.06
% RSD of entacapone (five standard injections)	0.42	0.46	0.51

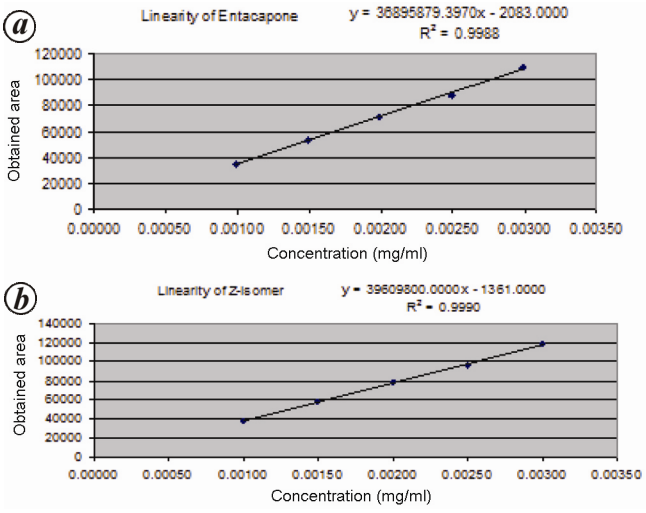

Figure 4. Linearity graphs of (***a***) entacapone and (***b***) *Z*-isomer of entacapone (impurity).

Detection limit (DL) was established from the standard deviation of the response and the slope. It can be expressed as: DL = $3.3\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. Stock solutions (50–150% concentration) were prepared for correlation coefficient and slope of regression line. The limit of detection was established at 0.01% level.

Quantification limit (QL) was established from the standard deviation of the response and the slope. It can be expressed as: QL = $10\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve. Stock solutions (50 to 150% concentration) were prepared for correlation coefficient and slope of regression line. The limit of quantification was established at 0.03% level.

This shows the reliability of an analysis with respect to variations in the composition of mobile phases A and B, and flow rate (Tables 9–12).

Table 12. Concentration variation in mobile phase A

System suitability parameters	Actual concentration (0.1% OPA)	Concentration	
		0.09% OPA	0.11% OPA
Entacapone retention time	6.78	6.79	6.78
Z-isomer retention time	6.33	6.34	6.33
USP place count	53,598	51,254	53,456
USP resolution between two adjacent peaks	3.2	3.1	3.3
USP tailing factor for entacapone	1.09	1.08	1.09
% RSD of entacapone (five standard injections)	0.42	0.5	0.38

Based on the development and validation results, this is a highly sensitive, accurate and robust stability-indicating method. It can be used for routine analysis of entacapone-related substances in drug products.

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