IJPSR (2015), Vol. 6, Issue 7 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 19 October 2014; received in revised form, 19 February, 2015; accepted, 28 February, 2015; published 01 July, 2015

QUANTIFICATION OF INTERMEDIATE, UNKNOWN IMPURITIES AND Z ISOMER IN ENTACAPONE API BY HPLC

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Keywords:

HPLC Related substance, Entacapone API, Z- isomer.

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ABSTRACT: Simple, linear and accurate method was developed for the quantification of Z-isomer impurity and 3, 4-dihydroxy-5-nitrobenzaldehyde intermediate in entacapone active pharmaceutical ingredient by using high performance liquid chromatography. Separation between impurities and Entacapone was achieved on Phenomenox Kinetex C18, (100 x 4.6) mm, 2.6 micron column. Mobile phase consisted of 0.1% Orthrophosphoric acid in water: Methanol in ratio 58:42:0.1 in isocratic mode for RSD of standard. Gradient method was used for sample preparation and blank with flow rate of 0.8 ml/min at 300 nm and column oven temperature at 50°C. RSD for standard preparation under system precision and percentage recovery was observed with in acceptance range. RSD for retention time was observed 0.05% which shows reproducibility during replicate injections. Limit of detection and limit of quantification was achieved at 0.1 ppm and 0.2 ppm level respectively which indicates the lowest level of detection and quantification. The linearity range was achieved from 0.2 ppm to 0.75 ppm level for entacapone, Z-isomer and intermediate. The method was applied for quantification of Z-isomer, intermediate and any unknown impurity of entacapone API.

INTRODUCTION: Manufacturer needs to provide documents related to quality or purity of that drug for approval of any pharmaceutical drug. Liquid chromatographic techniques are the excellent way for determination of purity and impurities in drug. High performance liquid chromatography (HPLC) is a worldwide accepted technique used for analysis of drug substance, drug product and quantification of known as well as unknown impurities at lower level [1]. Food and drug administration (FDA) highly appreciate the purity method of analysis by using HPLC, because this technique is well known for highly accurate, reproducible results.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(7).3076-82

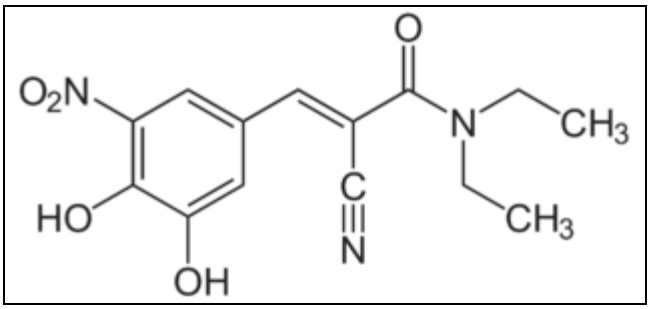
Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(7).3076-82

By using this technique we can separate drug related process impurities, degradation impurities as well as reactants. Entacapone API is nitrocatechol derivative and it is used for treatment of Parkinson's disease. Maximum recommended daily dose for adults is 2000 mg per day if necessary. Parkinson's disease is a neurodegenerative, slow progressive disorder, resting tremor, rigidity and postural reflex impairment with associated characteristic eosinophilic cytoplasmatic inclusions.

Entacapone should be used in combination with levodopa. It is film coated tablet containing 200 mg Entacapone API in one tablet. It is orally taken medicine which contains effect of a selective and reversible inhibitory effect on catechol-O-methyl transferase (COMT) enzyme. Entacapone consists of two isomeric forms E = trans-isomer and the Z = cis-isomer. The E-isomer was selected because it is easy for synthesis. Entacapone is rapidly absorbed

in the gastro-intestinal tract and undergoes extensive first pass metabolism. Entacapone is converted to its (cis)-isomer i.e. Z- entacapone, the main metabolite in plasma followed by direct glucuronidation to inactive glucuronide conjugates. Elimination of this conjugates is mainly via urine as glucuronide conjugates and Z- isomer [2]. A HPLC method for Entacapone was published by Dhawan et.al [3] worked for Entacapone tablets. Sultana et.al [4] extends their research work for Moxifloxacine in combination with Entacapone. These reference methods discuss about analytical purity method, but were found not suitable in terms of gradient equilibration and run time.



(2E)-2-CYANO-3-(3,4-DIHYDROXY-5-NITROPHENYL)-N,N-DIETHYLPROP-2-ENAMIDE [ENTACAPONE][5]

This work presents related substance quantification method by HPLC to quantify entacapone Z isomer, 3, 4 – dihydroxy -5-nitrobenzaldehyde as intermediate, degradation and unknown impurities present in drug.

Experimental:

HPLC grade methanol as a solvent (Spectrochem), orthrophosphoric acid AR grade (Rankem) and HPLC grade water (Merck) were used. Entacapone test sample was received from Ramdev Chemical as a free sample. Z- Isomer of Entacapone is procured from LGC Promochem India limited. HPLC instrument of make Shimadzu LC 2010C HT with a quaternary gradient pump system and a fixed dual wavelength UV detector having LC-solution software with auto sampler tray with cooling facility and column oven temperature compartment is available was used for measurements.

Development of method was initiated by keeping aim to develop simple gradient, easy setup of experimentation and cost effective short run time related substance HPLC method for entacapone API, in such a way that we will get accurate and reproducible results in short period of time. The referred USP method-2[5] method was not found suitable in terms of composition of mobile phase. Tekale et al[6] also extended the research for quantification from tablet. This work is in continuation of our research from assay method of entacapone by using HPLC technique[7].

During selection of HPLC column, Short length, lower micron size C18 column was selected to reduce run time and to achieve excellent separation with sharp peak shapes. Because of short length compound is eluted faster and C18 carbon load in column helps for separation and lower particle size column gives sharp peaks as well as more separation. Also short length column need less time for equilibration which can help us to reduce solvent consumption and save our analysis time. Column oven temperature study was performed in order to reduce the backpressure of column ensuring that it does not impact on separation.

Higher column oven temperature increase the kinetic energy of components by which we can achieve good and sharp peak. Some compounds have property to get retained in column and because of this property such compounds get stuck inside the column and when they are eluted it gives more tailing to peak. In this case higher column oven temperature increases kinetic energy of compound for early elution with good separation and symmetric peak shape will be obtained. Also it reduces viscosity of mobile phase which helps to reduce the back pressure of column.

In this proposed method mobile phase is also easy to setup, which is degassed solution of 0.1% orthtophosphoric acid in HPLC grade water as mobile phase-A and pure HPLC grade Methanol used as mobile phase-B. Gradient method was used for elution of all impurities and 5 micro liter injection volume was injected to avoid excess of sample loading on column. Since excess sample loading on column gives disturbance on baseline, hump and broad peak shape may not be observed.

Mobile phase and other working solutions were filtered through 0.45 micron membrane filter and degassed by sonication. Slowly the flow rate was

increased to achieve the column oven temperature and equilibrate column in mobile phase. Direct application of flow rate creates high pressure on column and may damage it. Diluent was injected as blank preparation. No peak was observed in blank preparation at retention time of entacapone means there is no any blank interference. Six replicate injections of standard preparation were injected for calculation of % RSD.

Chromatographic conditions:

Column name : Phenomenox Kinetex C18, (100 x 4.6) mm, 2.6 micron
Flow rate : 0.8 ml/min
Wavelength : 300 nm
Column Oven temperature: 50°C
Run time : 9 minutes (For standard in isocratic mode)

Injection volume : 5 µl
Diluent : Water: Methanol (50:50)

Mobile phase A:

Accurately pipette out and transfer 1.0 ml orthro phosphoric acid in 1000 ml HPLC grade water. Mix well and sonicate to degass it.

Mobile phase B:

Use pure and HPLC grade methanol as mobile phase-b, sonicate to degass it.

Gradient:

Time (mins)	Mobile phase-A	Mobile Phase-B
0.0	58	42
8	58	42
15	40	60
18	40	60
20	58	42
26	58	42

RESULTS AND DISCUSSION:

Chromatography:

Standard stock solution of entacapone was prepared by weighing accurately 55mg of entacapone standard in 100 ml standard volumetric flask. 50ml of methanol was added and sonicated to dissolve and volume was made up to mark with water. Further 3 ml of this solution was diluted to 100ml volumetric flask and made up to mark with diluent. From this again 3ml solution was diluted to 100ml volumetric flask and made up to mark with diluent. (0.5 ppm standard solution)

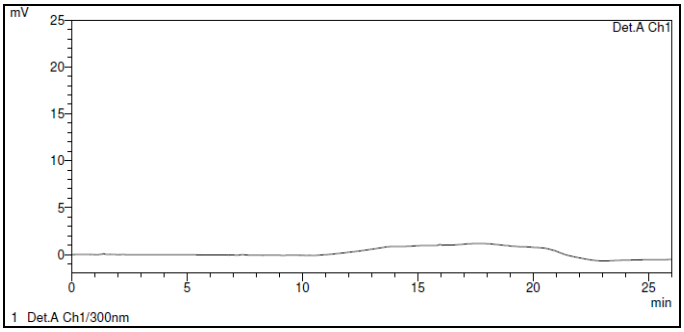


FIG.1: CHROMATOGRAM OF BLANK PREPARATION

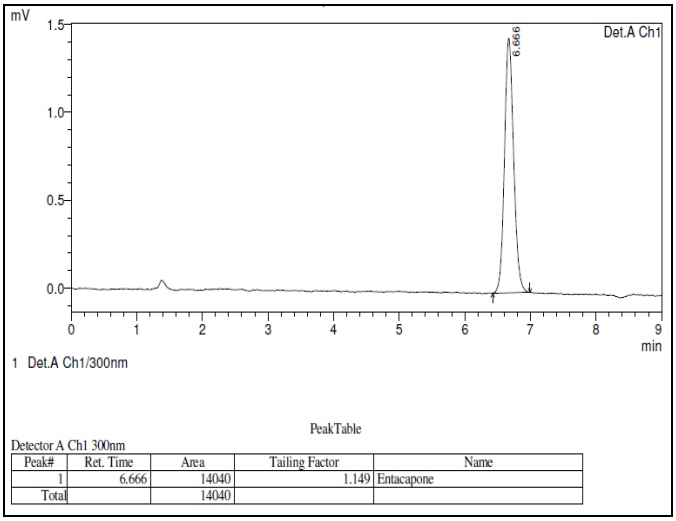


FIG.2: CHROMATOGRAM OF STANDARD PREPARATION

Fig.1 is blank preparation chromatogram, it is diluent injected in which test and standard solution was prepared. It is to ensure that there should not be any interference from diluent. From the standard chromatogram (**Fig. 2**) it was concluded that the Retention time for Entacapone was 6.6 ± 0.05 minutes and tailing factor was 1.14 which as per USP should not be more than 1.5.

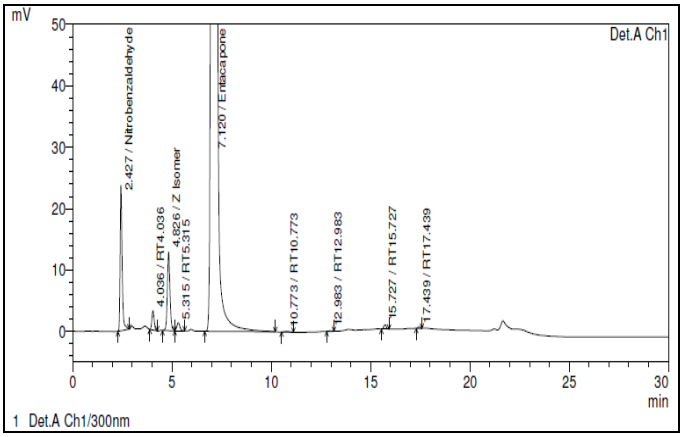


FIG.3: CHROMATOGRAM OF SPIKE PREPARATION

Fig 3 shows that impurity, intermediate as well as unknown impurities are also separated from entacapone peak. Where 3, 4-dihydroxy-5-nitrobenzaldehyde is an intermediate and Z isomer is impurity in entacapone. During method development it has to ensure that no any known as well unknown impurity especially any intermediate or raw material from which the product is synthesized should get merged with concern peak. If such peak gets merged then peak area of such peak will also get contributed in concerned peak area and incorrect results will be obtained. In the present work system precision performed by injecting six replicate injections of standard preparation and percent RSD was calculated. Table 1 refers to retention time and % RSD of standard area.

TABLE1: SYSTEM PRECISION

Sr.No	RT (min)	Area
1	6.691	14254
2	6.669	14146
3	6.677	14208
4	6.664	14017
5	6.659	14135
6	6.666	14040
Average	6.671	14132
% RSD	**0.17**	**0.34**

Linearity was performed from limit of quantification (LOQ) to 150% level with respect to test concentration level in which linearity range was covered from 0.20ppm to 0.75ppm level. It was concluded from linearity graph that test concentration is linear. Observed value of correlation coefficient is 0.99 for intermediate, Z isomer and Entacapone API. Linearity [8] is an important parameter in validation which needs to be performed to obtain test results which are directly proportional to the concentration of analyte in test sample.

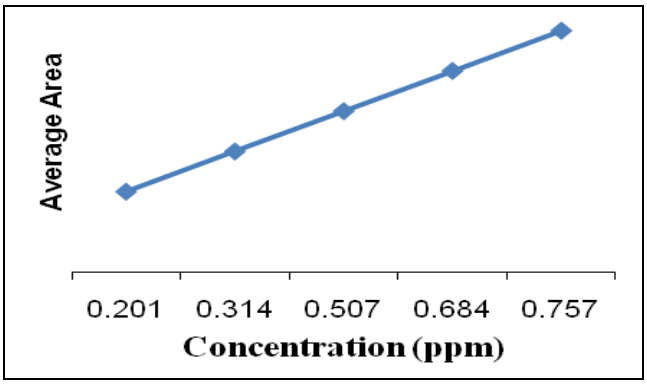


FIG.4: LINEARITY GRAPH FOR 3,4-DIHYDROXY-5-NITROBENZALDEHYDE (INTERMEDIATE)

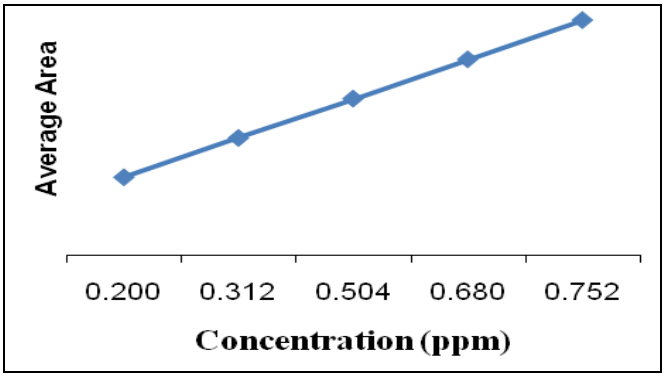


FIG. 5: LINEARITY GRAPH FOR Z ISOMER (IMPURITY)

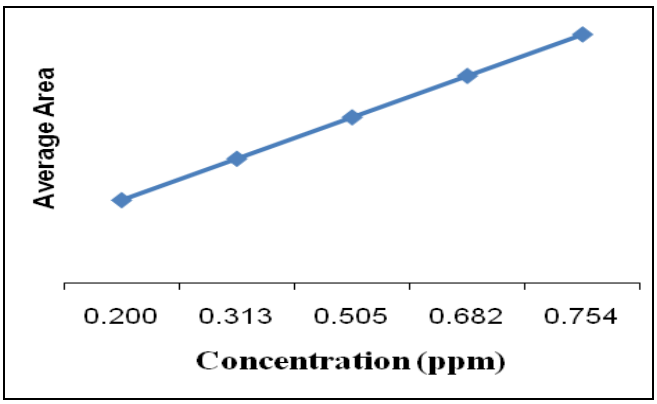


FIG. 6: LINEARITY GRAPH FOR ENTACAPONE (API)

Fig. 4 shows linearity graph for 3, 4-dihydroxy-5-nitrobenzaldehyde, **Fig. 5** shows linearity graph for Z isomer of entacapone and **Fig. 6** shows linearity graph of entacapone API that line passes through all five concentration points.

TABLE 2: LIMIT OF DETECTION (LOD AT 0.1 ppm LEVEL)

Sr. No	Intermediate	Z Isomer	Entacapone
1	1614	3464	3702
2	1558	3430	3755
3	1627	3417	3797
4	1540	3445	3777
5	1573	3391	3697
6	1629	3369	3736
Average	1590	3419	3744

Table 2 explains about lowest limit of detection (LOD) of intermediate, Z isomer and entacapone i.e. at 0.1 ppm level on visual detection technique. Six replicate injections of 0.1 ppm solution injected and observed consistent and average area tabulated in table 2. From this it was conclude that this method can be capable to detect presence of impurity at 0.1 ppm level.

TABLE 3: LIMIT OF QUANTIFICATION (LOQ AT 0.2 PPM LEVEL)

Sr. No	Intermediate	Z Isomer	Entacapone
1	3528	7217	5829
2	3364	7191	5695
3	3405	7051	5700
4	3350	7107	5671
5	3325	7192	5710
6	3351	7103	5746
Average	**3387**	**7144**	**5725**
% RSD	**2.2**	**0.9**	**1.0**

Table 3 explains about lowest limit of quantification (LOQ) of intermediate, Z isomer and entacapone i.e. at 0.2 ppm level. Six replicate injections of 0.2 ppm solution injected. Consistent, average area and relative standard deviation tabulated in **Table 3**. As per validation guidelines relative standard deviation for LOQ is not more that 10.0% and in this method LOQ achieve with in acceptance criteria. From this it was conclude that this method is capable to quantify compounds at 0.2 ppm level.

TABLE 4: ACCURACY STUDY FROM LOQ TO 150% LEVEL

Sr. No	Accuracy Level	Z Isomer	Intermediate
1	At LOQ (0.20 ppm)	103.2 %	90.6 %
2	At 100% (0.50 ppm)	101.1 %	94.9 %
3	At 150% (0.75 ppm)	103.8 %	93.8 %

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found [9].

This study is very important parameter during method validation. Result of this shows that whatever amount of compound we added that much amount should get recovered in our chromatographic method. It is the closeness of theoretical value against experimental value. **Table 4** shows accuracy values for Z isomer and intermediate at LOQ, 100% and at 150% level with respect to test concentration. These values are within acceptance criteria as per validation guidelines. From this we can conclude that this chromatographic method is capable to quantify presence of lower as well as higher amount of compounds present in entacapone API in such a way that we will get accurate results.

Fig.7 shows typical chromatogram of test preparation, where 25mg entacapone test sample dissolved in 50ml volumetric flask in such a way that to get 500ppm concentration of sample solution. This chromatogram indicates the separation of all known as well as unknown impurities present in entacapone API.

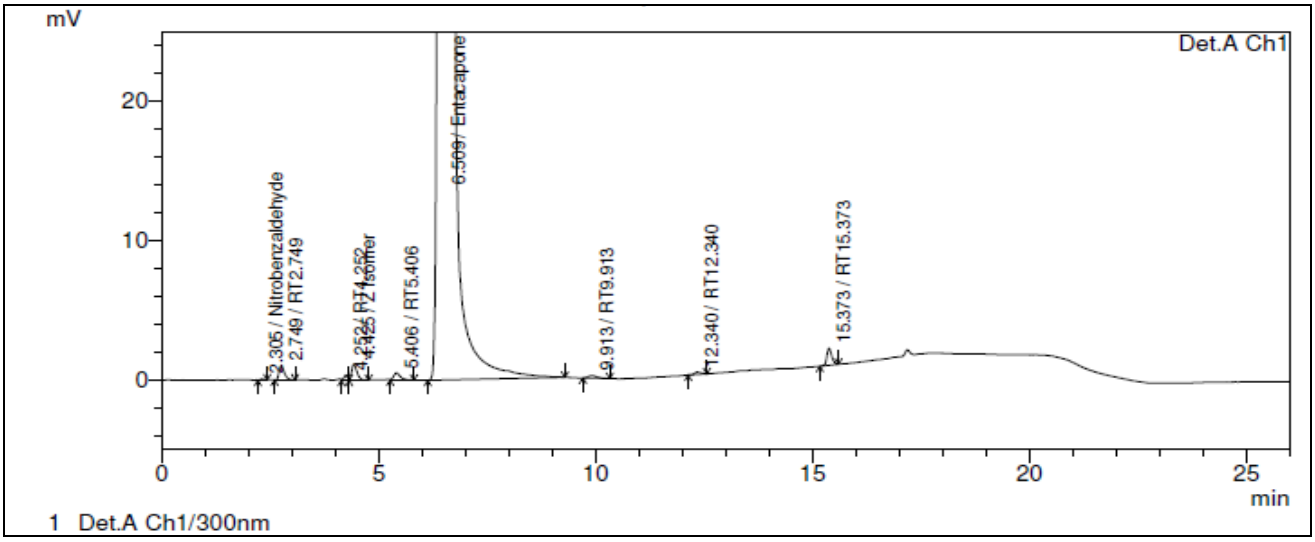


FIG. 7: TEST PREPARATION CHROMATOGRAM OF ENTACAPONE API.

Reproducibility of method was checked by performing method precision in which same test was prepared six times and each of the preparations was injected. Results were calculated against average area of six replicate injections of entacapone standard preparation. This is depicted in **Table 5** for comparative results.

TABLE 5: CALCULATION OF % W/W OF IMPURITIES UNDER METHOD PRECISION.

Sr. No	Intermediate (%w/w)	Z Isomer (%w/w)	Highest Unknown impurity (%w/w)	Total Impurities (%w/w)
1	0.003	0.058	0.056	0.24
2	0.003	0.058	0.056	0.24
3	0.003	0.059	0.057	0.25
4	0.003	0.058	0.056	0.24
5	0.003	0.059	0.057	0.24
6	0.003	0.058	0.055	0.24
Average	**0.003**	**0.058**	**0.056**	**0.24**

TABLE 6: DEGRADATION DATA

Impurity Type	RRT	As such test Preparation (% Area)	Acid Degradation Sample (%Area)	Base Degradation Sample (%Area)	Peroxide Degradation Sample (%Area)
Unknown	0.200	Not Detected	0.178	Not Detected	Not Detected
Unknown	0.354	Not Detected	0.169	0.252	0.163
Intermediate	0.383	0.004	0.042	0.191	0.475
Unknown	0.573	Not Detected	0.474	Not Detected	Not Detected
Unknown	0.679	0.019	0.157	Not Detected	0.157
Z Isomer	0.750	0.080	0.230	Not Detected	0.233
Entacapone	1.000	**99.673**	**98.467**	**99.163**	**98.735**

Degradation is also important criteria in method development. In this process, compound needs to be degraded by chemical treatment and formation of unknown impurities as well as increasing or decreasing trend of impurities to be monitor. Results obtained from this study can help to decide the storage condition and handling precaution of drug.

From **Table 6** it was concluded that intermediate and Z isomer shows increasing trend in all three i.e. acidic, basic and oxidative conditions. Acid degradation shows unknown impurity at RRT 0.573 which was not observed in any degradation condition. From this it was concluded that this method is capable to separate out degradation impurities.

Robustness of method was checked by changing mobile phase composition, column oven temperature and flow rate and found method is robust in all condition and there is no merging of any peaks and peak shapes is also found good. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

CONCLUSION: The elution of entacapone standard was carried out on Kinetex C18, 4.6 × 100 mm x 2.6 micron column HPLC column, at the mm x 2.6 micron column HPLC column, at the flow rate of 0.8 ml/min in isocratic mode for standard preparation and in gradient mode for blank and test sample analysis in which degassed solution of 0.1% orthtophosphoric acid in HPLC grade water as mobile phase-A and pure HPLC grade Methanol used as mobile phase-B with flow rate of 0.8 ml/min at 300 nm.

The method precision result shows reproducibility of method. Linearity graph plotted against average area Vs concentration which is found linear with correlation coefficient 0.99 for Z isomer, intermediate and entacapone. From LOD and LOQ results it was concluded that in this method lowest detection limit is 0.1 ppm and quantification limit is 0.2 ppm with respect to test concentration.

Degradation study clearly indicates that this chromatographic method is capable for separation and quantification of degradation, known as well as unknown impurities in entacapone sample. The method can be used successfully for identification and quantification of the active pharmaceutical

ingredient entacapone from pharmaceutical ingredient. Hence this method can be used for the routine analysis of entacapone.

ACKNOWLEDGEMENT: The author is very much thankful to Dr. Gujar, Principal Sinhgad Institute of Pharmacy, Wadgaon (BK) Pune for providing research laboratory as well as necessary facility.

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How to cite this article:
Aswale S, Aswale S, Zade S and There P: Quantification of Intermediate, Unknown Impurities and Z Isomer in Entacapone API by HPLC. Int J Pharm Sci Res 2015; 6(7): 3076-82.doi: 10.13040/IJPSR.0975-8232.6(7).3076-82.