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(Research Article)

Development and Validation of Stability Indicating HPLC Method for Combination Tablet Dosage Form of Efavirenz, Lamivudine and Tenofovir in Tablet

CH Venkat Reddiah[*1], P. Rama Devi[2], K. Mukkanti[3], P. Srinivasu [1]

[1] Analytical Development ,Unit-III,E.O.U,Block-B, Hetero Drugs Ltd.,Jeedimetla, Hyderabad-500055, India.
[2] Analytical Research and Development , HRF,Balanagar, Hyderabad-500018,India.
[3]Center for Chemical Science, Institute of Science and Technology, J.N.T.University, Hyderabad-500072, India..

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ABSTRACT

A novel rapid, sensitive and reproducible High performance liquid chromatographic method was developed for quantitative determination of Efavirenz,, Lamivudine and Tenofovir Disoproxil Fumarate in active pharmaceutical ingredients and its dosage forms. The synthetic nucleoside reverse transcriptase inhibitor analogues Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate form one of the fixed dosage combinations used in HIV. It belongs to a group of anti-HIV medicines called non-nucleoside reverse transcriptase inhibitors (NNRTIs). The method is applicable to the quantification of related compounds of Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate form one of the fixed dosage combinations. Chromatographic separation of drugs from the possible impurities and the degradation products was achieved on an ACE C18, 250 x 4.6 mm, 5.0µm column; the gradient elution achieved with in 120.0 min. Dilute Ammonium Acetate as mobile phase A and Degassed mixture of Acetonitrile and Methanol (40 : 60) as mobile phase B . The flow rate was 1.5 ml/min, and the detection was done at 265nm. The above developed HPLC method was further subjected to hydrolytic, oxidative, photolytic and thermal stress conditions. The performance of the method was validated according to the present ICH guidelines for specificity, limit of detection, limit of quantification, linearity, accuracy, precision, and ruggedness.

Key Words: Efavirenz, Lamivudine,Tenofovir , Force degradation, Validation

INTRODUCTION

Antiretroviral drugs like nucleoside reverse transcriptase inhibitors, non nucleoside reverse transcriptase inhibitors, and protease inhibitors are essential in the management of HIV infection. The synthetic non nucleoside reverse transcriptase inhibitor analogues Efavirenz, and nucleoside reverse transcriptase inhibitors Lamivudine and Tenofovir Disoproxil Fumarate form one of the fixed dosage combinations used in the effective management of HIV. Efavirenz (EFV), (4S)-6-chloro-4-(cyclopropylethynyl) -4-(trifluromethyl)-1-4-dihydro-2H-3,1-benzoxazin-2-one, is an antiretroviral drug which is a non-nucleoside reverse transcriptase inhibitor (NNRTI)[1,2]. EFV has been determined by UV spectroscopic[3] and RP-HPLC[4] methods in single and in combined dosage form. Tenofovir disoproxil fumarate (TDF), 9-((R)-2- (bis (((isopropoxycarbonyl)oxy) methoxy)phosphinyl)methoxy)propyl)adenine fumarate (1:1), is a nucleotide analogue reverse transcriptase inhibitor (nRTIs)[1,2].TDF has been determined in spiked human plasma by HPLC[5,6]. The estimation of TDF by RP-HPLC has been reported[4,7]. Lamivudine (LMI), (2R,cis)-4-amino-1-(2-(hydroxylmethyl-1,3-oxathiolan-5-yl)-(1H) pyrimidin-2-one, is nucleoside-reverse transciptase inhibitor (NRTI)[1,2]. It is an analogue of cytidine. The estimation of lamivudine using UV[3,8–10] spectroscopy and HPLC has been reported [7,11]. Although the combination of EFV,LMI and TDF is not available commercially in the market, it is in phase 3 clinical trial and the safety and efficacy of TDF in combination with LMI and FFV has already been report[12,13].This study revealed that once daily regimen containing EFV,TDF and LMI is virologically and immunologically effective, well tolerated and safe with benefits in the lipid profile in the majority of patient. Hence, the objective of the work is to develop new spectophotometric methods for estimating EFV,TDF and LMI in pharmaceutical formulation with good accuracy, simplicity, precision and economy.

MATERIAL AND METHODS

Chemicals and Materials

Methanol and Acetonitrile (HPLC grade) and Ammonium acetate and Glacial acetic acid (AR grade), were purchased from Spectrochem and E-Merck Limited respectively. In-house purified water (USP grade) was used throughout the study. Active pharmaceutical ingredients and its related

impurities (Fig.1) were procured from Hetero Drugs Ltd., India, commercially available.

Equipments

The High performance liquid chromatography (Waters) used was equipped with Photo diode array detector with gradient elution capacity and an auto sampler with data handling system (Empower software) on lenovo computer.

Chromatographic Conditions

The chromatographic separation was achieved using a gradient method on an ACE C18, 250 x 4.6 mm, 5-µm column; the gradient Liquid chromatographic method employs solution A and solution B as mobile phase. The solution A contains 1.54g of Ammonium acetate into a beaker containing 1000ml of water and mix. Adjust pH of the solution to 3.8±0.05 with dilute acetic acid. Filter the solution through 0.22µm membrane filter. The solution B contains is mixture of Acetonitrile and methanol in the ratio of (40 : 60) % v/v. The flow rate was 1.5 ml/min. The HPLC gradient program was set as Time/Mobile phase A/Mobile phase B. The column temperature was maintained at 30 °C, sample compartment temperature was maintained at 5°C and the detection wavelength was 265nm for identified and unidentified impurities. The injection volume of 10 µL was used.

Diluent

Prepare a degassed mixture of 0.1% v/v Orthophosphoric acid : Methanol (20 : 80) % v/v.

Gradient Program

Time (minutes)	Mobile phase- A (%v/v)	Mobile phase- B (%v/v)
0	100	0
10	95	5
30	70	30
50	70	30
70	50	50
80	40	60
90	20	80
105	15	85
110	100	0
120	100	0

Standard Solutions

Preparation of Lamivudine Resolution Solution

Accurately weighed and transferred about 10mg of the Lamivudine resolution mixture (containing Lamivudine and Lamivudine Diastereomer) into a 10ml volumetric flask, added 5ml of diluent and sonicated to dissolve. Diluted to volume with diluent and mixed.

Preparation of Efavirenz standard stock solution

Accurately weighed and transferred about 20mg of Efavirenz working standard into a 200 ml volumetric flask. Added about 120 ml of methanol and sonicated to dissolve. Diluted to volume with diluent and mixed.

Preparation of Lamivudine and Tenofovir disoproxil fumarate standard stock solution

Accurately weighed and transferred about 25mg of each Lamivudine working standard and Tenofovir disoproxil fumarate working standard into a 200ml volumetric flask. Added about 120 ml of diluent and sonicated to dissolve. Diluted to volume with diluent and mixed.

Preparation of Standard solution

Transfer 2.0 ml of Lamivudine and Tenofovir disoproxil fumarate standard stock solution and 5.0 ml of Efavirenz standard stock solution into a 50 ml volumetric flask, dilute to volume with diluent and mix

Sample Solutions

Accurately weighed and transferred tablets powder equivalent to about 100mg of Lamivudine into a 100ml volumetric flask, added about 60ml of diluent and sonicated for not less than 30minutes with occasional shaking (maintain the sonicator temperature between 20 to 25°C). Diluted to volume with diluent and mixed. Filtered a portion of the solution through 0.45µm membrane filter .

Degradation Studies

Specificity is the ability of method to measure the analyte response in the presence of its potential impurities and degradation products. The specificity of the developed RP-HPLC method of Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate was carried out in presence of its eight potential impurities, namely Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D. Forced degradation studies were performed on for Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate bulk drugs. Intentional degradation was attempted with stress conditions of UV light (254 nm), heat and humidity (105°C at 90 % RH), acid (1N HCl), base (0.1 N NaOH) and oxidation (3 % H2O2) to determine the ability of the proposed method to separate Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate from its impurities and degradation products generated during forced decomposition studies . For heat and light studies, study period was 7 days where as for acid, base and oxidation it was 24 hrs. Peak purity test was carried out on the stressed samples by using PDA. Related compounds studies were carried out for stress samples against qualified reference standard. Related compounds were also calculated for bulk sample by spiking with its impurities at its specification level (0.1%).

Accurately weighed and transferred tablets powder equivalent to about 100mg of Lamivudine into a 100 ml volumetric flask, added about 60 ml of diluent and sonicated for 30 minutes with occasional shaking (maintaining the sonicator temperature between 20 to 25°C). Diluted to volume with diluent and mixed. Filtered a portion of the solution through 0.45µm membrane filter.

Method Validation

System and Method Precision

The system precision is indicated by the repeatability of multiple injections and indicates the performance of the HPLC instrument under the prescribed chromatographic

conditions. The variance of the values obtained is represented as the percent relative standard deviation (% RSD). A working standard solution of Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate and its related compounds was consecutively injected six times under the same analytical conditions. The % RSD of peak areas, difference of retention times, tailing factor (T) column efficiency (N) and resolution (R) are calculated. The intermediate precision of the method was also evaluated using one unspiked sample and 6 independent sample preparations spiked with a 100% of the target concentrations as defined by the method. The samples were injected using a different instrument and column.

Linearity

The linearity is determined by the ability of the method to obtain test results, which are directly proportional to the concentration of the compounds of interest in the sample. Stock solutions were serially diluted to produce solutions containing concentration levels from QL to 150% with respect to impurity specification limits of 0.1 %. The calibration curve was drawn by plotting the peak areas of Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate; Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D versus its corresponding concentrations. The % RSD value of the slope and Y intercept of the calibration curve was calculated.

Quantification limit (QL) and Detection Limit (DL)

The lower end of the linear range was considered to be the QL for the method. The QL concentrations were determined by injecting diluted standard solution to a level such that % RSD was not more than 10%, precision study was also carried at the QL level by injecting six individual preparations of Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate; Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D.

Accuracy

Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate; sample solution was spiked with impurity standard solutions containing Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D at three concentration levels corresponding to QL 100% and 150% of analyte concentration. The % recovery is the amount of the compound of interest analyzed as a percentage of the theoretical amount present in the medium wa alculated from the slope and the Y-intercept of the calibration s c curve.

Robustness

Deliberate variations in critical method parameters were done to assess the robustness of the related compounds method to evaluate method reliability. The flow rate of the mobile phase was 1.5ml/min, to study the effect of flow rate on the resolution; it was changed by 0.1 unit from 1.4 to 1.6ml/min. The effect of column temperature on resolution was studied at 25 and 35 °C instead of 30 °C. The pH of Buffer Mobile phase 3.6 and 4.0 instead of 3.8.

Solution Stability

The stability of the analyte was established for standard and sample solutions under conditions as prescribed in the method. The purpose of this procedure was to determine the time during which the standard and sample solutions remain stable. In this validation three solutions were studied: Stock standard solution, Working standard solution and Sample solution.

RESULTS AND DISCUSSIONS

Method development and optimization

The main aim of the chromatographic method is to achieve the separation of precursors, intermediates and the main components Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate. From the UV profiling it was found that the suitable wavelength for the Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate drugs and its related impurities is 265 nm. Hence it was concluded anticipating the possible base line interferences at lower wavelength 265 nm was selected as the detection wavelength for the quantification of Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate, its identified and unidentified impurities. When developing a reversed phase method for basic compounds, like Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate, you can expect a more robust method when using acidic mobile phases. Based on the experimental data and the opted wavelength it was found ammonium acetate buffer is suiable. The chromatographic separation was achieved on an ACE C18 250 x 4.6 mm, 5 um column. The gradient liquid chromatographic method employs solution A and Solution B as mobile phase. Mobile phase A contains 1.54 g of Ammonium acetate a beaker containing 1000 ml of water and mix, Adjust pH of the solution to 3.8±0.05 with dilute acetic acid.and mobile phase B is mixture of HPLC grade methanol : Acetonitrile (60:40).

The flow rate was 1.5 ml/min. The HPLC gradient program was set as Time / mobile phase A/ mobile phase B. The column temperature was maintained at 30 °C, sample compartment temperature is maintained at 5 °C and the detection wavelength was 265 nm for identified and unidentified impurities. The injection volume 10µL. The peak shape of Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate, were found to be symmetric and well separated by its potential process impurities and degradants. In the optimized conditions, Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate; Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D were well separated with a resolution greater than 1.5 and the typical retention times for Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate; Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D were about 86.4, 9.92, 65.92, 4.15, 25.51, 55.06, 67.13, 74.34, 82.02, 87.18 and 95.65 respectively. The system suitability results were tabulated and the developed method for Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate and its impurities was found to be specific (Table 1)

Results of Forced Degradation

Forced degradation samples were analyzed with a sample concentration of 1000 mg/ml of lamivudine equivalent with above mentioned chromatographic conditions using a PDA detector to monitor the homogeneity and purity of the Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate peaks. Degradation was not observed under stress condition like, heat and humidity (105 °C and 90 % RH for 7 days) oxidative (3 % H2O2 at RT for 24 hours) and light exposure in solid state and liquid state. Very mild degradation of drug material was observed during acid hydrolysis (1 N HCl 24 hours at 80 °C) however the drug is more susceptible to base hydrolysis (0.1 N NaOH 24 hours at 60 °C) . The RS studies were carried out for the stress samples against a Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate qualified reference standard. The mass balance (%assay + % sum of all related compounds + % sum of all degradants) were calculated for all of the stressed samples and were found to be more than 95 %. Peak purity test results obtained from PDA confirm that the Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate peaks were homogeneous and pure in all analyzed stress samples, which confirms the stability indicating power of the developed method.

Results of Method Validation

Precision

The injection (system) precision was evaluated by performing six replicate injections for its related compounds at 100 % working standard concentration. The % relative standard deviation of 6 injections was calculated, the % RSD for Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D were found to be 2.81, 0.46, 0.79, 0.48, 0.12, 0.51, 0.79 and 1.10% respectively. The RSDs of the % recovery values meet the requirement of not more than 10% for all impurities. (Table 2)

Linearity

For all eight impurities, a linear calibration curve was obtained ranging from QL to 0.15 %. The analytical data and linearity results for Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D were tabulated in (Table 2).

The coefficient of determination (r2) is 0.99972, 0.99969, 0.99974, 0.99904, 0.9963, 0.9996, 0.9994 and 0.9989 respectively, which meets the specification for the r2 value of not more than 0.99, confirming the linearity of the method.

Accuracy

The related compounds of Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate can also be determined accurately over a concentration range varying from QL to 150 % of their respective target analyte concentrations when in Efavirenz, Lamivudine and Tenofovir Disoproxil Fumarate sample solution. The percentage recovery for the related compounds Lamivudine Carboxylic acid, Mono-POC-PMPA, ipr-POC-PMPA, n-POC-POC-PMPA, Tenofovir mixed dimer, Efavirenz Related compound-A, Tenofovir dimer, Efavirenz Related compound-D were ranged from 95.9 to 104.8 (Table 2).

Robustness

In all the deliberate varied conditions (flow rate and column compartment temperature) the resolution between Lamivudine, Lamivudine diastereomer and its impurities was greater than 1.5, illustrating the robustness of method.

Solution Stability

The Stock standard solution, Working standard solution and Sample solution were prepared as per the method, after dispensing an amount for the testing of initial time , the solutions were stored in volumetric flasks and kept in refrigerator (5±3°C) prior to the testing at each time interval of 1st week, 2nd week, 3rd week and 4th week for Stock standard solution and 24 hours and 48 hours, for Working standard solution and Sample solution, the flasks were taken out of the refrigerator, allowed to equilibrate to room temperature before use. The % recovery of each analyte meets the requirement of 90 to 110% after 2nd day for Stock standard solution; however working standard is stable up to 2nd day. No extra peaks detected, no peaks disappeared and no peak areas are increased or decreased by more than the respective QL level after 48 hours in case of sample solution. Therefore sample solution was found to be stable for 48 hours. However working standard and sample stored at room temperature showed a stability of 24 hours.

CONCLUSION

A stability indicating HPLC related compounds method was developed for the quantification of, Efaviren, Lamivudine, Tenofovir Disoproxil Fumarate and its potential impurities in active pharmaceutical ingredients and its dosage forms. The developed method is specific, precise, accurate, linear and robust for, Efavirenz, Lamivudine, Tenofovir Disoproxil Fumarate and its impurities. Degradation products formed during forced decomposition studies were very well separated from analyte peak, which demonstrates that the developed method was specific and stability indicating. This method can be used to carry out the analysis of Efavirenz, Lamivudine, Tenofovir Disoproxil Fumarate drug product in regular quality check and stability samples.

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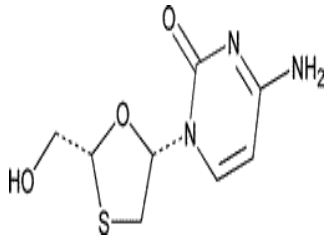
Table 1: Forced degradation results

Impurities (% Area Normalization)	Stress Condition					
	HCl	NaOH	H2O2	UV	Thermal	Humidity
Lamivudine Carboxylic acid(imp 1)	ND	ND	ND	ND	ND	ND
Mono-POC-PMPA (imp 2)*	2.187	1.285	0.742	0.658	0.627	0.674
Lamivudine	12.14	12.08	12.11	10.75	10.63	10.74
ipr-POC-PMPA (imp 3)*	ND	ND	ND	ND	ND	ND
n-POC-POC-PMPA (imp 4)*	ND	ND	ND	ND	ND	ND
Tenofovir mixed dimer (imp 5)*	ND	ND	ND	ND	ND	ND
Efavirenz Related compound-A (imp 6)	ND	ND	ND	ND	ND	ND
Tenofovir dimer (imp 7)*	ND	ND	ND	ND	ND	ND
Tenofovir disoproxil	67.30	67.33	67.41	66.76	66.79	66.79
Efavirenz	87.51	87.55	87.60	87.11	87.09	87.13
Efavirenz Related compound-D (imp 8)	ND	ND	ND	ND	ND	ND

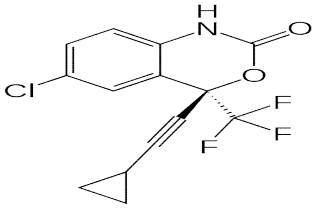
ND: Not detected *Process impurities

Table 2: Summary of method validation results

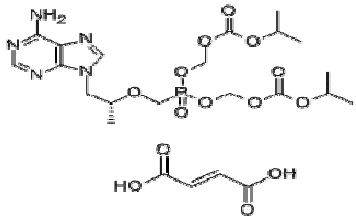
Validation Parameter	IMP 1	IMP 2	IMP 3	IMP 4	IMP 5	IMP 6	IMP 7	IMP 8
System Precision								
% RSD of peak area	2.74	1.34	0.90	1.72	1.02	2.79	2.03	0.86
% Difference of Retention time (last two std)	0.001	0.001	0.000	0.001	0.000	0.001	0.000	0.000
% Difference of Retention time (last std and check std)	0.001	0.000	0.000	0.001	0.000	0.001	0.001	0.001
Resolution	2.4	--	--	--	--	--	--	--
Tailing Factor	0.82	0.92	1.05	1.24	1.14	1.05	1.12	0.9
Column efficiency	32198	22616	6215	12902	20895	12093	235422	10970
Linearity								
Slope	5416.27	10080..42	9660.91	8672.27	9679	3198.09	13037.13	6372.85
Intercept	-96.08	1324.39	-12.63	77.68	-184.8	75.26	-201.55	321.77
r2	0.99972	0.99969	0.99974	0.99904	0.9963	0.9996	0.9994	0.9989
RRF	1.200	1.0	1.0	1.0	1.0	1.02	1.0	0.824
Accuracy Mean % Recovery at QL	100.6%	101.1%	100.4%	101.5%	100.4%	101.3%	101.5%	99.2%
50%	98.0%	95.9 %	101.9 %	100.2 %	100.5 %	98.8 %	104.8 %	98.8 %
100 %	99.1%	102.2 %	102.8 %	103.8 %	100.1 %	101.9 %	103.9 %	100.6 %
150 % of target	99.7%	102.4 %	101.3 %	102.1 %	96.3 %	101.9 %	97.7 %	99.4 %
Intermediate Method Precision								
% RSD	2.81	0.46	0.79	0.48	0.12	0.51	0.79	1.10
Quantitation limit(µg/ml)	0.456	0.526	0.766	0.379	0.374	0.651	0.128	0.478
Detection limit(µg/ml)	0.138	0.159	0.232	0.114	0.113	0.197	0.038	0.144
Stability of Solutions Working Standard Stock , Std Solutions (Room temp) and Sample Solution (5±3°C) (Room temp)	0 hours to 48 hours stable	0 hours to 48 hours stable	0 hours to 48 hours stable	0 hours to 48 hours stable	0 hours to 48 hours stable	0 hours to 48 hours stable	0 hours to 48 hours stable	0 hours to 48 hours Stable
Filter Variability Difference (Centrifuged Vs 0.45 µ PVDF) and (Centrifuged Vs 0.45 µ Nylon)	0.031 0.032							



Lamivudine



Efavirenz



Tenofovir DF

Fig. 1: Lamivudine, Efavirenz, Tenofovir Disoproxil fumarate and its related compounds structures.

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Corresponding Author:
CH Venkat Reddiah , Analytical Development ,Unit-III,E.O.U,Block-B, Hetero Drugs Ltd.,Jeedimetla, Hyderabad-500055, India.
E mail ID: venkat.re2007@gmail.com