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*Research Article*

# SIMULTANEOUS DETERMINATION OF ATORVASTATIN, EZETIMIBE AND FENOFIBRATE IN TABLETS BY PLANAR CHROMATOGRAPHY WITH CONFORMATION BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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**ABSTRACT**

A new high-throughput high-performance thin-layer chromatographic- mass spectrometry (HPTLC-MS) method was developed to separate and quantify atorvastatin (AT), ezetamibe (EZ) and fenofibrate (FN) in solid pharmaceutical formulations. Separation was performed on silica gel $60F_{254}$ plates using a saturated mixture of toluene: chloroform: ethyl acetate: acetic acid (5.0:3.0:1.6:0.4 v/v) as mobile phase. After chromatography, densitometric detection was carried out by measuring the UV-absorbance at 254 nm for AT, EZ and FN. The $R_f$ values were (0.46±0.01), (0.57±0.01) and (0.84±0.01) for AT, EZ and FN respectively. The method exhibited good linearity over a dynamic range of 50-350 ng/band for AT, 50-350 ng/band for EZ and 1000-5000 ng/band for FN. Recoveries were between 98.98% and 101.26% at three different concentration levels with an intermediate precision (RSD) of the three compounds ranging from 0.3% to 1.5%. The repeatability (RSD) of all the substances was between 0.2% and 1.6%. The validated lowest limit of detection (LOD) was 3.69 ng /band, 18.31 ng /band and 12.22 ng /band whereas lowest limit of quantification (LOQ) was 37.49 ng /band, 61.05 ng /band and 40.67 ng /band for AT, EZ and FN respectively. MS confirmation was accomplished by ion trap mass spectrometry in positive electrospray ionization full scan mode for EZ and FN and in negative mode for AT. The HPTLC-MS facilitated the separation and determination of the compounds understudy in one analytical run with reduced analysis cost, good chromatographic selectivity and proves to be a reliable alternative for routine analysis of pharmaceutical formulations.

**Keywords:** Atorvastatin, Ezetimibe, Fenofibrate, HPTLC-MS.

## 1. INTRODUCTION

Atorvastatin calcium (AT) is the calcium salt (2:1) trihydrate of [R-(R*, R*)]-2-(4-fluorophenyl)-b, d-dihydroxy-5-(1-methyl ethyl) - 3- phenyl4 [(phenylamino) carbonyl] lH pyrrole- heptanoic acid. It is an inhibitor of 3-hydroxy- 3- methyl glutaryl- coenzyme A (HMGCoA) reductase. AT is the most efficacious of the currently available HMG-CoA reductase inhibitors in terms of lowering plasma cholesterol levels by suppressing the hepatic production of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) cholesterol [1]. Ezetimibe (EZ) is [(3*R*, 4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone]. EZ[2, 3] inhibits the absorption of cholesterol, decreasing the delivery of intestinal cholesterol to the liver. It is a selective cholesterol absorption inhibitor used in the treatment of primary hypercholesterolemia. It inhibits the absorption

of biliary and dietary cholesterol from small intestine without affecting absorption of fat soluble vitamins, triglycerides and bile acids. After oral administration, EZ is metabolized into its glucuronide in the liver and small intestine, which is also active in prevention of absorption of cholesterol. EZ does not have significant pharmacokinetic interactions with other lipid lowering drugs as it does not influence the activity of cyotochrome P450 [4]. Fenofibrate (FN), 1-methylethyl 2-[4-(4-chlorobenzoyl) phe-noxy]-2-methylpropanoate, is used as antihyperlipidemic drug[5]. FN activates lipoprotein lipase, which reduces triglycerides and increases HDL cholesterol. It exerts a variable but generally modest LDL cholesterol- lowering effect[6].

High-performance thin-layer chromatography (HPTLC) is very fast and convenient method to separate samples. In the past unknown substances were scraped off from the TLC/HPTLC plate, eluted into a tube and transferred into the mass spectrometer. Now a very convenient and universal TLC-MS Interface is available which can semi-automatically extract zones of interest and direct them online into any brand of HPLC-MS system. The interface is quickly and easily connected (by two fittings) to any LC coupled mass spectrometer without adjustments or mass spectrometer modifications. Questioned substances are directly extracted from a TLC/HPTLC plate and sensitive mass spectrometric signals are obtained within a minute per substance zone. The interface extracts the complete substance zone with its depth profile and thus allows detections comparable to HPLC down to the pg/zone range. The interface has been proven to be one of the most reliable and versatile interfaces for TLC/HPTLC-MS coupling.

The TLC-MS interface (CAMAG) extraction head has two connections on the topside, one inlet and one outlet. On the bottom surface there is a cutting edge seal with a height of about the thickness of the platelayer. When the extraction head is pressed onto a foil or glass plate the cutting edge seal cuts into the adsorption layer and creates a leakage free seal. In bypass position (**Figure 1(a))** solvent flows directly to MS-System. With the help of a laser crosshair, the extractor head is easily positioned on a selected zone position. After lowering the piston, when the valve is switched to extract position (**Figure 1(b)).** The solvent passes through the extraction head, elutes the sample and transports it through the integrated frit to the MS-System.

Literature survey revealed that there are many methods like UV-Spectrophotometric, high performance liquid chromatography (HPLC), HPTLC, liquid chromatography-mass spectrometry (LC-MS), ultra performance liquid chromatography (UPLC), gas chromatography-mass spectrometry (GC-MS) and FT-Raman Spectroscopy[7-32] for individual and combined determination of AT, EZ and FN. However, there is no HPTLC-MS method reported for simultaneous estimation of AT, EZ and FN in their combined dosage form. Therefore the objective of the present work is to develop a simple HPTLC method which could accomplish the simultaneous separation and detection of AT, EZ and FN in combined tablet dosage form.

## 2 EXPERIMENTAL

### 2.1 MATERIALS

Analytically pure samples of AT, EZ and FN were kind gift from Sipra labs, Hyderabad, India, and were used without further purification. The pharmaceutical dosage form used in this study was Lorlip EZ tablets (Unichem laboratories Ltd, Mumbai, India) labeled to contain 10 mg of AT, 10 mg of EZ and 200 mg FN and purchased from a local pharmacy. All chemicals and reagents used were of chromatographic grade and purchased from Merck, India.

### 2.2 Standard Solution

Standard stock solutions of AT, EZ and FN were prepared separately (1000 ng/µL) stored at 4°C until the analysis is performed. From the standard stock solutions, ten different concentration levels of mixed standard solutions were prepared by diluting suitable volumes with methanol in appropriate volumetric flasks.

### 2.3 Sample Solution

The tablets (10) were weighed accurately and finely powdered. A quantity of powder equivalent to (10 mg AT 10 mg EZ and 200 mg FN) was weighed and transferred to a 100 mL volumetric flask containing about 50 mL methanol. The solution was ultrasonicated for 5 min, filtered through a Whatman No. 41 and subsequently made up to the volume with methanol.

### 2.4 HPTLC

TLC was performed on 10 ×10 cm HPTLC plates pre coated with 60F–254 (With 0.25 mm thickness; Merck, Darmstadt, Germany) and the plates were washed with methanol before use. The sample and mixed standard solutions were applied as bands of 4 mm wide and 10 mm apart using Linomat 5 sample applicator (Muttenz, Switzerland, supplied by Anchrom technologists, Mumbai) equipped with a 100 µL syringe. A constant application of 6 µL/sec

was used. The mobile phase consisted of a saturated mixture of toluene: chloroform: ethyl acetate: acetic acid (5.0:3.0:1.6:0.4 v/v), chromatography was performed using 10 mL of mobile phase in a 10 × 10 cm twin trough glass chamber with linear ascending development. The optimized chamber saturation time for mobile phase was 20 min at room temperature with a chromatographic run length of 8.5 cm. Subsequent to the development, the TLC plates were dried in a current of air with the help of a dryer in wooden chamber with adequate ventilation. Densitometric scanning was performed with Camag TLC scanner III in the absorbance–reflectance mode at 254 nm with a slit dimension of 3.0 mm × 0.45 mm and a scanning speed of 20 mm/sec. All the instruments were operated by WINCATS software (V 143 CAMAG) resident in the system. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 200 and 400 nm and the concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Further, for digital documentation, the digistore 2 documentation system (CAMAG) consisting of illuminator, Reprostar 3 and digital camera power shot G2 (Canon, Tokyo, Japan) was used.

**2.5 HPTLC-MS**

After scanning the plate, the exact position of the separated bands were marked with a pencil. With the help of a laser crosshair, the extractor head could be easily positioned on a selected zone position. After lowering the piston, the valve switched to extraction position. Now, the solvent pumped with a flow rate of 0.1 mL/min through the extraction head, eluted the sample and transported the same through the integrated frit to the MS–system. The elution was accomplished as circular zones of 4mm diameter from the plate in one minute and methanol was used as the extraction solvent. The following MS parameters were optimized in the $ESI^+$ mode: source temperature 300°C, capillary voltage 1.25 kV, HV lens 3.4 kV, capillary current 17.15 nA, skimmer voltage 44 V, nitrogen as nebulizing gas (30 Psi) and as drying gas at a flow rate of 7.0 L/min.

**2.6 Method Validation**

The method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and specificity as per the International Conference on Harmonization (ICH) guidelines[33].

***2.6.1* Linearity**

From the mixed standard solutions 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 µL were spotted separately on HPTLC plate to obtain a final concentration of 50-350 ng /band of AT, 50-350 ng /band of EZ and 1000-5000 ng /band of FN. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves.

***2.6.2* Precision**

The precision of the method was studied with the help of repeatability and intermediate precision analysis. Repeatability was performed by analyzing three different concentrations of AT, EZ and FN for six times on the same day while the intermediate precision of the method was checked by separation studies on three different days.

***2.6.3* Accuracy**

Accuracy of the proposed method was determined by spiking pre-analyzed samples with known amounts of standard drug solution. Standard AT, EZ and FN corresponding to 50%, 100% and 150% have been added to the preanalysed tablet sample solution. Three determinations were performed at each level of recovery.

***2.6.4* Specificity**

The specificity of the method was determined by analyzing standard drug and test samples. The peak for AT, EZ and FN test sample were confirmed by comparing the $R_f$ value and the spectrum of the peak with that of the standard. The peak purity of AT, EZ and FN was determined by comparing the spectrum at three different regions of the spot *i.e.* peak start (S), peak apex (M) and the peak end (E). Additionally, peak identity and purity was confirmed by MS.

**3 RESULTS AND DISCUSSIONS**

**3.1 Method optimization and wavelength selection**

Mobile phase optimization plays a major role in accomplishing the desired separation profiles. Thus, different mobile phases containing various ratios of toluene, chloroform, ethyl acetate and acetic acid were examined. However, only toluene: chloroform: ethyl acetate: acetic acid (5.0:3.0:1.6:0.4 v/v) offered the best separations with well resolved zones but ethyl acetate content is always crucial and any change in the ratio presented would drastically alter the chromatographic profiles. On the other hand, glacial acetic acid helped to sharpen the peak. Chamber saturation for 20 min with the mobile phase

facilitated the best chromatographic behavior with well defined bands with selective retention factors for atorvastatin (0.46±0.01), ezetimibe (0.57±0.01) and fenofibrate (0.84±0.01) respectively as depicted in the chromatogram **(Figure 2)** and also as a video image (**Figure 3)**. After chromatography, the optimum wavelength for detection and quantification was 254 nm when scanned at 254 nm and confirmed by UV spectra of AT, EZ and FN (**Figure 4)**.

### 3.2 Method Validation

#### 3.2.1 Linearity

The analyte response was linear ($r^2$ = 0.999 for AT, 0.998 EZ and 0.999 for FN) over the concentration range between 50**-**350 ng /band for AT, 50-350 ng /band EZ and 1000-5000 ng /band for FN the results were shown in **Table 1**. Calibration curves were constructed as described and expressed acceptable accuracy and precision over a wide concentration range. The LOD was found to be 3.69 ng /band for AT, 18.31 ng /band for EZ and 12.22 ng /band for FN. The LOQ for AT, EZ and FN were found to be 37.49 ng /band, 61.05 ng /band and 40.67 ng /band respectively as depicted in **Table 1**.

#### 3.2.2 Precision

Intra- and inter-day variation in estimation of AT, EZ, and FN (**Table 2**) showed that the RSD was <2% during the analysis. These low values of RSD show that the precision of the method is good.

#### 3.2.3 Accuracy

The study of accuracy reveals the positive or negative influence of additives that are usually present in the dosage forms on the quantification parameters. The recovery study data presented in **Table 3** indicates that the accuracy of the quantification of AT, EZ, and FN was more than 98%.

#### 3.2.4 Specificity

The compound identification was established by $R_f$ and confirmed by a comparison with UV spectra of the standard sample (**Figure 4)** further, the peak purity was also assumed using the UV range. Also the adequate selectivity and separation power was given by MS. Through the elution-based interface, sample bands were directly eluted from the plate into the mass spectrometer without any post-chromatographic protocol. The mass spectrum acquired in the m/z range from 0 to 800, show at the $ESI^+$ mass spectra of EZ and FN in which the $[M+Na]^+$ ions were detected at m/z 431.8 (**Figure 5(a))** and m/z 382.8 (**Figure 5(b))** and $[M-H]^-$ ion was detected at m/z 557.3 (**Figure 5(c))** for AT. Thus, the identity of the bands and an adequate chromatographic selectivity were confirmed.

### 3.3 Analysis of a formulation

The results obtained for the amount of AT, EZ and FN in tablets as against the label claims were in good agreement suggesting that there is no interference from any of the excipients which are generally present in tablets. The drug content was found to be 99.05±0.13 % when analyzed in triplicate. It may therefore be inferred that AT, EZ and FN had no quantifiable additional impurities in the marketed formulations analyzed by use of this method. The good performance of the method was indicative because of its suitability for routine analysis of AT, EZ and FN in pharmaceutical dosage forms.

## 4 CONCLUSION

With the onset of stringent quality regulations for globalization, pharmaceutical industry now requires sensitive and reliable analytical methods to ensure the product quality. In this context HPTLC-MS is a reliable alternative and complementary to other chromatographic methods. Therefore, this simple, rapid and high through put HPTLC-MS method for the separation and determination of AT, EZ and FN in formulations has been developed, validated and the potential utility of the same has been discussed. The presented HPTLC-MS method with an extraction head facilitated a quantitative extractability of all the drugs from silica gel phases with required analytical response and adequate sensitivity. The method also facilitated a simultaneous separation of all the three compounds on a single silica gel plate with a simple mobile phase. The current study showed that the new hyphenation technique could be successfully employed not only for separation of drug molecules but also for the drug impurity profiling as well where the detection could be accomplished with comparable sensitives as in other methods like HPLC-MS. This new convenient and universal HPTLC- MS interface that facilitated complete substance zone extraction with its depth profile allows detections comparable to other methods.

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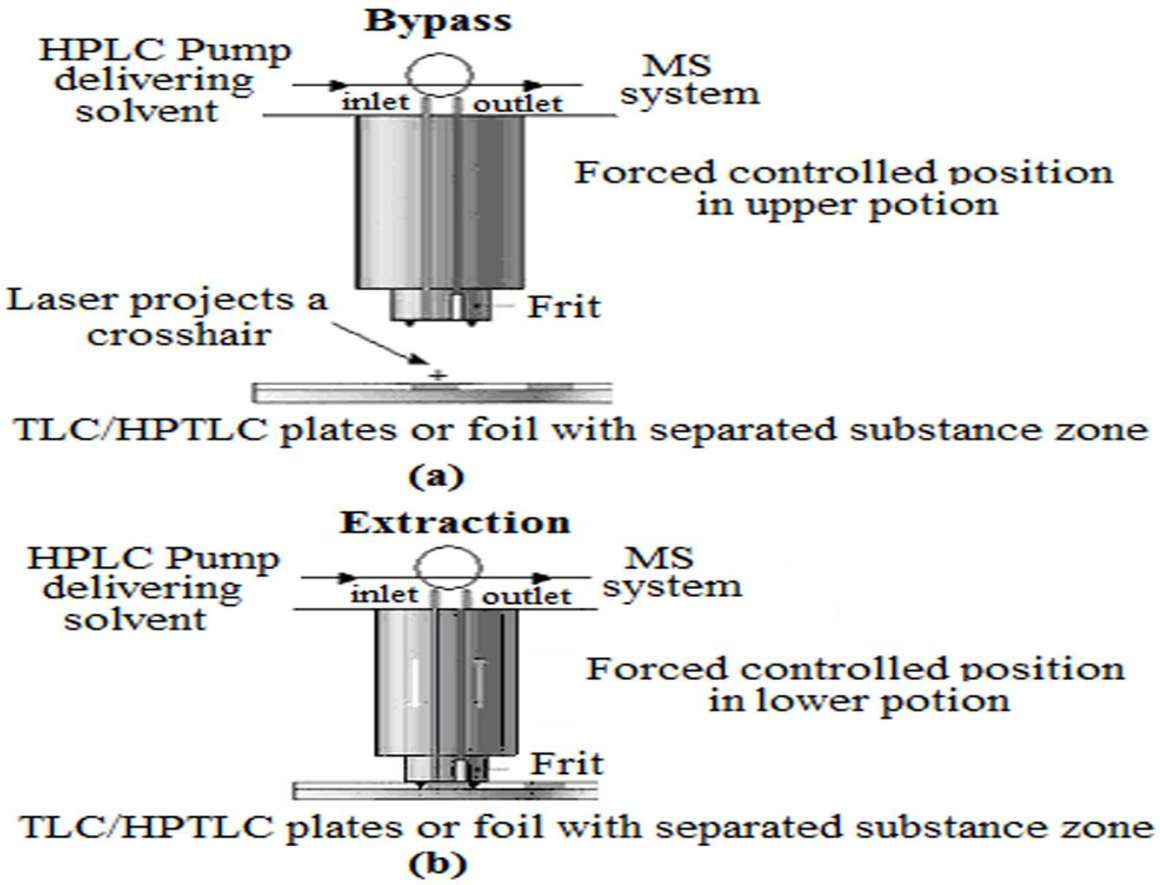


**Fig. 1: (a) TLC-MS interface in bypass position, (b) TLC-MS interface in extraction position**

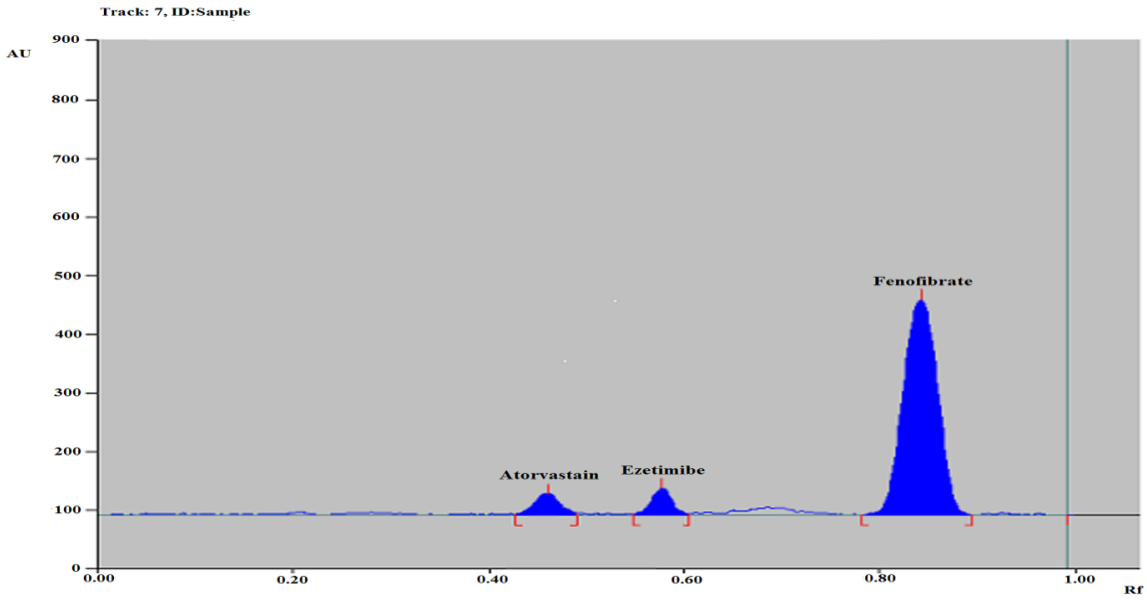


**Fig. 2: Representative HPTLC Chromatogram obtained from AT, EZ and FN of 10 ng /band,10 ng /band and 1000 ng /band and $R_f$ value (0.46±0.01), (0.57±0.01) and (0.84±0.01) respectively with toluene: chloroform: ethyl acetate: acetic acid (5.0:3.0:1.6:0.4** v/v/v/v)

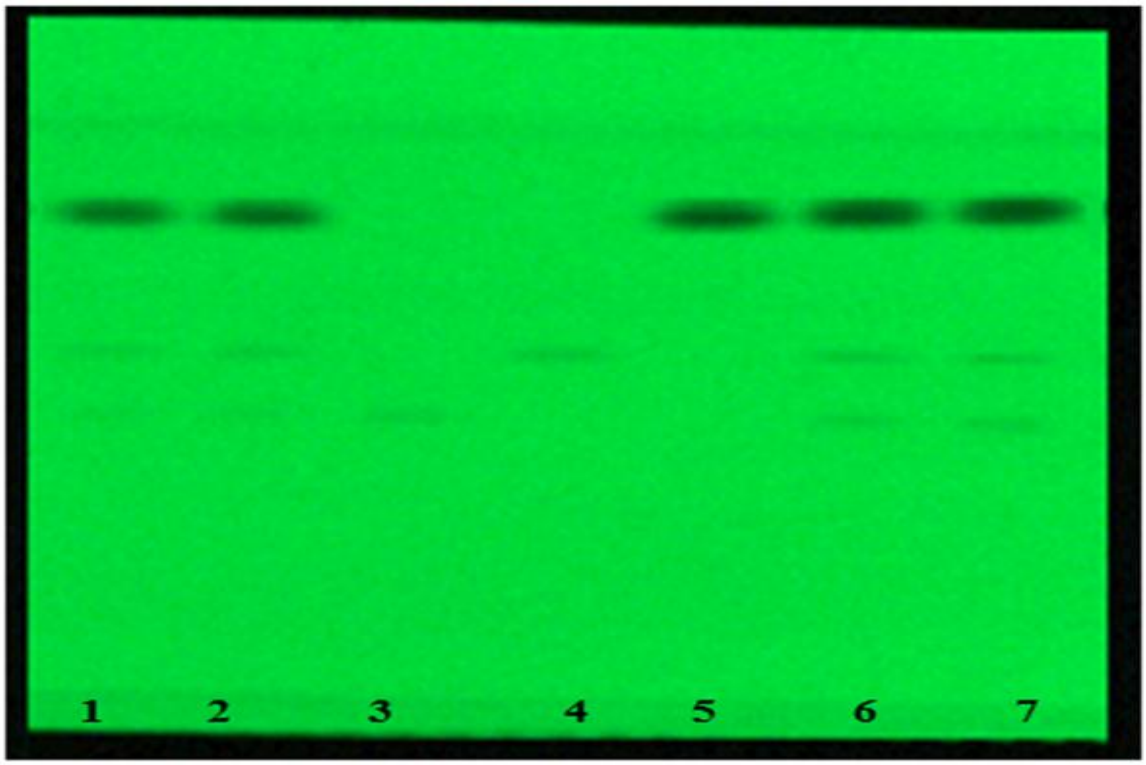


**Fig. 3: Photo documentation of mixture standards (track 1-2); AT (tack 3); EZ (track 4); FN (track 5); sample (track 6-7); at 254nm**

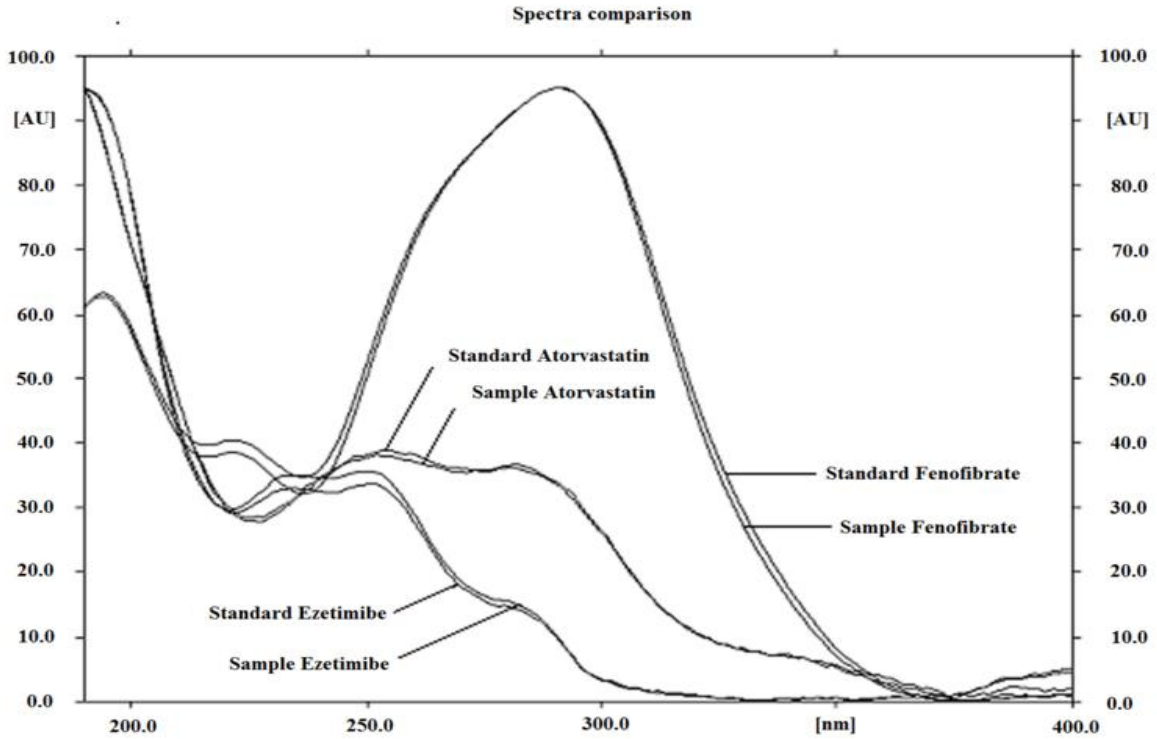


**Fig. 4: Insitu UV spectra of AT, EZ and FN in standard and sample**

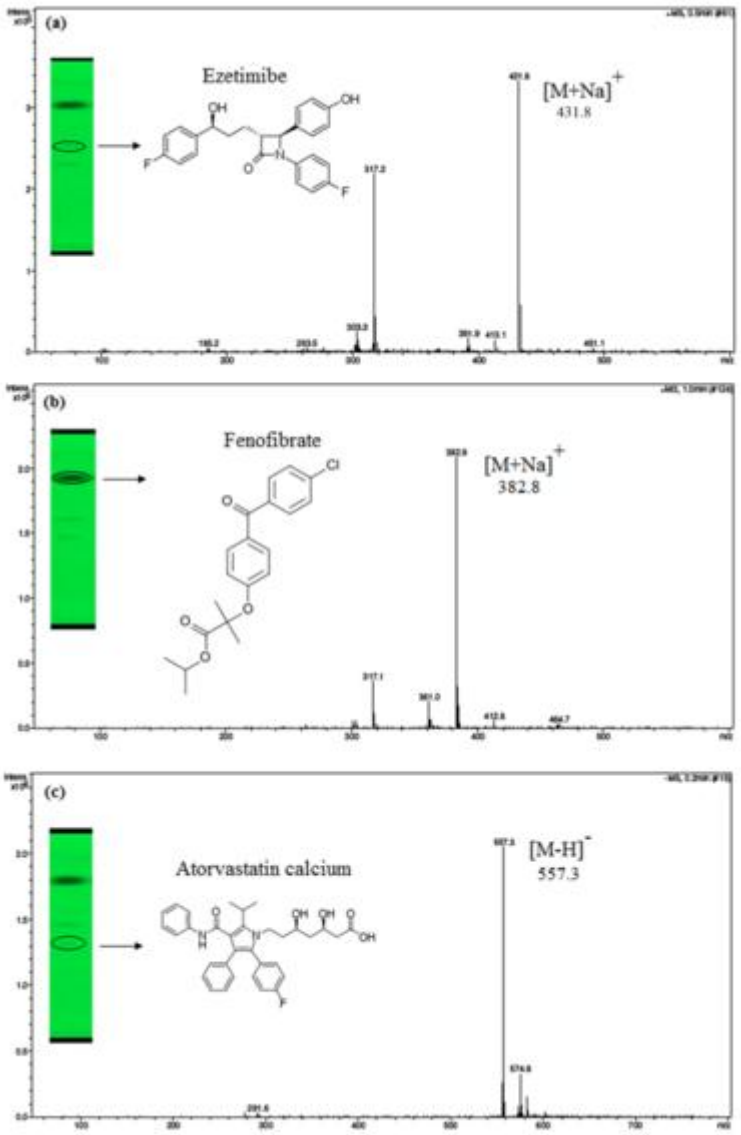


**Fig. 5: Mass spectrum of (a) EZ (b) FN (c) AT obtained by HPTLC-MS**

**Table 1: Calibration studies (n=6)**

| Parameters | AT | EZ | FN |
|---|---|---|---|
| Linear range (ng /band) | 50-350 | 50-350 | **1000-5000** |
| Correlation coefficient (r) ± SD | 0.999±0.0004 | 0.998±0.0002 | 0.999±0.0007 |
| Slope±SD | 2.351±0.65 | 2.601±0.83 | 31.8±0.92 |
| Confidence limit of slope [b] | 3.583-24.83 | 39.77-38.27 | 28.15-26.39 |
| Intercept±SD | 27.85±1.8 | 37.56±3.27 | 15.40±2.46 |
| Confidence limit of intercept [b] | 97.96-94.46 | 1019.4-1013.4 | 3983.3-3979.05 |
| LOD (ng /band) | 3.69 | 18.31 | 12.22 |
| LOD (ng /band) | 37.49 | 61.05 | 40.67 |

b= 95% Confidence interval

**Table 2: Precision studies (n=6)**

| Analyte | Amount (ng /band) | Intermediate precision | | Repeatability | |
|---|---|---|---|---|---|
| | | Mean Area (AU) ±SD | %RSD | Mean Area (AU) ±SD | %RSD |
| AT | 50 | 148.28± 6.3 | 1.5 | 148.14± 4.1 | 1.1 |
| | 150 | 385.30± 5.6 | 0.5 | 384.20± 4.6 | 0.4 |
| | 250 | 616.12± 5.9 | 0.4 | 616.08± 4.3 | 0.2 |
| EZ | 50 | 152.30± 5.0 | 1.5 | 151.22± 5.2 | 1.6 |
| | 150 | 444.78± 4.0 | 0.3 | 444.38± 3.9 | 0.3 |
| | 250 | 708.14± 8.9 | 0.5 | 708.32± 7.8 | 0.4 |
| | 1000 | 3038.30± 35.8 | 0.5 | 3034.01± 34.3 | 0.4 |
| FN | 2000 | 4773.70± 50.8 | 0.4 | 4778.22± 45.3 | 0.3 |
| | 3000 | 6424.94±77.9 | 0.5 | 6429.38±71.8 | 0.4 |

**Table 3: Recovery studies (n=6)**

| Drug | Amount taken (ng) | Amount added (ng) | Total amount found | % Recovery | % RSD |
|---|---|---|---|---|---|
| AT | 100 | 50 | 148.7 | 99.72 | 0.5 |
| | 100 | 100 | 198.4 | 99.48 | 0.3. |
| | 100 | 150 | 252.8 | 100.44 | 0.4 |
| | 100 | 50 | 148.1 | 99.66 | 0.3 |
| EZ | 100 | 100 | 197.4 | 99.19 | 0.3 |
| | 100 | 250 | 348.6 | 99.57 | 0.4 |
| | 2000 | 1000 | 2998.9 | 99.80 | 0.4 |
| FN | 2000 | 2000 | 4002.5 | 101.26 | 0.3 |
| | 2000 | 3000 | 4997.3 | 98.98 | 0.4 |

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