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STABILITY INDICATING LC-MS METHOD DEVELOPMENT AND VALIDATION FOR PRAVASTATIN

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ABSTRACT
A Simple, rapid, presice and accurate reversed phase stability indicating HPLC and LC-QTOF-MS/MS method was developed and validated for determination pravastatin in drug substance. The drug was subjected to acid (1N HCL), neutral and alkaline (1N NAOH) hydrolytic conditions at 80ºC as well as thermal studies. Photolysis was carried out in 0.1N HCL, water and 0.1 N NaOH at room temperature. Stress studies were performed on 1mg/ml solution of drug, starting with milder conditions followed by stronger conditions so as to get sufficient degradation around 5-20%.the method has shown adequate separation from pravastatin and its degradation products. The separation was achieved on a water's C-18 (4.6x250mm,5µm) column using 0.01M Ammonium acetate pH (3.0):acetonitrile as a mobile phase in gradient elution mode by LC at flow rate of 1.0ml/min and UV detection at 238 nm. The degradation products were characterized by LC-MS/MS and its fragmentation pathways were proposed. The pravastatin was degraded to thirteen degradation products, P-1 to P-13 which are formed under different conditions. The linearity of proposed method was investigated in the range of 50-500ug/ml having correlation coefficient r2 =0.9999.The intraday and interday precision was found to be less than <1%.Percentage recovery was shown to be 99-100%.As proposed method could effectively separate the drug from its degradation products, it can be employed as stability indicating method.

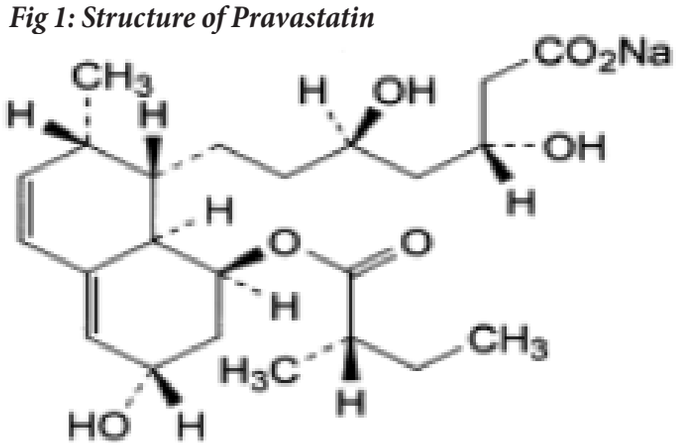
Key Words:
Pravastatin, LC-QTOF-MS/MS, Validation.

INTRODUCTION:

Pravastatin is a lipid regulating drug which is used to reduce LDL-cholesterol,apolipoprotein B, and triglycerides, and to increase HDL cholesterol in the treatment of hyperlipidaemias, including hypercholesterolemia, combined (mixed) hyperlipidaemia(-type IIa or IIb hyperlipoproteinaemias), hypertriglyceridaemia (type IV),dysbetalipoproteinaemia (type III), and post-transplantation hyperlipidaemia.[1] The drug stability investigation represents an important subject in drug quality evaluation many environmental conditions such as heat, light or the chemical suscepatibilty of substances to hydrolysis or oxidation can take part in pharmaceutical stability[2-3]. Analytical method for Pravastatin is official in, British Pharmacopoeia(BP2010),European Pharmacopoeia (EP2010 or EP2009), United States Pharmacopeia (USP32) and Japanese Pharmacopoeia 2006 (JP2006)[4-8]. Earlier Pravastatin has been determined by polarographic pulse polarography[9] and capillary electrophoresis (CE)[10] in tablets, and HPLC-UV[11-16] and HPLC-MS[17-20], HPLC-MS-MS[21,22] GC-MS[23] in urine, plasma and other matrix.High throughput methods (HPLC and CE) have been also developed todetermine pravastatin in production media[24]. Reviews of analytical methods for the quantitative determination of HMG-CoA reductase inhibitors, including pravastatin, in biological samples and in pharmaceutical formulations have been also published[25,26].

Up to date, no LC-MS stability indicating analytical method applied to a systematic study focused on pravastatin degradation have been described in the literature. For this reason, the aim of this paper is to study the hydrolytic behaviour of pravastatin in different pHs and temperatures and to develop an HPLC analytical method for determination of pravastatin in the presence of its hydrolytic degradation product(s). The novelty of this work is based on the description of a new analytical method, suitable for monitoring the purity of drug substance, and the obtained results can be helpful to assure the quality, safety and effectiveness of pharmacotherapy.

Fig 1: Structure of Pravastatin



MATERIALS AND METHODS:

Chemicals/ Reagents and Solvents:

Pravastatin was obtained from gift sample from IICT, Hyderabad. Acetonitrile, Ammonium acetate, Sodium hydroxide, Hydrochloric acid were purchased from Merck (Darmstadt, Germany). All reagents used were at least of analytical grade except Acetonitrile which was HPLC grade. High purity water was prepared by passage through a Millipore milli-Q plus system (Milford MA, USA) and was used to prepare all solutions.

Instrumentation and Equipments:

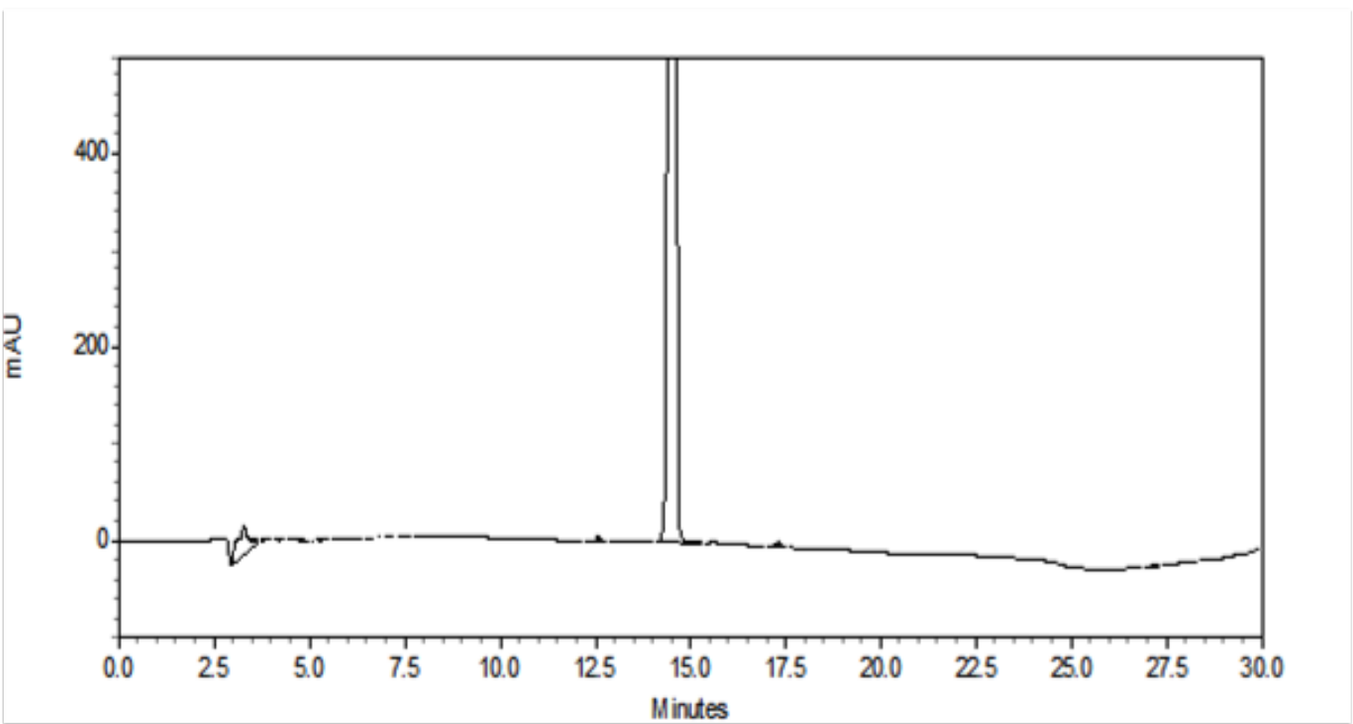
The LC system used for method degradation study was Shimadzu separation module consisting of binary pump (LC-20AD); Photo Diode array (PDA) Detector (SPD-M20A) Degasser (DGU-20A3).The output signal was monitored and processed using LC-Solution software. All PH measurements were done on pH-meter (Metrohm schweiz AG, 780 PH meter, Germany with Epson printer lx-300t, weighing was done Sartorius balance (CD 225D, 22308105 Germany). Oil bath with temperature controller (Seelbach, Germany) were used for solution degradation studies), Incubator (IB-05 G). Photolytic studies were carried out in a photo stability chamber (KBWF240), WTC Binder Germany)

For LC-MS Analysis, Agilent 1200 series LC instrument (Agilent technologies, USA) which is coupled to a quadrapole time of flight (Q-TOF) mass spectrometer Q-TOF LC-MS 6510 Series classic G6510 Agilent Technologies USA) equipped with an electro spray ionization (ESI) source. The data acquisition was carried out with mass haunter workstation software.

ANALYTICAL METHOD DEVELOPMENT

LC-MS analysis for the separation of degradation products and drug in HPLC and LC-QTOF-MS/MS:

Fig 2:Chromatogram of standard sample (pravastatin)



A Waters C_{18} (4.6mm x 250mm, 5µm) was used for chromatographic separation . The mobile phase composed of Ammonium acetate pH3.0:Acetonitrile in a gradient elution mode by LC at flow rate 1.0 mL/min with run time 30mins. Mobile phase and sample solution were filtered through a 0.45µm membrane filter and degassed.The detection of drugs was carried out at 238nm with retention time 14.5.

Preparation of different solutions and samples

1) 0.01M Ammonium acetate, pH 3.0:

Accurately weighed 0.308 gm. of CH3COO(NH4) in beaker dissolved and volume made up to 1000 ml with milli Q water, finally the solution was sonicated. pH of solution was adjusted with the help of dilute acetic acid. Finally solution was filtered through 0.45 µm filter paper and was transferred to mobile phase container.

2) 1N HCL:

8.8 ml accurately measured amount of HCL (33%) analytical grade was diluted to dissolve with of Milli Q water, was sonicated to mix them was made upto 100 ml was filtered through what man no 41 filter paper.

3) 1 N NAOH:
4.1 gm accurately weighed amount of NAOH was diluted with Milli Q water was sonicated to mix and was made up to 100 ml of Milli Q water and was filtered through what man no 41 filter paper

4) Sample diluent:
Acetonitrile was used to dilute the samples.

5) Procedure for conducting stress (FD) studies:
All stress decomposition studies were performed at an initial drug concentration of 1 mg/ml. Stress studies were carried out under the conditions of dry heat, hydrolysis, oxidation and photolysis as mentioned in ICH Q1A (R2). Generally, forced degradation conditions are severe than accelerated testing. The drug was subjected to forced degradation like acid hydrolysis, base hydrolysis, neutral hydrolysis, thermal decomposition, photolytic degradation. The progress of degradation at different conditions was monitored by direct ESI-MS analysis at different time points. At each point, the sample solution was neutralized, if needed, and diluted 10 times using solvent (methanol) so that the final concentration of the drug was 100 ppm prior to MS analysis.
The specific stress conditions were as follows:

RESULTS AND DISCUSSION:
a) Acidic degradation study:
Acid degradation was performed at 1N HCL at for 48hrs. Fig shows the LC chromatogram obtained in acidic condition. It shows 2 clear peaks representing the elution of an unknown compound at RT 20.1mins, DP-1 and the drug at RT 20.4mins, respectively. Total two degradant products were formed p-1 p-2 fig.

b) Alkaline degradation study:
The drug showed sufficient degradation after 48 hrs when refluxed at 80 c in 1N NAOH forming one degradant product.

c) Neutral degradation study:
Under the neutral condition (water), pravastatin was degraded at 80 c for 48hrs.Total one degradant product is formed.

d) Photo degradation study:
The drug showed no degradation when exposed to photolytic acid; base neutral conditions. Pravastatin is stable in all conditions for about 10-15 days.

e) Thermal degradation study:
Drug was spread over Petri plate dish and exposed to dry heat 60 c 24hrs in an oven then from that powder an accurately weighed quantity of pravastatin (5mg) transferred to 50 ml volumetric flask and dissolved in 30 ml of mobile phase. Flask was sonicated for 5min and volume was made up to mark with mobile phase to get 100ug/ml of pravastatin further dilution was carried out by diluting 1ml of solution In 10ml volumetric flask with mobile phase to get 10 ug/ml pravastatin The drug showed no degradation when exposed to thermal decomposition.pravstatin is stable in thermal decomposition.

Fig 3: Chromatogram of acid degradation study

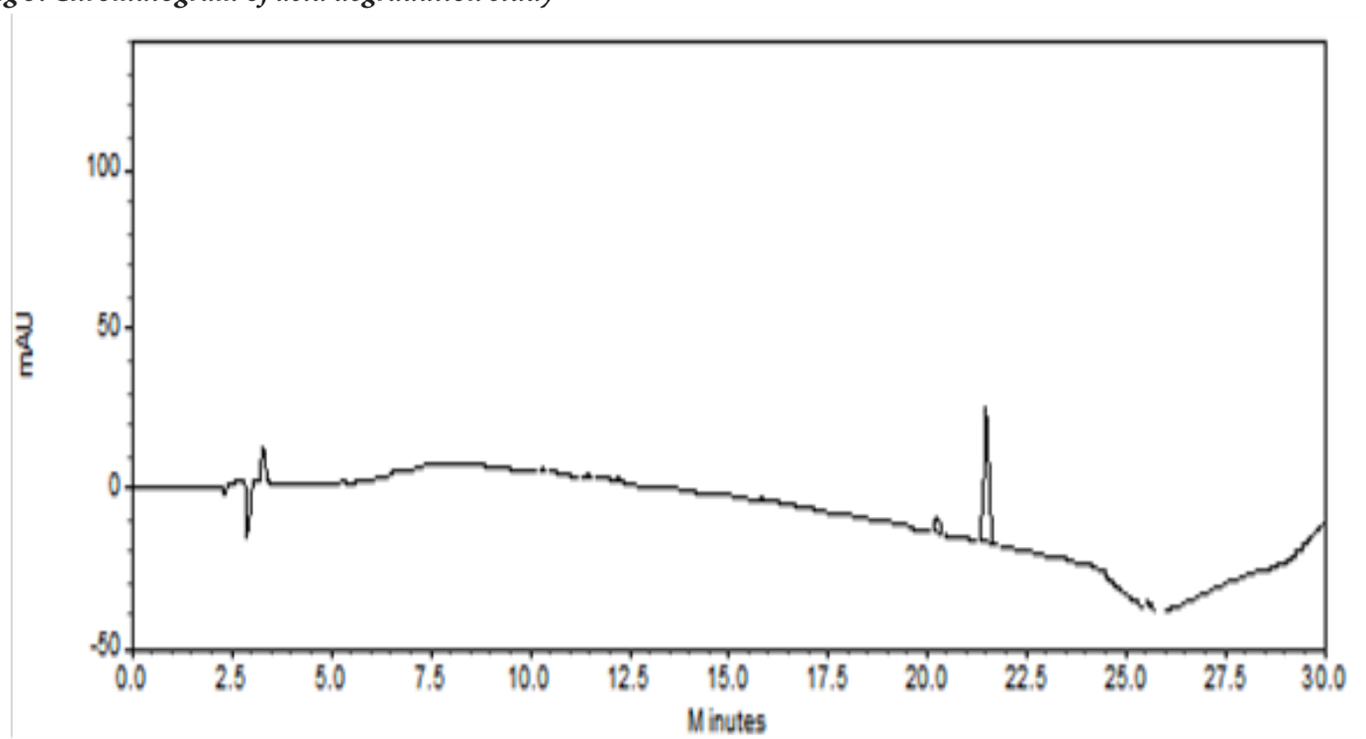


Fig 4: Chromatogram of alkaline degradation study

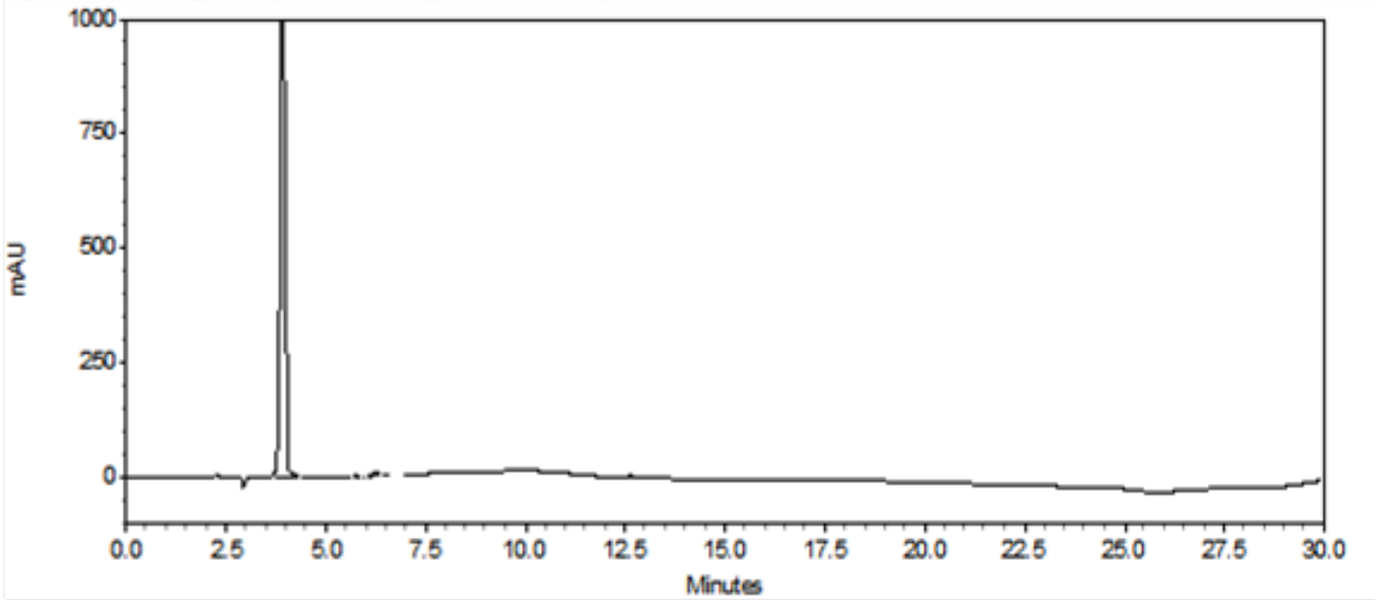


Fig 5: Chromatogram of neutral degradation study

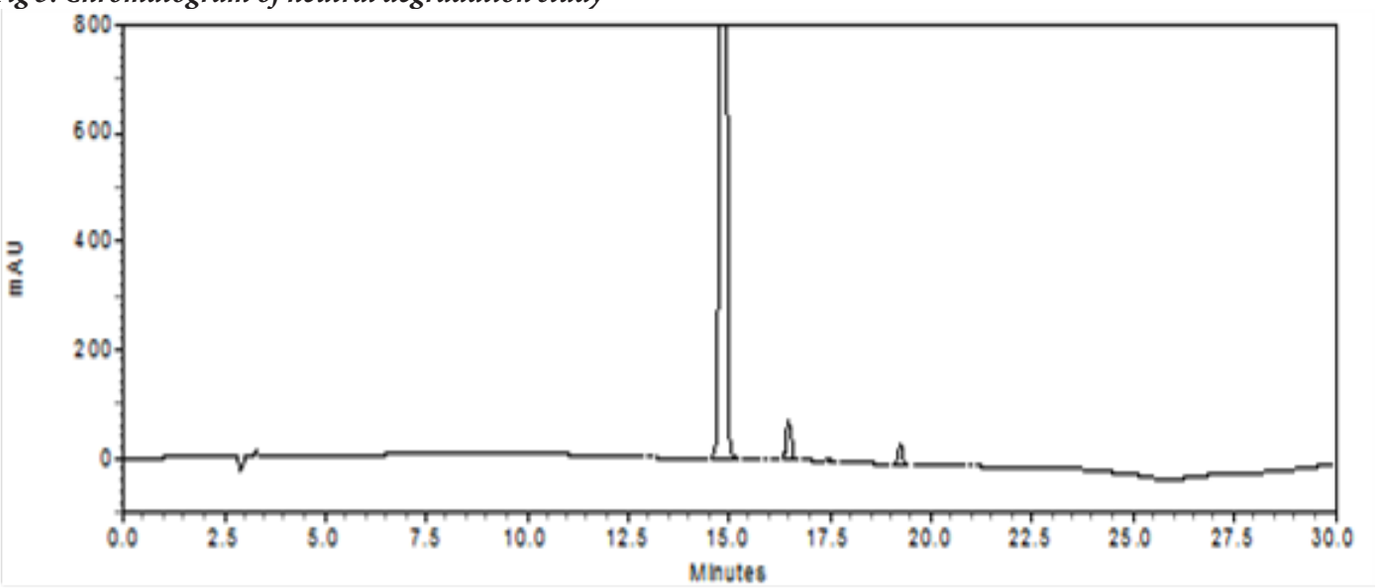


Fig 6:Chromatogram of photo degradation study

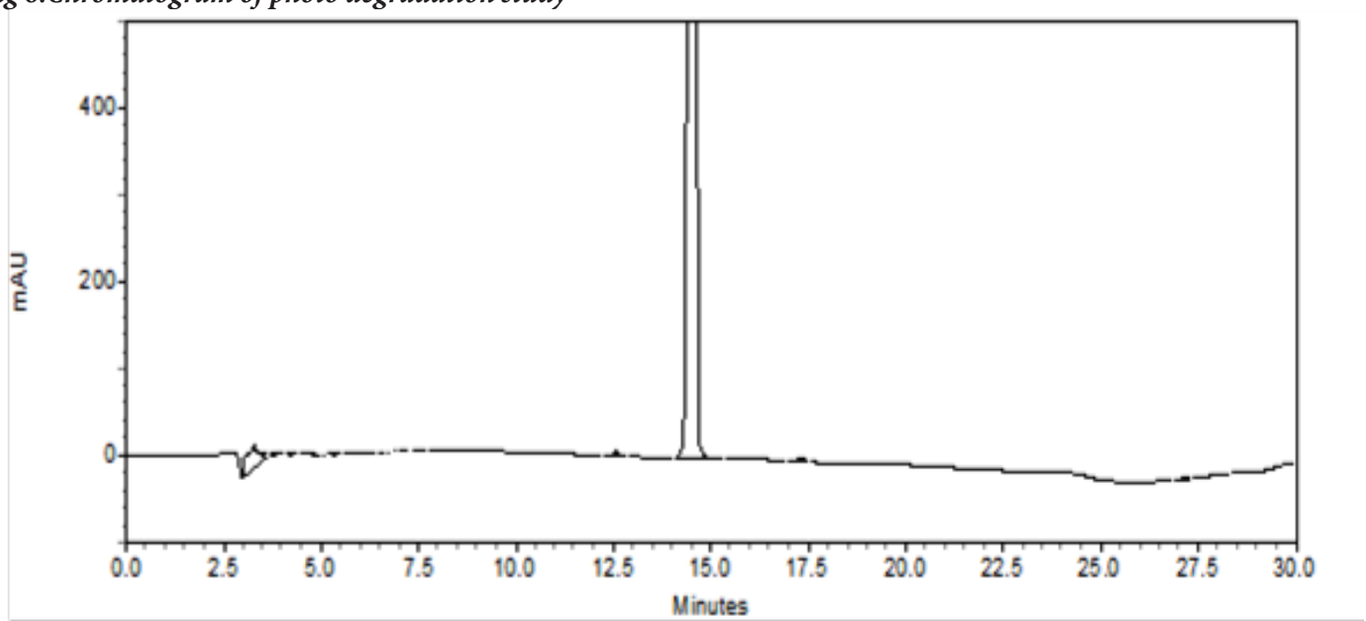


Fig 7: Chromatogram of thermal degradation study

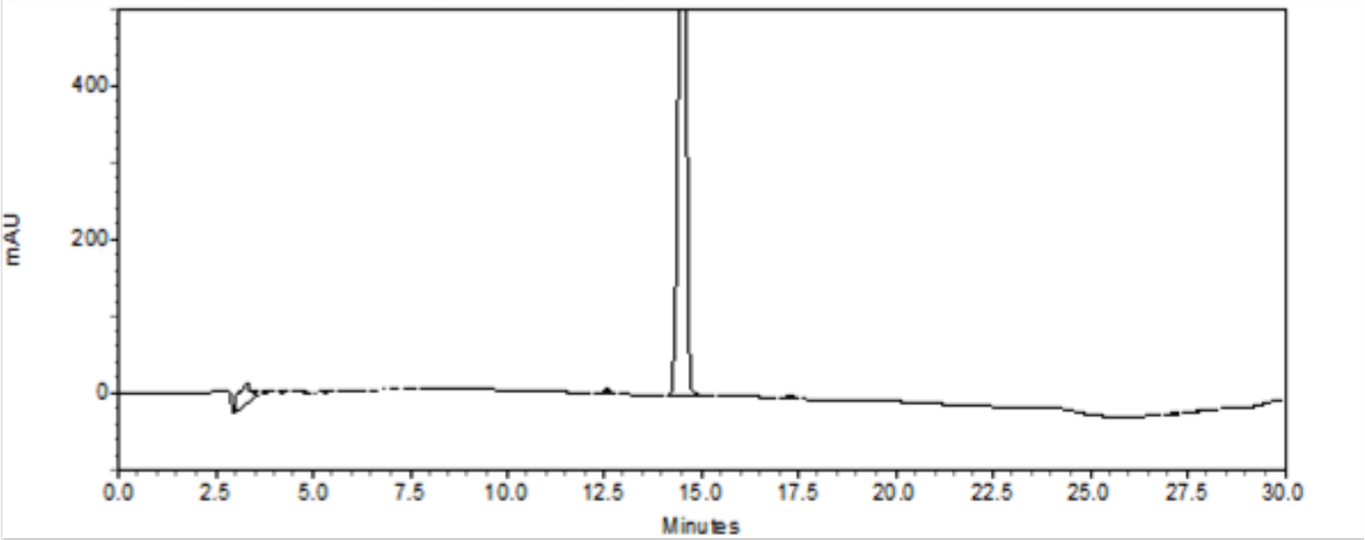


Table 1: Optimized stress condition:

SNO	Stress condition	Exposure		Duration
1	Hydrolysis			
2	Acid	1 N HCL	80 C	48HRS
3	Base	1N NaOH	80 C	48 HRS
4	Neutral	H20	80 C	48HRS
5	Photolysis(UV chamber)	-		10 DAYS
6	Thermal		60 C oven	24HRS

ESI-MS analysis of Pravastatin:

ESI tandem mass spectrometry has been established as a powerful technique for structural elucidation of protonated or deprotonated drug molecules, degradation products, impurities and metabolites. ESI Mass spectrum of pravastatin was recorded on QTOF Mass Spectrometer (QSTAR-XL-Applied Biosystems/MDS Sciex, Foster City, CA, USA)

Fig8: LC-MS/MS Study of molecular ion peak of m/z

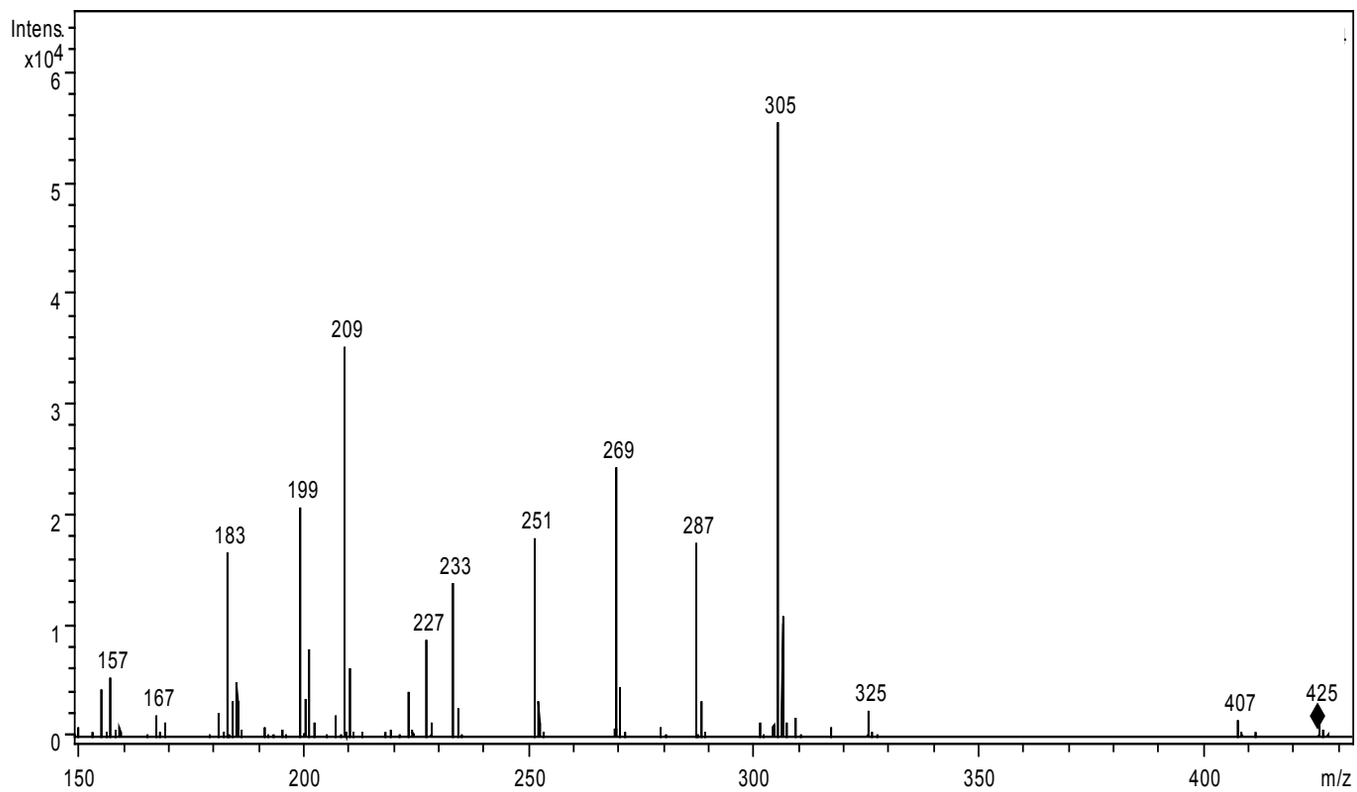
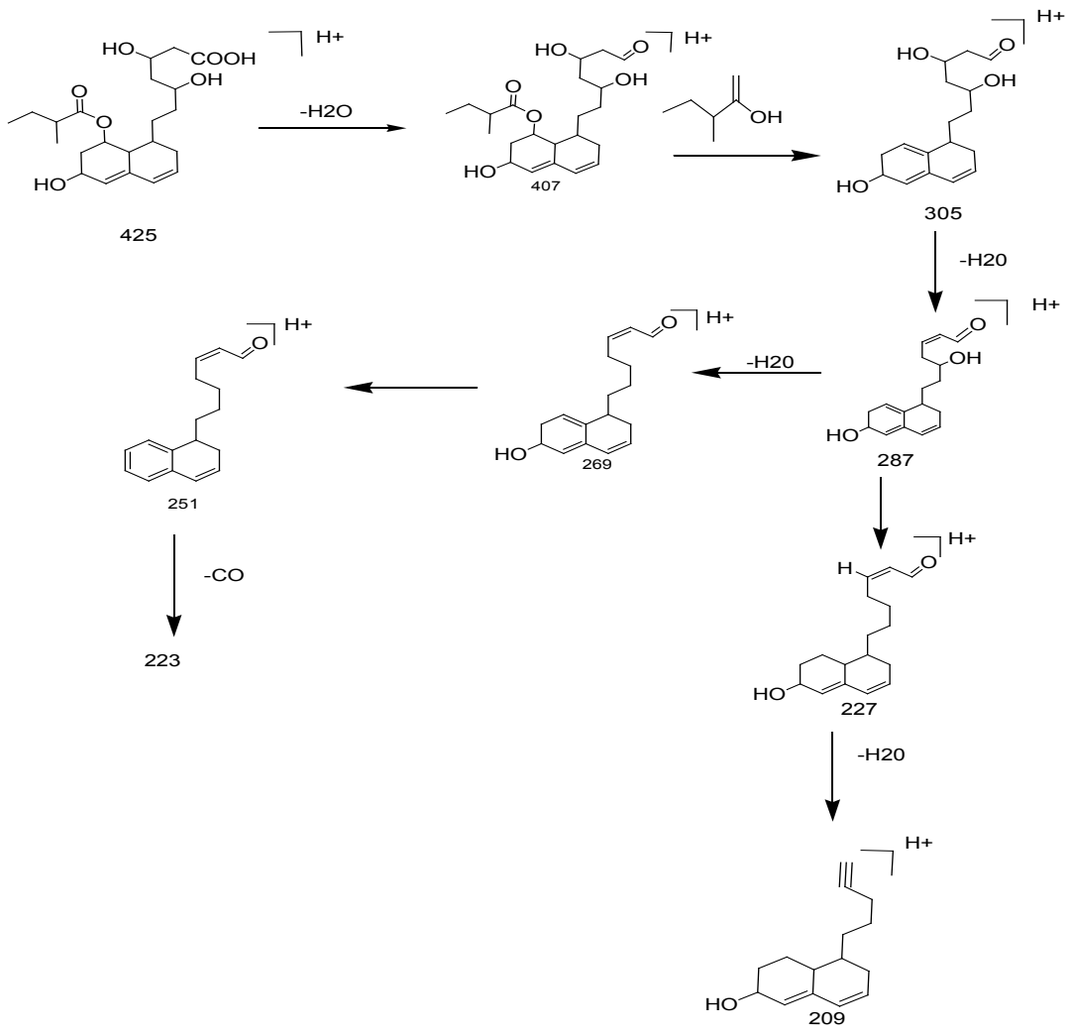


Table 2: Data on conditions of formation and retention times of degradation products and its molecular weight[M+H]+

DEGRADATION PRODUCT CODE	RETENTION TIME(MINUTES)	m/z value of [M+H]+ion	Condition(s) of formation
PRA	14.5	425	Drug peak
p-A	20.1	407	ACID
P-B	20.4	389	ACID
P-C	2.6	345	BASE
P-D	16.5	-	NEUTRAL
P-E	19.5	-	NEUTRAL



Scheme 1.Proposed Fragmentation Pathway of pravastatin [M+H]+ Degradation Products

Table 3: Calibration Curve Readings of Pravastatin

Conc.	±SD Peak Area (mA* ug/ml)					RSD (%)
(ug/ml)	A1	A2	A3	Av. A		
50	1176568	1169032	1170920	1172173	3921.219	0.334526
100	2376752	2370382	2368173	2371769	4454.507	0.187814
200	4932707	4723391	4743564	4799887	51466.6	0.405611
300	7075126	7081573	7040902	7065867	21859.31	0.309365
400	9366140	9363876	9366140	9365385	1307.121	0.013957
500	11660178	11650618	11671011	11660602	10203.12	0.087501

Fig 9:calibration curve of pravastatin

On the basis of calibration curve the range of linearity was found to be 50-500 µg/ml. for pravastatin.

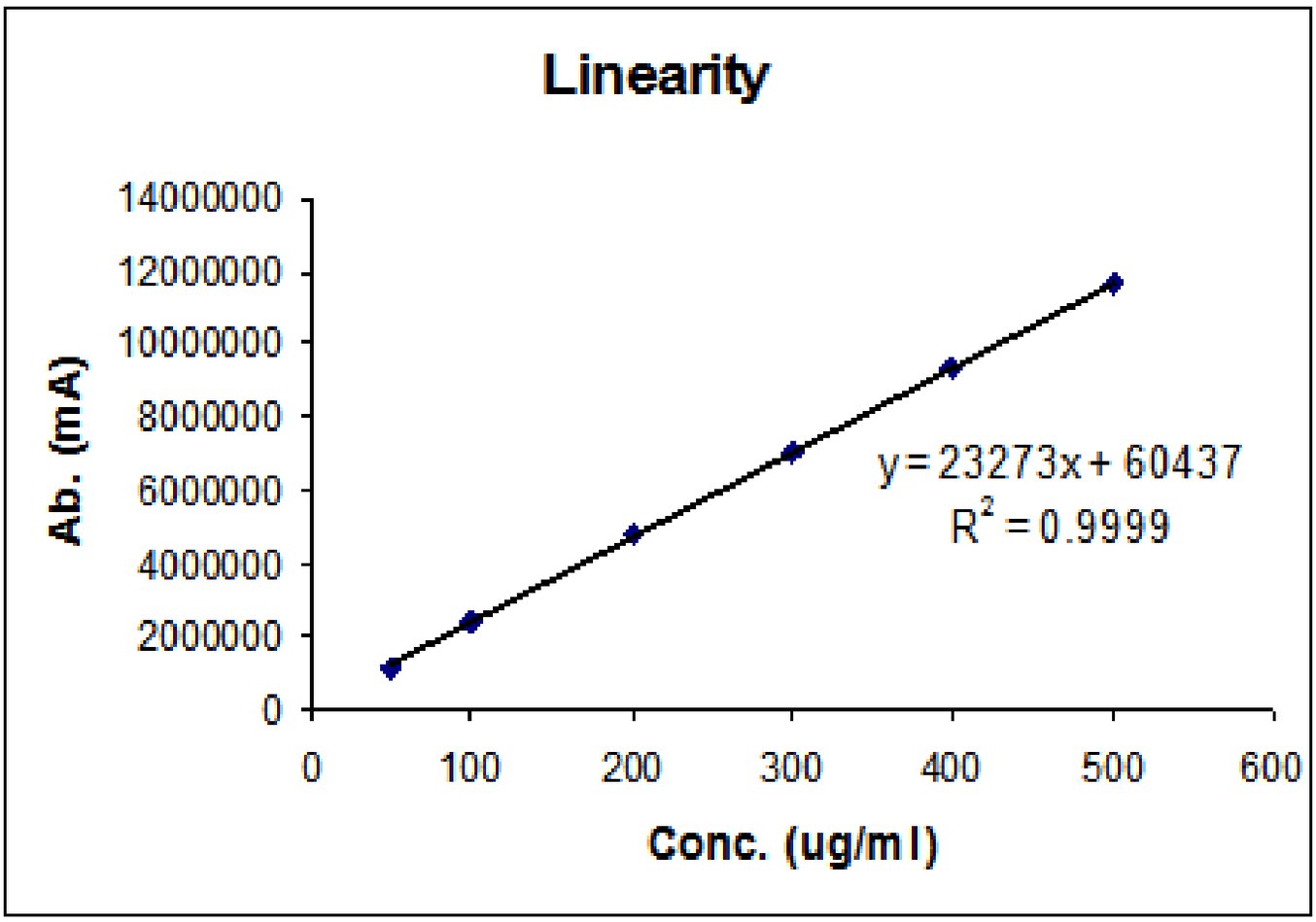


Table 5: Intra-day studies (n=3).

DRUG	Conc. (µg/ml)	Intra-day precision	Inter-day precision
		Found ± SD (µg/ml), RSD (%)	Found ± SD (µg/ml), RSD (%)
	100	99.31 ± 0.19, 0.19	98.45 ± 0.17, 0.17
	300	301.01 ± 0.93, 0.31	303.09 ± 0.63, 0.21
	500	498.43 ± 0.43, 0.08	498.45 ± 0.34, 0.06

Validation of Developed Stability Indicating Method:

The method was successfully validated as per ICH guidelineQ2 (R1): Validation of analytical procedures: text and methodology. The method was validated and parameters were Specificty, linearity, range, accuracy, precision, recovery, robustness.

Specificity

The specificity of the method was evaluated by determining peak purity for pravastatin in a mixture of stressed samples using the PDA detector in which purity angle was less than purity threshold. This indicates that peak was pure. The mass detector also showed an excellent mass purity for pravastatin.

Linearity:

Calibration curves were constructed by analysis of working standard solutions of pravastatin with at least 6 different concentrations in the range between 50-500 ug/ml each concentration was injected and measured in triplicate (n=3) standard calibration curves were plotted by taking area on y axis and nominal concentrations of drug on x axis. Results obtained from the regression analysis of data are given in table.

Accuracy:

Accuracy show that recovery of added drug, obtained from the difference between peak areas of unfortified and fortified samples, was acceptable at all the tested concentrations. Recovery was within the range of100 ± 2%, which indicates accuracy of method.

Table 4: Recovery Data for pravastatin.

Spiked conc. (ug/ml)	Calculated spiked conc. (ug/ml)				±SD	RSD %	Recovery %
	C1	C2	C3	Mean C			
150	150.3424	150.8938	150.8938	150.71	0.3184	0.2112	100.4733
250	250.8602	249.5712	249.5282	249.9865	0.76	0.30	99.99461
350	350.8032	350.7718	350.7718	350.7822	0.0182	0.0052	100.2235

Precision

The relative standard deviation (RSD) data obtained on analysis of the samples on the same day (n=3) and on consecutive days (n=3). As evident, the RSD values were <1% demonstrating that the method was sufficiently precise. Almost similar RT was obtained when the method was tried on two different columns showing intermediate precision of the method.

Table 6: Intermediate precision studies.

Parameters		RT IMT
Column	column –1	3.65
	column –2	3.87
Analyst	Analyst I	3.98
	Analyst II	3.45

Robustness

The sample was analyzed using proposed method after a deliberate change in detection wavelength for estimation by ±2 nm. Results are as shown in table 7.

Table 7: Robustness study for pravastatin

S. No.	Change in wavelength (± 2 nm)	% Estimation
1.	288	98.49
2.	290	98.62
3.	292	98.83
Mean		98.65
S.D.		0.1709
%R.S.D.		0.1733

CONCLUSION:

Developed a selective and validated stability indicating LC-MS method for pravastatin on C18 column, which could separate the drug and its degradations products formed under a variety of stress conditions. This method was then employed in LC-QTOF-ESI-MS/MS instrument to characterize the degradation products of pravastatin under hydrolytic, thermal and photolytic degradation conditions.

Indirectly the study highlights the benefit of the use of ICH Stress testing approach in establishment of complete degradation products of drugs.

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