Stability indicating HPTLC Method for the Estimation of Pitavastatin Calcium in presence of Acid induced Degradation Product.

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Abstract: Pitavastatin Ca is a HMG-CoA reductase inhibitor, blood cholesterol lowering agent. An approach for the stress degradation was successfully applied for the development of stability indicating HPTLC method for the determination of Pitavastatin Ca in the presence of its degradation product on the plates precoated with silica gel 60 F_{254}. The mobile phase used was Chloroform: Methanol in the ratio of 8:2 v/v. The drug showed considerable absorbance at 244nm. Stress testing of Pitavastatin Ca was carried out according to the international conference of harmonization (ICH) guideline Q1A (R2). The drug was subjected to acid, base, neutral hydrolysis, oxidation, thermal degradation and photolysis. There was no interference between the drug peak and peak of product of degradation; therefore the method was specific for the determination of Pitavastatin Ca in the presence of the degradation product. This system showed a peak for Pitavastatin Ca at Rf value of 0.44 ± 0.02. The data of linear regression analysis indicated a good linear relationship over the range of 200–1000 ng/band concentrations. The method was validated for robustness, precision and recovery. The LOD and LOQ were 16.23 and 49.20 ng/band, respectively. Under various stressed conditions, Pitavastatin Ca showed degradation product only under acidic hydrolysis at Rf value of 0.70 ± 0.02.
Keywords: Pitavastatin Calcium, high-performance thin layer chromatographic (HPTLC) method, Stability-Indicating Method.

Introduction

Pitavastatin (PIT), (3R, 5S, 6E)-7-[2-cyclopropyl-4-(pfluorophenyl)-3-quinolyl]-3, 5-dihydroxy-6-heptenoic acid, is a novel, fully synthetic statin, which has a more potent cholesterol-lowering action than other drugs in its class. PIT is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, used as the calcium salt in the treatment of hyperlipidemia and can reduce the risk of cardiovascular diseases in everyday medical practice [1,2], Based on the preclinical findings, PIT has been widely used as a first-line agent to lipid-modifying therapies [3].

Metabolism of Pitavastatin by the cytochrome P450 (CYP) system is minimal, principally through CYP 2C9, with little involvement of the CYP 3A4 isoenzyme, potentially reducing the risk of drug-drug interactions between pitavastatin and other drugs known to inhibit CYP enzymes [4,5].
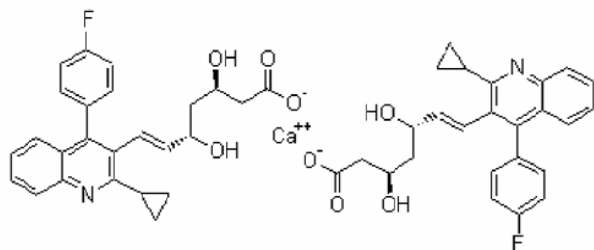
Fig.1: Structure of Pitavastatin Calcium

Literature search reveals following methods reported viz. Validated Stability Indicating UPLC Method[6,] Simple LC–MS/MS methods for simultaneous determination of Pitavastatin and its lactone metabolite in human plasma and urine[7,] Determination of pitavastatin in human plasma via HPLC-ESI-MS/MS[8,] Determination of two HMG-CoA reductase inhibitors, pravastatin and pitavastatin, in plasma samples via.LC-MS[9,] Simultaneous Determination of Pitavastatin Ca and Ezetimibe by Liquid Chromatography[10], HPLC Determination of Pitavastatin Calcium in Pharmaceutical Dosage Forms[11,12], Quantification of Pitavastatin Calcium in Pharmaceutical Dosage Forms by HPTLC[13,14], Novel spectrophotometric method for the assay of Pitavastatin calcium in pharmaceutical formulations[15,16], However, there is no stability-indicating HPTLC method reported so far for the quantification of Pitavastatin Ca in the presence of its degraded products. In the current work, we have developed and validated stability indicating HPTLC method for estimation of Pitavastatin Ca as per ICH guidelines[17]. Intensive stress studies are carried out and the method could resolve degradation product from the response of Pitavastatin Calcium.

The present active pharmaceutical Ingredient (API) stability test guideline Q1A (R2) issued by international conference on harmonization (ICH) [18] suggests that stress studies should be carried out on active pharmaceutical ingredient (API) to establish its inherent stability characteristics. The stability indicating method should ensure separation of degradation products.

Experimental

Standards and chemicals

Standard Pitavastatin Ca was procured from Ind-Swift Limited, Mumbai. Aluminum sheets precoated with silica gel (60 F_{254}, 20 cm × 20 cm with 250 µm layer thickness) were purchased from E-Merck, Darmstadt, Merck (Germany). Methanol (AR grade), Chloroform (AR grade) were purchased from S. D. fine chemical Laboratories, Mumbai. Hydrochloric acid (HCl), hydrogen peroxide (H_2O_2, 30% v/v) and sodium hydroxide (NaOH) were purchased from LOBA CHEMIE PVT. LTD. Mumbai.

Chromatographic state and instrumentation

Chromatographic separation of drug was performed on Aluminum plates precoated with silica gel 60 F_{254} , (10 cm × 10 cm with 250 µm layer thickness).Samples were applied on the plate as a band with 4 mm width using Camag 100 µL sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland). The mobile phase was composed of Chloroform: Methanol (8:2 v/v). 20 cm × 10 cm CAMAG twin trough glass chamber was used for linear ascending development of TLC plate under 15 min saturation conditions and 10 mL of organic solvent was used per run, migration distance was 90 mm. Densitometric scanning was performed using Camag TLC scanner 3 in the range of 400-200 nm, operated by winCATS software (Version 1.4.3, Camag), slit dimensions were 3.00 x 0.45 mm and Deuterium lamp was used as a radiation source.

Selection of detection wavelength

From the standard stock solution further dilutions were done using methanol and scanned over the range of 200 – 400 nm and the spectra was obtained. It was observed that the drug showed considerable absorbance at 244 nm.
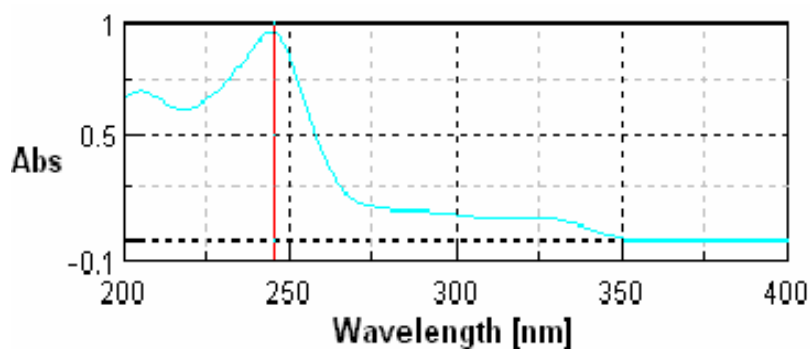
Fig.2: UV Spectra Pitavastatin Ca (10µg/ml)

Preparation of Standard stock solution

Standard stock solution of Pitavastatin Ca was prepared by dissolving 10 mg of drug in 10 ml of methanol to get concentration of 1000µg/ml. From the standard stock solution, working standard solution was prepared to contain 100µg/ml of Pitavastatin Ca.

Stress degradation study of bulk drug

Stress degradation studies were carried under condition of acid/ base/ neutral hydrolysis, oxidation, dry heat and photolysis. For each study, samples were prepared as follows

1. The blank subjected to stress in the same manner as the drug solution

2. Working standard solution of Pitavastatin Ca subjected to stress condition.

Dry heat and photolytic degradation were carried out in solid state. 6µL of the resultant solution was then applied at TLC plate and densitogram was developed

Degradation under alkali catalyzed hydrolytic condition

To 1 mL of 1000 µg.mL^{-1}solution of Pitavastatin Ca, 1mL of 0.1 N NaOH was added. The volume was made up to 10 mL with methanol. The above solution was kept for 2 hours at room temperature.

Degradation under acid catalyzed hydrolytic condition

To 1 mL of 1000 µg.mL^{-1}solution of Pitavastatin, 1mL of 0.1N HCL was added. The volume was made upto 10 mL with methanol. The above solution was kept for 30minutes at room temperature.
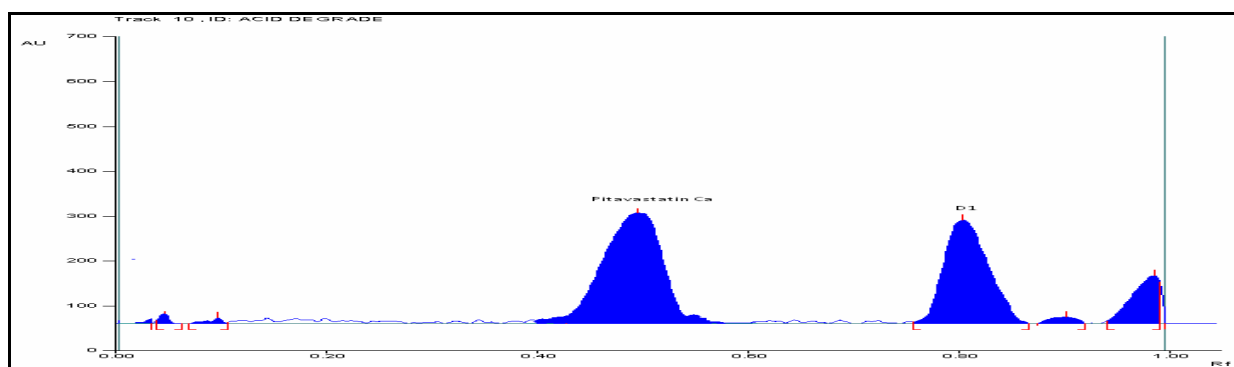

Fig.3: Representative Densitogram of acid induced degradation of Pitavastatin Ca, 600ng/band, D1= Degradation Product
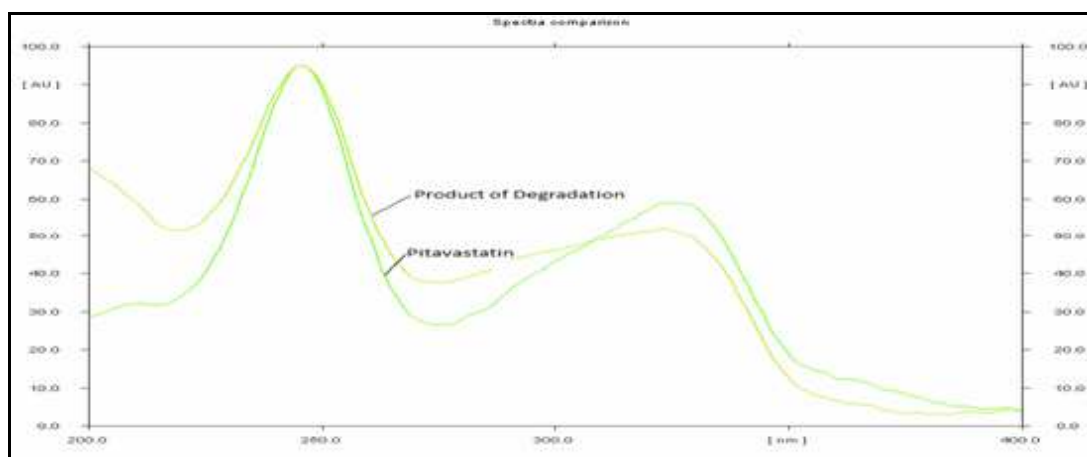
Fig.4: Overlain UV spectra of Pitavastatin Ca and product formed after acid catalyzed degradation

Degradation under neutral hydrolytic condition

To 1 mL of 1000 µg.mL^{-1}solution of Pitavastatin Ca, 1mL of distilled water was added. The volume was made upto 10 mL with methanol. The above solution was kept for 30minutes at room temperature.

Degradation under oxidative condition

To 1 mL of 1000 µg.mL^{-1}solution of Pitavastatin Ca, 1 mL of 3% H_2O_2 was added. The volume was made upto 10 mL with methanol. The above solution was kept for 2 hours at room temperature.

Degradation under dry heat

Dry heat studies were performed by keeping drug sample in oven (80^0 C) for a period of 6 hours.

Photo-degradation studies

The photo degradation study of the drug was carried out by exposing the drug to UV light providing illumination of NLT 200 watt hr/m^2, after UV light exposure drug color was changed and showed higher degradation, hence fresh drug was exposed to cool white fluorescence light of NLT 1.2million Lux-Hrs.

Assay of Tablet Formulation

Twenty tablets (2mg Pitavastatin Ca/tablet) were accurately weighed and powdered. From the powder, an amount equivalent to 5mg of Pitavastatin Ca was accurately weighed and transferred to 25 mL volumetric flask. Methanol was added in the volumetric flask and the resultant mixture was sonicated for 10 min at room temperature to disperse the powder completely. The resultant mixture was centrifuged and then filtered through 125 mm Whatman filter to get the clear solution of Pitavastatin Ca (200µg/ml). 2µL of this solution were applied on 5 X 10 cm pre-coated TLC plate as a band of length 4 mm for the assay of Pitavastatin Ca. The percentage assay was calculated upon extrapolation from standard curve.
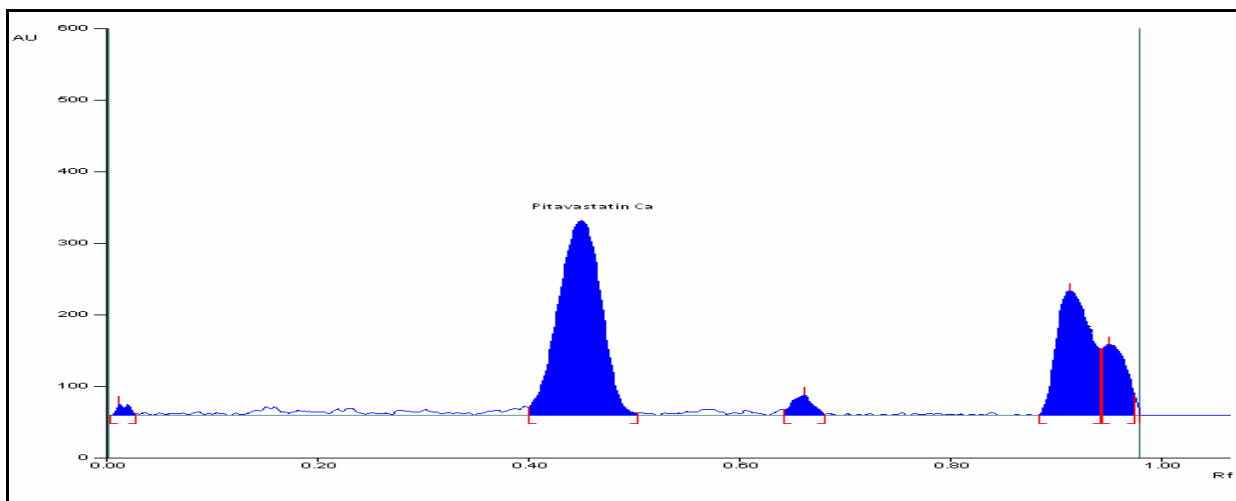
Fig.5: Representative Densitogram of Pitavastatin Ca tablet 600ng/band

Table 1: Summarized results of stress degradation of Pitavastatin Ca

Stress Degradation Condition	Percent recovery (%)	Percent degraded (%)	Rf of degradation product	Peak purity	
				r(s,m)	r(m,e)
Initial	100	-	-	0.9998	0.9992
Base (0.1 N NaOH, kept for 2 hrs)	79.67	20.33	-	0.9975	0.9986
Acid (0.1 N HCl, Kept for 30 minutes)	85.82	14.18	0.70	0.9996	0.9998
H_2O_2 3% (kept for 2hrs)	96.6	3.4	-	0.9987	0.9984
Water (Kept for 30 minutes)	96.7	3.3	-	0.9992	0.9984
Heat dry (80^{0}C, 6 hrs)	73.97	26.03	-	0.9980	0.9981
Photo stability (UV, 200 watt hrs/square meter	20.65	79.35	-	0.9997	0.9994
Florescence , 1.2 million Lux. Hrs)	76.57	23.43		0.9998	0.9995

Result

Validation of method

The method validation was done as described by the ICH guidelines.

Calibration curve of Pitavastatin Ca

Stock solution of Pitavastatin Ca (1 mg mL-1) was prepared in methanol. This solution as further used to prepare range of solution containing five different concentrations. Five replicates per concentration were spotted. The linearity (relationship between peak area and concentration) was determined by analyzing five solutions over the concentration range of 200-1000 ng/band for Pitavastatin Ca. Prepared solutions were stored at lower temparature until use. The spotted plate was developed as mentioned in previous section. Linearity equation and Regression coefficient was found to be y = 8.012x + 2159 and r² = 0.990 respectively.
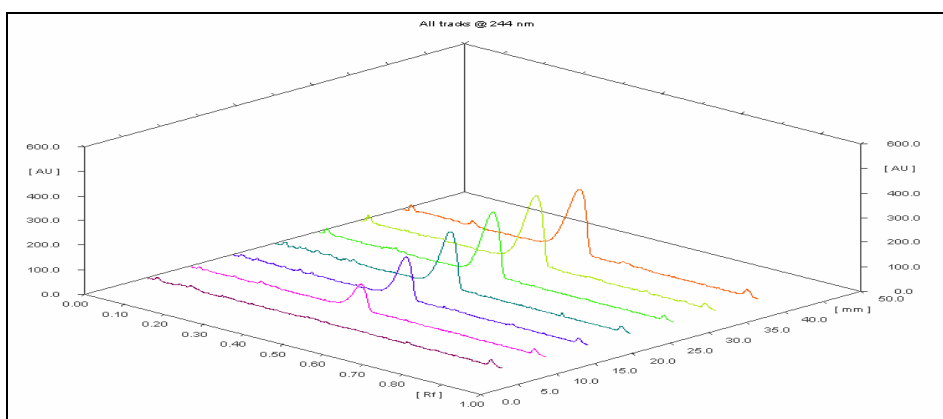
Fig.6: Densitogram of linearity of Pitavastatin Ca (200-1000 ng/band)

The method sensitivity was estimated with respect to limit of detection (LOD), limit of quantification (LOQ) and correlation coefficient. Working solutions containing 200–1000 ng of Pitavastatin Ca were spotted on TLC aluminum plates. In order to evaluate LOD and LOQ, calibration curve was used and were evaluated by using equation: LOD = 3.3 δ/S, LOQ = 10 δ/S respectively, where, S = the slope of the calibration curve, δ = standard deviation of regression line. The LOD and LOQ were estimated as 3and 10 times of the noise level, correspondingly. The Intra- and inter-day variation for the estimation of Pitavastatin Ca was evaluated for method precision. It was achieved by using concentration level of 600ng spot-1. Repeated analyses were carried out in a same day for intra-day analysis while the same practice was repeated next day for inter-day analysis. Intra- and inter-day analyses were performed to check the repeatability and reproducibility of the method, respectively and results were statistically evaluated in terms of % R.S.D. In order to check the robustness, following parameters were intentionally changed within the range of ± 2% at 200, 400 and 600 ng/ spot concentration level; mobile phase composition, chamber saturation time, time from spotting to development, time from development to scanning. The accuracy of the method was assessed by adding standard drug to sample at three different levels 80, 100 and 120 %. Basic concentration of sample chosen was 600ng/band of Pitavastatin Ca standard solution.

Table 2: Summary of validation study

Sr.No.	Validation parameters	Pitavastatin Ca
1.	Linearity Equation (r2) Range	Y=8.012x+1986 $R^2 = 0.99$ 200- 1000ng/band
2.	Precision (% RSD) Interday Intraday	1.077% 0.74%
3.	Accuracy	% Recovery
	80	99.48
	100	102.3
	120	101.9
4.	Limit of Detection	16.23ng/band
5.	Limit of Quantitation	49.20 ng/band
6.	Specificity	Specific
7.	Robustness	Robust

Characterization of acid induced degradation product by LCMS

During stress degradation studies, since a well resolved product peak was observed under acid induced degradation conditions, the objective of the next part of the study was to optimize the conditions so as to obtain complete conversion of Pitavastatin to degradation product. The next steps planned were isolation, purification and characterization of the product. Acid induced degradation was observed to commence within 15 min and HPTLC studies over a prolonged period indicated absence of Pitavastatin after about 24 hrs.
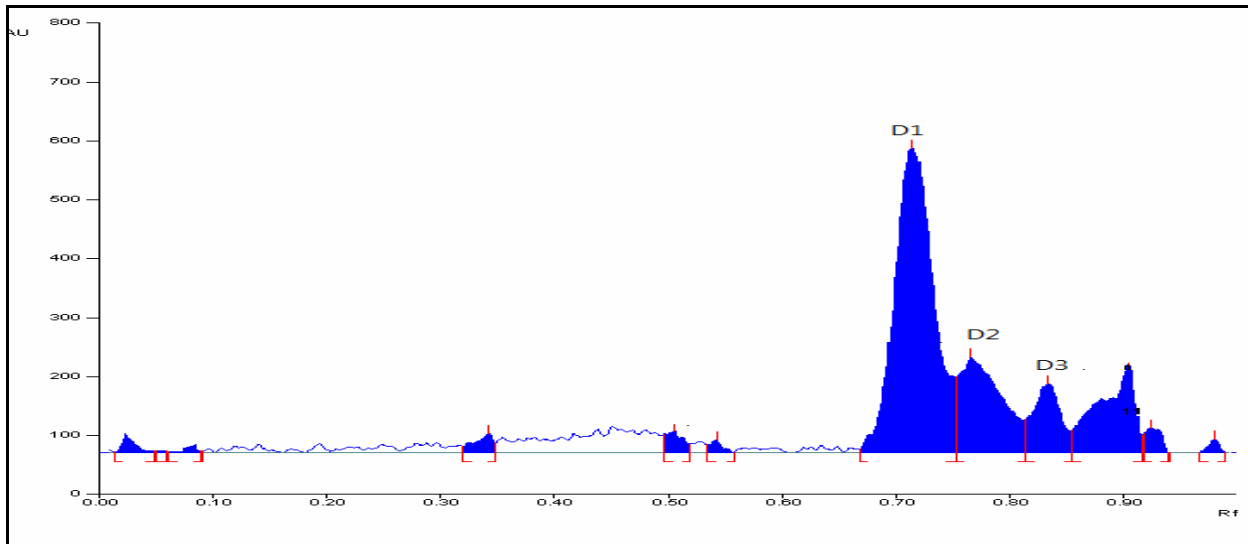
Fig.7: Representative Densitogram of acid induced degradation of Pitavastatin Ca (24 hrs exposure), 600ng/band, D1, D2, D3= Degradation Products
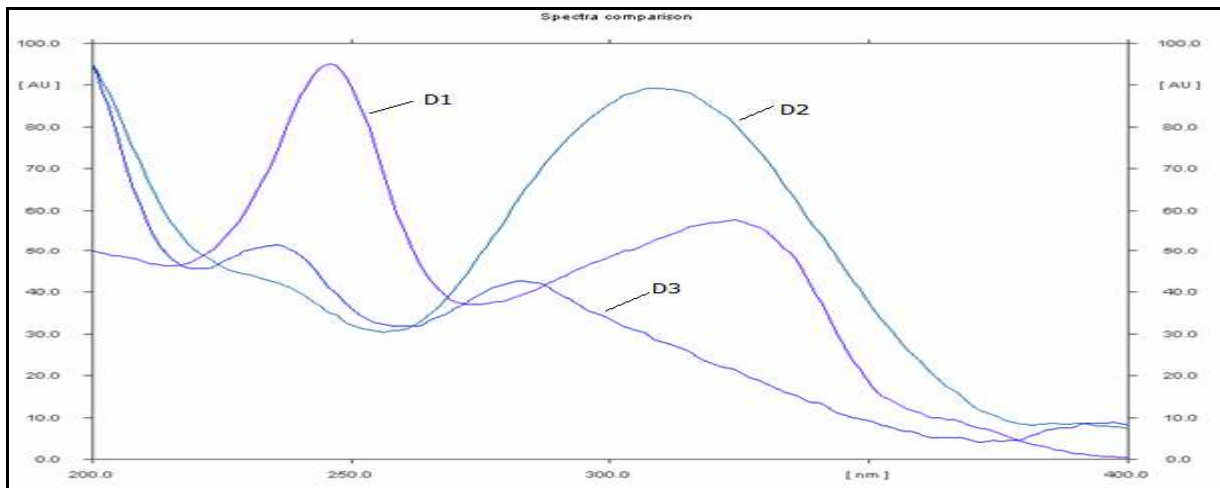

Fig.8: Overlain UV spectra of acid induced degradation products of Pitavastatin Ca

Since no single product was achieved, it became necessary to resolve the products on HPLC-MS system with a view to monitor the mass of the product which would help in characterizing it. Hence chromatographic conditions were developed using HPLC system with Diode array detector.

Chromatographic Conditions:

The mobile phase consisting of acetonitrile: water in the ratio of 80:20 *v/v*, was filtered through 0.45μ membrane filter, sonicated and was pumped from the solvent reservoir. Separation was achieved on C_{18} column. The flow rate of mobile phase was maintained at 1ml/min and the response was monitored between 200 to 400 nm with a run time of 20 min. The volume of injection loop was 20μl. The column and the HPLC systems were kept at ambient temperature.

Instrument: Jasco HPLC system comprising: Model PU 2080 Plus pump, Rheodyne sample injection port, Grace C_{18} Columns, MD 2010 PDA detector, Borwin- PDA software (version 1.5)
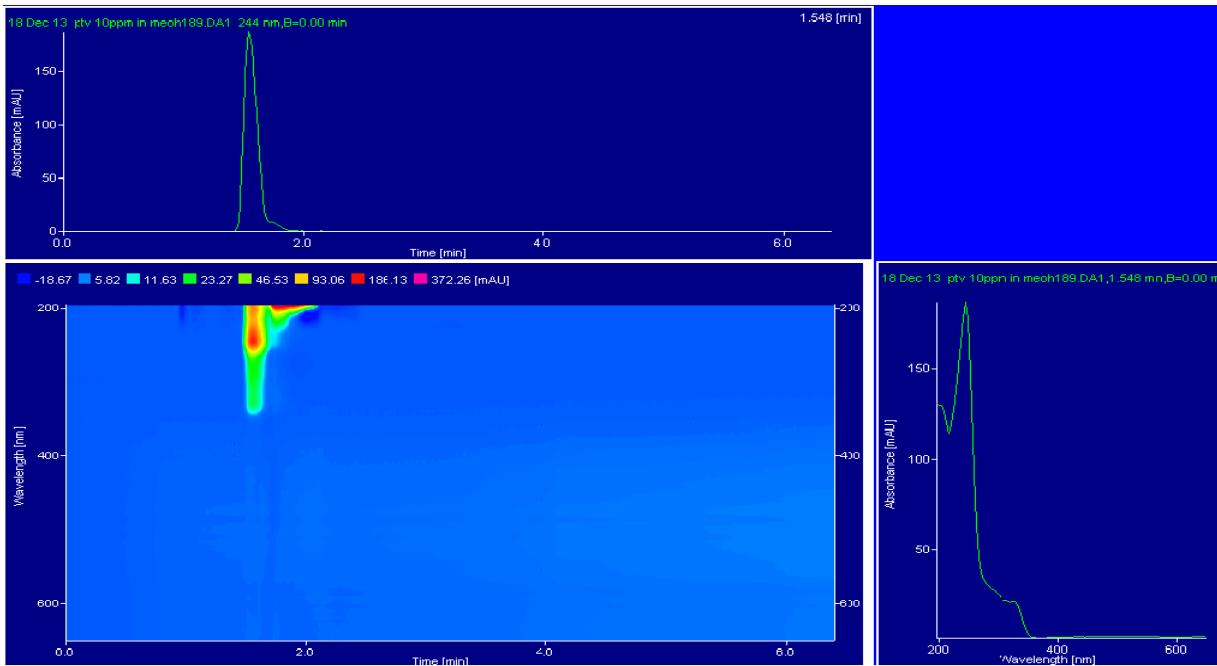
Fig.10: Chromatogram of Pitavastatin Ca 10µg/ml

The retention time of Pitavastatin Ca was 1.548 min. Its UV spectrum showed λ = 244 nm which matches its reported λ value.
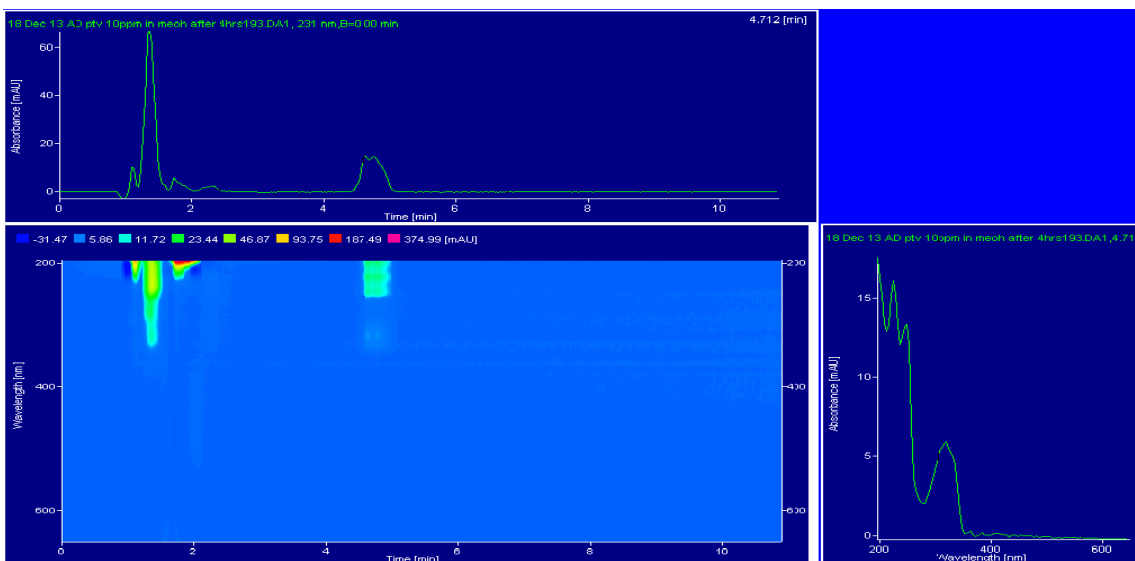

Fig.11: Chromatogram of acid induced Pitavastatin Ca 10µg/ml after 4hrs

The peak area of Pitavastatin Ca was significantly reduced after 4 hrs. as is observed in the above chromatogram (Rt = 1.548 min). The peak for product of degradation is observed at 4.712 min. Its UV spectrum showed λ = 221 nm, 243 nm and 315 nm.

This sample (exposed to acid conditions for 4 hrs) was also analysed simultaneously on LC-MS system at National Chemical Laboratory, Pune. The details of chromatographic conditions are as follows :

1. Make of LC-MS INSTRUMENT : Thermo Fischer Scientific

2. Model : Q Exactive(LC-MS system)

3. Ionisation technique : Electrospray Ionization

4. Column used: Hypersil Gold 150x4.6mm, 8um

   Pitavastatin elutes at 3.56 min and the degradation product at 4.54 min. The mass for Pitavastatin Ca was 422 units which matches the reported value.
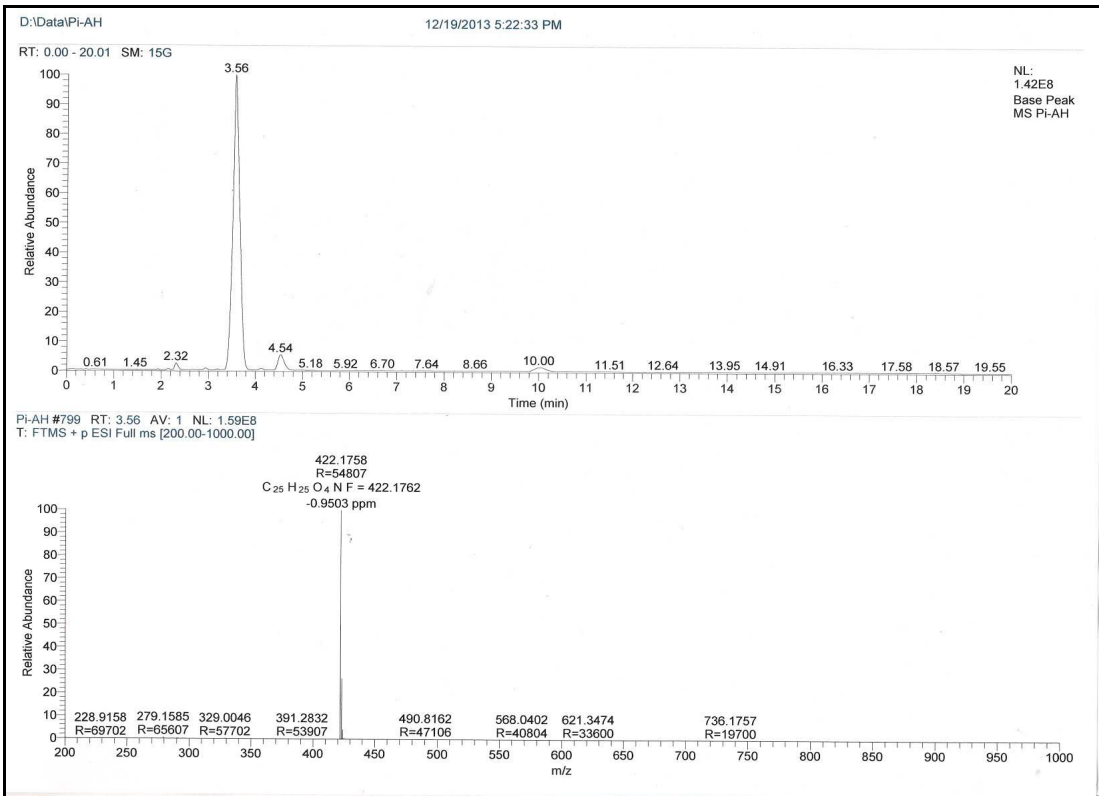
Whereas for the product peak at 4.54 min, the mass is 404.
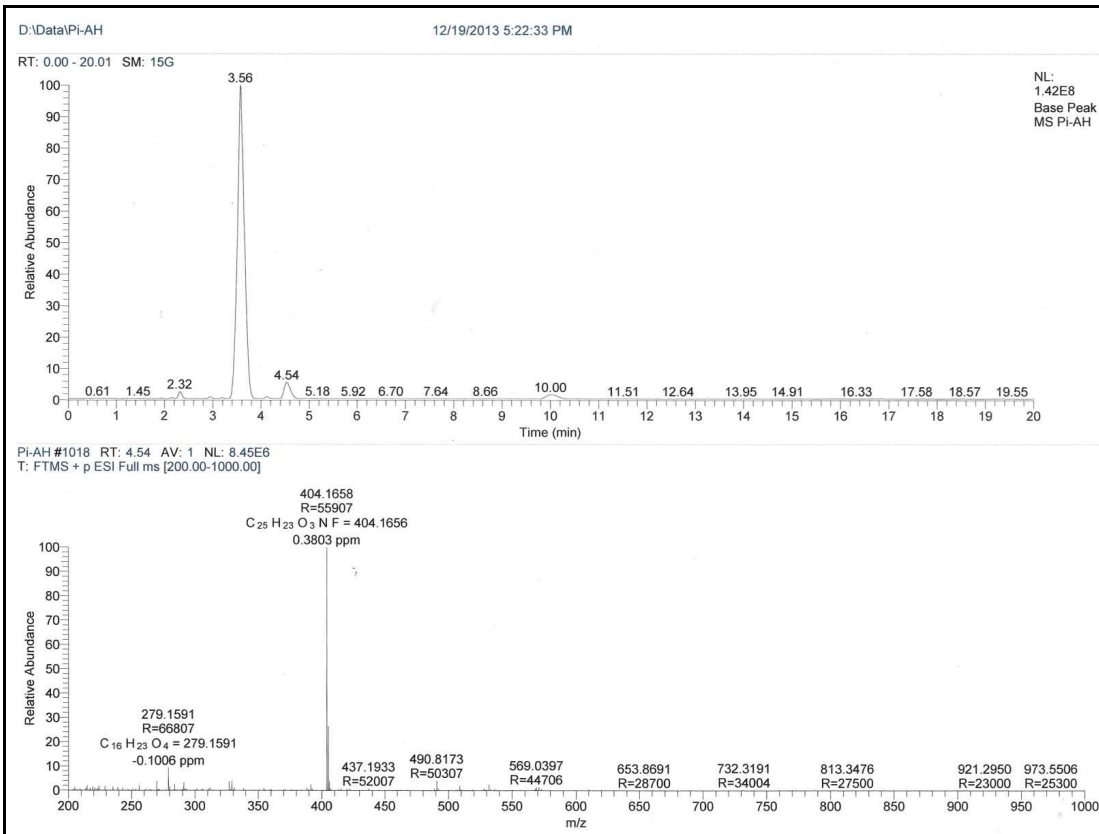
Results of LCMS study

The mass difference of 18 units is obtained between Pitavastatin Ca and the product, indicating loss of one water molecule. The product shows λ of 315 nm which may be due to increase in the length of conjugation during dehydration step.

Acknowledgements:

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