



## **Development and validation of HPTLC method for simultaneous estimation of atorvastatin calcium and Vitamin D<sub>3</sub> in pharmaceutical dosage form**

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### **ABSTRACT**

HPTLC method was developed and validated for simultaneous estimation of Atorvastatin calcium and Vitamin D<sub>3</sub> in their combined dosage form. Separation was achieved on aluminum plates precoated with silica gel 60F254 as stationary phase and mixture of Toluene: Ethyl acetate: Methanol: Glacial Acetic Acid (7:2:1:0.1 v/v/v/v) as a mobile phase. For simultaneous estimation of both drugs, photometric evaluation was performed at 262 nm. The drugs were satisfactorily resolved with R<sub>f</sub> values of 0.38±0.02 and 0.83±0.02 for Atorvastatin calcium and Vitamin D<sub>3</sub> respectively. The accuracy and reliability of the method was assessed by evaluation of linearity (600-1400 ng/spot for both Atorvastatin calcium and Vitamin D<sub>3</sub>), precision (intra-day and inter-day)%RSD values were always less than 2.00 and accuracy (98.75±0.43% for Atorvastatin calcium and 98.79 ±0.33% for Vitamin D<sub>3</sub>).

**Keywords:** Atorvastatin calcium, Vitamin D<sub>3</sub>, HPTLC method



### **INTRODUCTION**

Atorvastatin calcium (Figure 1) is a choice of drug for treatment of Hyperlipidemia. Atorvastatin when given in combination with Vitamin D<sub>3</sub> (Figure 2) acts synergistically and reduce LDL and total cholesterol levels. The concurrent prescription of Vitamin D<sub>3</sub> along with Atorvastatin might reduce dosage requirements of the drug. Vitamin D<sub>3</sub> may also lessen the risk of adverse effects of Atorvastatin including myopathy. Since Vitamin D<sub>3</sub> has mild HMG-CoA reductase activity, it will work synergistically<sup>[1,2]</sup>. Atorvastatin calcium is official in IP and JP<sup>[3,4]</sup> while Vitamin D<sub>3</sub> is official in IP, JP and USP<sup>[3-5]</sup>. Several methods like Spectrophotometric method, First Derivative Spectrophotometric method, Colorimetry, Hydrometry, HPLC, HPTLC etc. are reported for estimation of Atorvastatin calcium individually as well as for its simultaneous estimation with other drugs in pharmaceutical dosage form<sup>[6-12]</sup>.

However, extensive literature review revealed no chromatographic or spectrophotometric method for simultaneous estimation of Atorvastatin Calcium and Vitamin D<sub>3</sub>. So, the objective of present study is to develop and validate a HPTLC method for the simultaneous estimation of Atorvastatin calcium and Vitamin D<sub>3</sub> in pharmaceutical dosage form. It

is validated as per International Conference on Harmonisation (ICH) guidelines<sup>[13]</sup>.

### **MATERIALS AND METHODS**

**Material and reagents:** Atorvastatin calcium and Vitamin D<sub>3</sub> were kindly gifted by ZydusCadila Ltd., Ahmedabad, Gujarat, India. All reagents used to prepare solution as well as mobile phase were of analytical grade. (Methanol and Ethanol from RFCL Ltd., New Delhi and Ethyl acetate, Toluene and Glacial acetic acid from Finar chemical Ltd., Ahmedabad). The tablet samples were obtained from local market (Lipicure D10 tablets from Intas pharmaceuticals Ltd. and Atorsave D10 tablets from Eris Lifesciences Ltd.)

**Instrumentation and chromatographic conditions:** A Camag HPTLC system equipped with a semi-automatic spotter Linomat IV, twin trough plate development chamber, TLC scanner III and CATS4 software was used. Pre-coated silica gel 60 F254 TLC plates (20 × 20 cm<sup>2</sup>, layer thickness 0.2 mm) (E. Merck, Darmstadt, Germany) was used as stationary phase. The standard and formulation samples of ATR and VITD in mixture were spotted on Pre-coated TLC plates in form of narrow bands of lengths 5 mm. Samples were applied under continuous drying

stream of nitrogen gas at constant application rate of 10 sec/ $\mu$ L. The mobile phase consisted of Toluene: Ethyl acetate: Methanol: Glacial acetic acid (7: 2: 1: 0.1 v/v/v/v). Linear ascending development was carried out in twin trough chamber (10  $\times$  10 cm). The optimized chamber saturation time for mobile phase was 20 min at  $28 \pm 2.0$  °C; the length of chromatogram run was 80 mm and TLC plates were air dried. Densitometric scanning was performed on CAMAG TLC scanner III in Absorbance/Reflectance mode and operated by CATS4 software. The source of radiation utilized was deuterium lamp. The spots were analyzed at a wavelength of 262 nm. The slits dimensions used in the analysis were length and width of 5 mm and 0.1 mm respectively with a scanning rate of 100 mm/sec. Evaluation was performed using linear regression analysis via peak areas.

#### Standard solutions and calibration curves:

Standard stock solution of ATR and VITD each was prepared by dissolving 10 mg of drug in 10 ml of ethanol respectively. 1 ml of ATR solution + 1 ml of VITD solution were added in 10 ml volumetric flask and volume was made up to the mark to get the combined standard solution of ATR and VITD. Calibration was done by applying this combined standard solution ranging from 2-12  $\mu$ L by Hamilton syringe with the help of Linomat IV semiautomatic spotter on TLC plate that gave concentration 600-1400 ng/spot for both ATR and VITD. From the developed plates calibration curve was plotted as peak areas versus corresponding concentration.

#### Method Validation

**Linearity:** Linearity responses for ATR and VITD were assessed in the concentration range of 600-1400 ng/spot for both.

**Repeatability:** Repeatability of measurement of peak area or peak height was determined by measuring the same spot (800 ng/spot) for peak area or peak height without changing the position of the plate. Repeatability of sample application was assessed by spotting 8  $\mu$ L (800 ng/spot) of standard drug solution six times on a TLC plate, followed by development of the plate and photometrically analyzing it.

**Precision:** Precision of the method was determined in the terms of intra-day precision and inter-day variation (%RSD). Intra-day precision (%RSD) was assessed by analyzing standard drug solutions within the calibration range, three times on the same day. Inter-day precision (%RSD) was assessed by analyzing standard drug solutions within the calibration range on three different days.

**Accuracy:** To the pre-analysed sample a known amount of standard solution of pure drug (ATR and VITD) was spiked at three different levels. These solutions were subjected to re-analysis by the proposed method.

**Sensitivity:** The sensitivity of measurement of ATR and VITD by the use of proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantification (LOQ). LOD was calculated practically while LOQ was calculated by equation.

#### Analysis of ATR and VITD in marketed formulation:

Ten tablets were weighed and powdered. Powder equivalent to 100 mg of Atorvastatin calcium & 10,000 I.U. (250  $\mu$ g) of Vitamin D<sub>3</sub> was transferred to 50 mL volumetric flask. 25 mL of ethanol was added, mixed thoroughly and sonicated for 45 minutes. The solution was filtered through Whatman filter paper No. 41 & diluted to 50 mL with ethanol (T<sub>a</sub>). Aliquot (1mL) was transferred in 10mL volumetric flask and volume was made up to the mark with ethanol (T<sub>b</sub>). 5 $\mu$ L of T<sub>b</sub> and 160  $\mu$ L of T<sub>a</sub> for Atorsave D 10 and 3  $\mu$ L of T<sub>b</sub> and 160  $\mu$ L of T<sub>a</sub> for Lipicure D 10 were spotted in duplicate along with standard solution.

#### RESULTS AND DISCUSSION

Due to its versatility HPTLC technique was found suitable for analysis of Atorvastatin calcium and Vitamin D<sub>3</sub>. Best results were obtained with a mixture of toluene: ethyl acetate: methanol: glacial acetic acid (7:2:1:0.1 v/v/v) and R<sub>F</sub> value of Atorvastatin and Vitamin D<sub>3</sub> were  $0.38 \pm 0.05$  and  $0.83 \pm 0.05$  respectively (Figure 1). It was observed that pre-saturation of TLC chamber with mobile phase for 20 min ensures good reproducibility and peak shape. Photometric evaluation was performed at 262 nm, the wavelength of maximum absorbance of Vitamin D<sub>3</sub>. Quantitative determinations of Atorvastatin and Vitamin D<sub>3</sub> were made by considering the peak areas from chromatograms and regression line equation using optimized conditions. Developed HPTLC method was validated in terms of linearity, limit of detection, limit of quantification, precision, accuracy and specificity.

#### Method Validation:

**Linearity:** The linearity range for both Atorvastatin and Vitamin D<sub>3</sub> was found to be **600-1400 ng/spot** (Table 1) and (Table 2) respectively. Calibration curves were prepared (Figure 5 and Figure 6) and chromatogram is shown in Figure 2. Regression for calibration curve of Atorvastatin was found to be **0.9911** and for Vitamin D<sub>3</sub> was

found to be **0.9902**. The regression line equation is as follows:

$$y = 3.888x + 963.4 \text{ for Atorvastatin}$$

$$y = 3.473x + 523.1 \text{ for Vitamin D}_3$$

Where, y= Corresponding peak area

x= Concentration in ng

#### Repeatability/Replication:

**Repeatability of peak area measurement:** The data for repeatability of peak area measurement is represented in (Table 3) is based on six-time measurement of a same spot (800 ng/spot) of Atorvastatin and Vitamin D<sub>3</sub>. The %RSD for peak area was found to be **0.78%** and **0.65%** for Atorvastatin and Vitamin D<sub>3</sub> respectively.

**Repeatability of sample application:** The data for repeatability of sample application based on six-time application of 8µL sample is represented in Table 4. The %RSD for peak area was found to be **1.60%** and **0.88%** for Atorvastatin and Vitamin D<sub>3</sub> respectively.

#### Precision:

**Intraday precision:** The data for intraday precision of method are summarized in (Table 5). The %RSD for intraday precision was found to be **0.88 - 1.43%** and **1.21 - 1.48%** for Atorvastatin and Vitamin D<sub>3</sub> respectively.

**Interday precision:** The data for interday precision of method are summarized in (Table 6). The %RSD for interday precision was found to be **1.44 - 1.58%** and **1.61 - 1.99%** for Atorvastatin and Vitamin D<sub>3</sub> respectively.

**Accuracy:** Accuracy of the method was evaluated by calculating recovery of ATR and VITD by

standard addition method at 3 different levels. The percentage recovery was found to be **98.75±0.43** for ATR (Table 7) and **98.79±0.33** for VITD (Table 8) ensuring that the method is accurate.

**Sensitivity:** LOD was determined practically and was found to be **100 ng/spot** for both Atorvastatin calcium and Vitamin D<sub>3</sub>. LOQ was calculated by using formula mentioned in ICH guidelines and were found to be **358.40 ng/spot** and **430.33 ng/spot** for Atorvastatin calcium and Vitamin D<sub>3</sub> respectively.

**Analysis of ATR and VITD in marketed formulation:** The method was applied to marketed formulation and percentage assay for Atorvastatin calcium and Vitamin D<sub>3</sub> was found to be 102.71±0.042% and 121.71±4.54% respectively for LIPICURE D 10 Tablets and 100.62±2.75% and 113.95±4.16% for ATORSAVE D 10 Tablets. (Table 9)

#### CONCLUSION

The developed HPTLC method is simple, reproducible, precise and accurate and can be used for simultaneous determination of ATR and VITD in tablets.

#### ACKNOWLEDGEMENT

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TABLE 1: CALIBRATION CURVE DATA OF ATORVASTATIN CALCIUM

Sr. No.	Standard ID	Std. Conc. (ng/spot)	Average Area (n=5) Mean ± S.D	%RSD
1	Std-1	600	3280.02±63.14	1.92
2	Std-2	800	3973.62±46.23	<b>1.16</b>
3	Std-3	1000	4977.84±58.89	1.18
4	Std-4	1200	5742.76±101.03	1.76
5	Std-5	1400	6283.62±121.40	<b>1.93</b>

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 TABLE 2: CALIBRATION CURVE DATA OF VITAMIN D<sub>3</sub>

Sr. No.	Standard ID	Std. Conc. (ng/spot)	Average Area (n=5) Mean ± S.D	%RSD
1	Std-1	600	2535.92±47.11	1.86
2	Std-2	800	3328.98±45.35	1.36
3	Std-3	1000	4019.96±72.52	1.80
4	Std-4	1200	4844.26±52.15	<b>1.07</b>
5	Std-5	1400	5251.26±99.85	<b>1.90</b>

TABLE 3: REPEATABILITY OF SCANNER PEAK AREA MEASUREMENT

Sr. No	Peak Area of Atorvastatin Calcium	Peak Area of Vitamin D <sub>3</sub>
1	4312.41	3922.61
2	4377.45	3934.02
3	4375.16	3916.75
4	4386.58	3916.97
5	4365.31	3984.36
6	4311.47	3940.98
<b>Mean</b>	4354.73	3935.95
<b>S.D.</b>	33.83	25.61
<b>%RSD</b>	0.78	0.65

TABLE 4: REPEATABILITY OF SAMPLE APPLICATION

Sr. No	Peak Area of Atorvastatin Calcium	Peak Area of Vitamin D <sub>3</sub>
1	4598.56	3429.15
2	4668.98	3348.65
3	4471.73	3398.82
4	4639.78	3393.94
5	4561.83	3388.07
6	4522.43	3428.42
<b>Mean</b>	4577.22	3397.84
<b>S.D.</b>	73.70	29.82
<b>%RSD</b>	1.60	0.88

TABLE 5: INTRADAY PRECISION

Sr. No.	Atorvastatin calcium			Vitamin D <sub>3</sub>		
	Std. Conc. (ng/spot)	Average Area (n=3) Mean ± SD	%RSD	Std. Conc. (ng/spot)	Average Area (n=3) Mean ± SD	%RSD
1	600	3319.03±47.45	<b>1.43</b>	600	2517.77±33.17	1.32
2	1000	5008.83±17.13	<b>0.34</b>	1000	4057.80±60.16	<b>1.48</b>
3	1400	6287.63±55.14	0.88	1400	5194.17±62.91	<b>1.21</b>

TABLE 6: INTERDAY PRECISION

Sr. No.	Atorvastatin calcium			Vitamin D <sub>3</sub>		
	Std. Conc. (ng/spot)	Average Area (n=3) Mean ± SD	%RSD	Std. Conc. (ng/spot)	Average Area (n=3) Mean ± SD	%RSD
1	600	3272.03±47.20	<b>1.44</b>	600	2560.67±46.05	1.79
2	1000	4958.80±72.82	1.47	1000	4003.43±79.91	<b>1.99</b>
3	1400	6342.43±100.36	<b>1.58</b>	1400	5302.40±85.61	<b>1.61</b>

TABLE 7: RECOVERY FOR ATORVASTATIN CALCIUM

Amount of (Formulation) spotted (ng/spot)	Amount (std) spiked (ng/spot)	Total Amount (ng/spot)	Spiked amount recovered(n=3)± SD	% Recovery Mean ± SD (n=3)
600	480	1080	471.68±2.59	98.27±0.54
600	600	1200	593.36±3.31	98.89±0.55
600	720	1320	713.47±2.84	99.09±0.39
<b>%Average Recovery ± SD:</b>			<b>98.75±0.43</b>	

TABLE 8: RECOVERY FOR VITAMIN D<sub>3</sub>

Amount of (Formulation) spotted (ng/spot)	Amount (std) spiked (ng/spot)	Total Amount (ng/spot)	Spiked amount recovered(n=3)± SD	% Recovery Mean ± SD (n=3)
600	480	1080	473.25±6.05	98.59±1.26
600	600	1200	591.63±5.52	98.61±0.92
600	720	1320	714.07±4.76	99.18±0.66
<b>%Average Recovery ± SD:</b>			<b>98.79±0.33</b>	

TABLE 9: ASSAY RESULTS

Formulation	LIPICURE D 10		ATORSAVE D 10	
	Atorvastatin calcium	Vitamin D <sub>3</sub>	Atorvastatin calcium	Vitamin D <sub>3</sub>
Amount obtained (ng) ± SD (n=2)	613.035±0.25	973.67±35.62	1006.17±27.57	911.63±33.26
<b>% Assay ± SD</b>	102.17±0.042	<sup>a</sup> 121.71±4.54	100.62±2.75	<sup>a</sup> 113.95±4.16

a=Overages allowed in Vitamin D<sup>[14,15,16,17]</sup>

TABLE 10: VALIDATION PARAMETERS

Parameters	Atorvastatin calcium	Vitamin D <sub>3</sub>
Linearity range	600-1400 ng/spot	600-1400 ng/spot
Correlation Coefficient (r)	0.9955	0.9951
Repeatability		
a. Repeatability of peak area measurement	0.78%	0.65%
b. Repeatability of sample application	1.60%	0.88%
Intraday precision (% RSD)	0.34-1.43%	1.21-1.48%
Interday precision (% RSD)	1.44-1.58%	1.61-1.99%
Accuracy (% Recovery)	98.75±0.43	98.79±0.33
LOD	100 ng/spot	100 ng/spot
LOQ	358.40 ng/spot	430.33 ng/spot

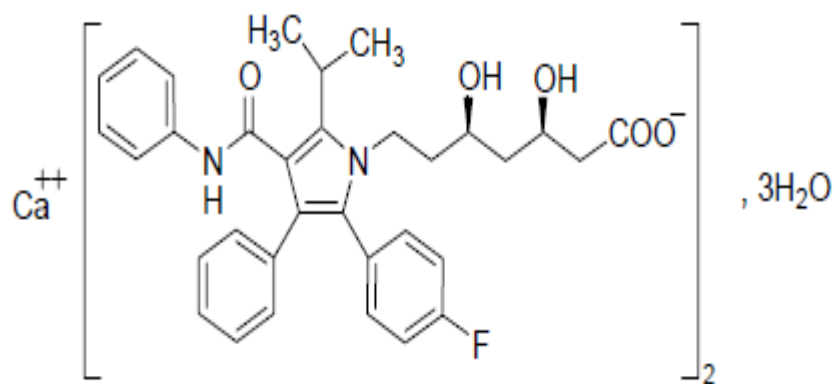
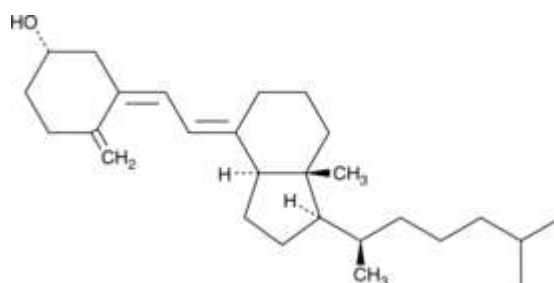


Figure 1: Structure of Atorvastatin calcium

Figure 2: Structure of Vitamin D<sub>3</sub>

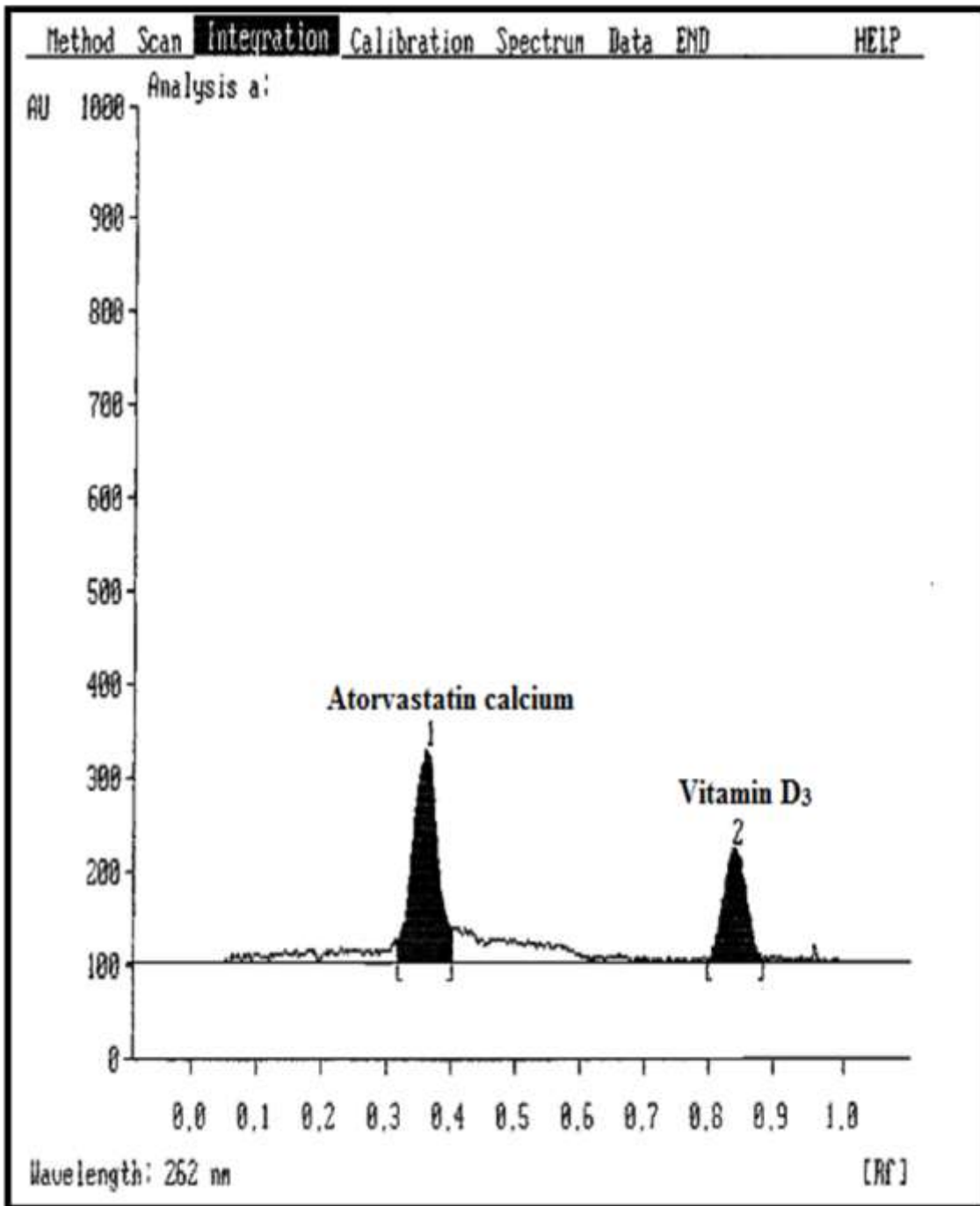


Figure 3: Chromatogram showing resolution between Atorvastatin calcium ( $R_f = 0.38 \pm 0.05$ ) and Vitamin D<sub>3</sub> ( $R_f = 0.83 \pm 0.05$ )

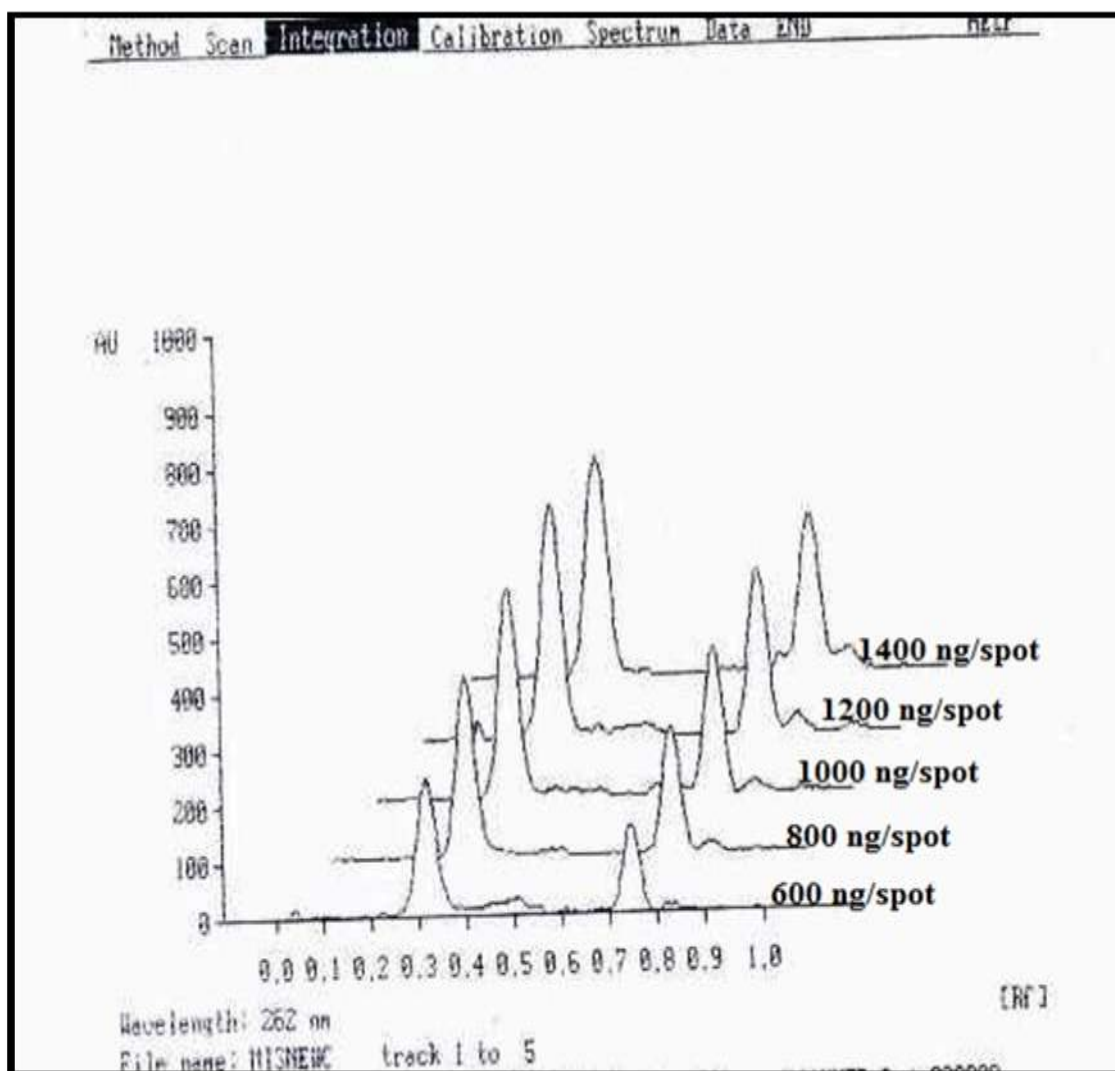


Figure 4: Chromatogram of standard Atorvastatin calcium & Vitamin D<sub>3</sub> over linearity range

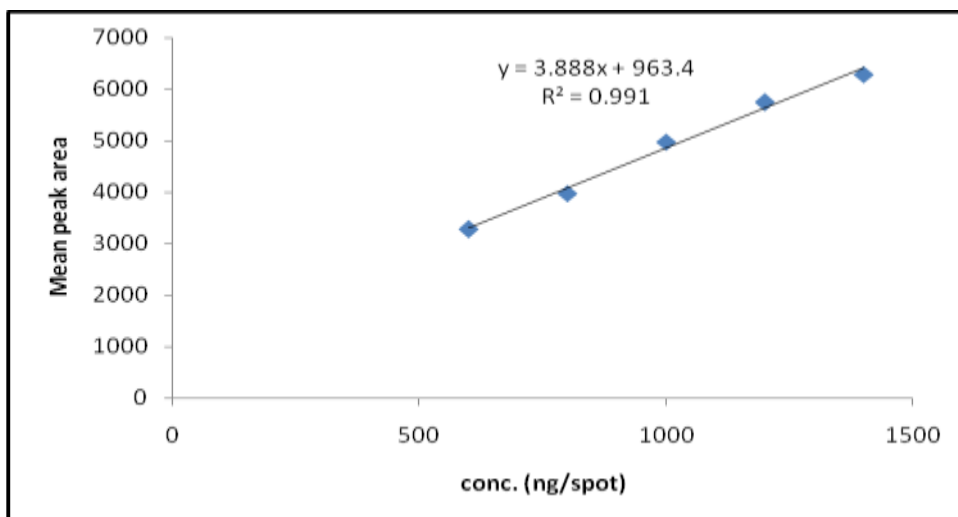


Figure 5: Calibration curve of standard Atorvastatin calcium (n=5)



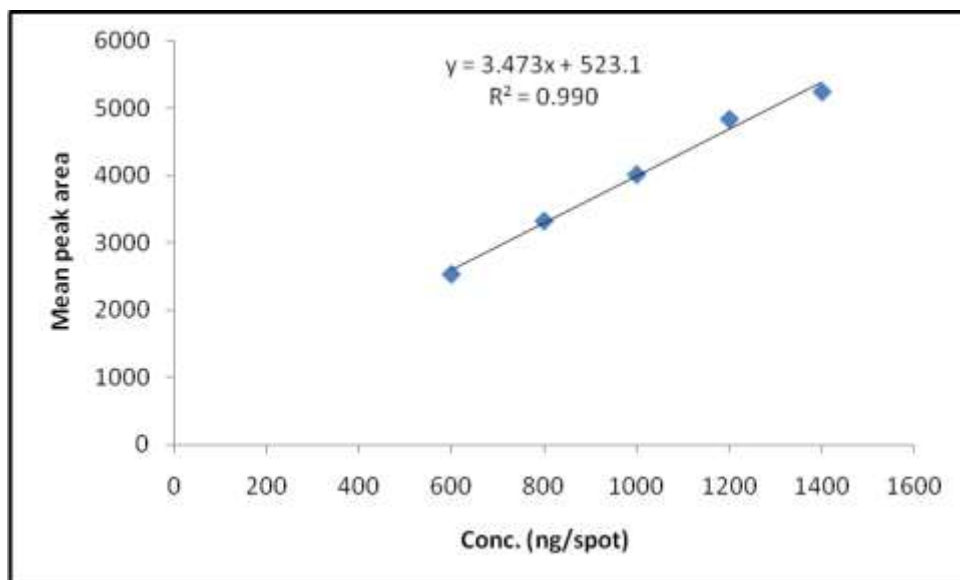


Figure 6: Calibration curve of standard Vitamin D<sub>3</sub> (n=5)

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