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Analytical Method Development and Validation for Simultaneous Estimation of Asparin and Atrovastatin Calcium in Combind Dosage Form by Using RP-HPLC Method

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A B S T R A C T

A simple, Accurate, precise method was developed for the simultaneous estimation of the Aspirin and Atorvastatin calcium in Tablet dosage form. Chromatogram was run through Agilent C18, 4.6 mmx150mm, 5µm. Mobile phase containing Methanol and Phosphater buffer in the ratio of 70:30 was pumped through column at a flow rate of 0.8ml/min. Optimized wavelength for Aspirine and Atorvastatin was 254nm. Retention time of Aspirine and Atorvastatin calcium were found to be 2.972min and 3.548 min. %RSD of the Aspirine and Atorvastatin calcium were and found to be 0.24 and 0.28 respectively. %assay was obtained for Aspirin and Atorvastatin within the limits. LOD, LOQ values are obtained from Aspirin and Atorvastatin within the limits respectively.

Keywords: Aspirin, Atorvastatin, RP-HPLC

A R T I C L E I N F O

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1. Introduction

Aspirin is chemically named as (3R, 5S, 6E)-7-[4-(4-fluorophenyl)-2-(N-methylmethanesulfonamido)-6-(propan-2-yl) pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoicacid[1]. Rosuvastatin is a competitive inhibitor of the

enzyme HMG-CoA reductase, having a mechanism of action similar to that of other statins. Its approximate elimination half life is 19 h and its time to peak plasma concentration is reached in 3–5 h following oral administration[2]. Putative beneficial effects of rosuvastatin therapy on chronic heart failure may be negated by increases in collagen turnover markers as well as a reduction in plasma coenzyme Q10 levels in patients with chronic heart failure [3]. Atorvastatin is chemically named7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl) -5-(propan-2-yl)-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoate. Atorvastatin selectively and competitively inhibits the hepatic enzyme HMG-CoA reductase. As HMG-CoA reductase is responsible for converting HMG-CoA to mevalonate in the cholesterol biosynthesis pathway, this results in a subsequent decrease in hepatic cholesterol levels [4]. Decreased hepatic cholesterol levels stimulates upregulation of hepatic LDL-C receptors which increases hepatic uptake of LDL-C and reduces serum LDL-C concentrations [5].

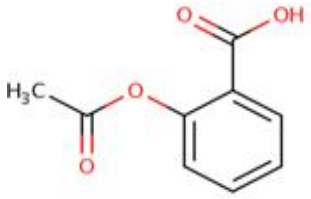


Figure 1: Structure of Aspirin

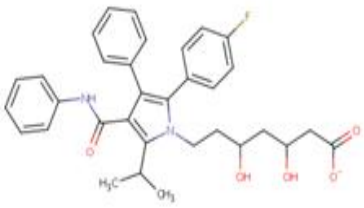


Figure 2: Structure of Atorvastatin

2. Materials and Methods

Preparation of standard solution:

10 mg of Aspirinand 10mg of Atorvastatin were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicate to dissolve it completely and the volume was made up to the mark with the same solvent to give a concentration of 1000 µg/ml. (Stock solution) Further 0.2 and 0.1 ml were pipette out from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give a concentration of 20 µg/ml and 10µg/ml respectively[7].

Preparation of sample solution:

10 Tablets of contents were weighed and triturated in glass mortar. The quantity of powder equivalent to 10 mg of active ingredient present in Aspirin and Atorvastatin was transferred into a 10 ml clean dry volumetric flask, 7 ml of diluent was added to it and was shaken by mechanical stirrer and sonicate for about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent to give a concentration of 1000 µg/ml and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution)[8]. 0.2 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark to give the respective concentrations as par with standard solution. The solution was filtered through 0.45 µm filter before injecting into HPLC system.

Preparation of Placebo:

The amount of powdered inactive ingredient supposed to be present in 10 tablets were accurately weighed and transferred in to 10 ml volumetric flask, 7 ml of diluent was added and shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes and was diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for dilution. 0.1 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 µm filter before injecting into HPLC system.

Linearity:

Preparation of sample stock solution: About 20 mg of Aspirin and 10 mg of Atorvastatin samples was weighed in to 10ml volumetric flask, it was dissolved with diluent and the volume was made up to the mark with same diluent (2000µg/ml of Aspirin and 1000µg/ml of Atorvastatin)[12].

Preparation of Level – I (20µg/ml of Aspirin &10µg/ml of Atorvastatin)

0.1ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–II (40 µg/ml of Aspirin &15.0 µg/ml of Atorvastatin)

0.2ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–III (60 µg/ml of Aspirin &20 µg/ml of Atorvastatin)

0.3ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–IV (80 µg/ml of Aspirin &25 µg/ml of Atorvastatin)

0.4ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

Preparation of Level–V (100 µg/ml of Aspirin &30 µg/ml of Atorvastatin).

0.5ml of stock solution had taken in 10ml of volumetric flask diluted up to the mark with diluent.

10µl of each level were injected into the system and recorded the peak response.

Procedure:

Each level solution was injected into the chromatographic system and the peak area was measured. A graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) was plotted and the correlation coefficient was calculated.

The linearity of the method was demonstrated over the concentration range of 10-100µg / ml. Aliquots of five levels were prepared from sample solution and labeled as solution 1, 2, 3, 4 and 5 respectively.

Acceptance criteria

1. Correlation Coefficient should be not less than 0.9990.
2. % RSD of peak areas for Solution 1, 2, 3, 4 and 5 should be not more than 2.0 %.

Precision

Preparation of stock solution:

10 mg of Aspirin and 10mg of Atorvastatin were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added and sonicate to dissolve it completely. The volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution) .Further 0.2 ml and 0.2 ml was pipette out from the above stock solutions into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 20 µg/ml and 20 µg/ml respectively.

Procedure:

The standard solution was injected for five times and the areas for all five injections were measured in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits

Acceptance criteria

% Relative standard deviation of peak areas and R_t should not be more than 2.0 %.

Intermediate Precision/Ruggedness

a. To evaluate the intermediate precision (also known as Ruggedness) of the method.
b. Precision was performed on different day by using different make column of same Dimensions.

Preparation of stock solution:

10 mg of Aspirin and 10mg of Atorvastatin accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added ,sonicate to dissolve it completely and the volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution) Further 0.2ml and 0.1ml were pipette out from the above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 20µg/ml and 10 µg/ml respectively[13].

Procedure:

The standard solution was injected for five times and the area was measured for all five injections in HPLC. The %RSD for the area and R_t of five replicate injections was found to be within the specified limits.

Acceptance criteria

% Relative standard deviation of peak areas and R_t should not be more than 2.0 %

Accuracy

Assay was performed in triplicate for various concentrations of Aspirin and Atorvastatin equivalent to 50, 100, and 150 % of the standard amount was injected into the HPLC system as per the test procedure.

Preparation of Standard stock solution:

10 mg of Aspirin and 10mg of Atorvastatin accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicate to dissolve it completely and volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution)

Preparation Sample solutions:

Preparation of 50% solution (15 µg/ml of Aspirin and 90µg/ml of Atorvastatin): From the above stock solutions take 0.15 ml and 0.9 ml into 10 ml dry volumetric flask, make up to the mark with diluent**.**

Preparation of 100% solution (30 µg/ml of Aspirin and 180 µg/ml of Atorvastatin):

From the above stock solutions take 0.3 ml and 1.8 ml into 10 ml dry volumetric flask, make up to the mark with diluent

Preparation of 150% solution (45 µg/ml of Aspirin and 270 µg/ml of Atorvastatin):

From the above stock solutions take 0.45 ml and 2.7 ml into 10 ml dry volumetric flask, make up to the mark with diluent. These solutions were filtered through 0.45µ membrane and then each concentration; three replicate injections were made under the optimized conditions

Procedure:

The standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions were injected. The amount found and amount added for Aspirin and Atorvastatin individual recovery and mean recovery values were calculated and the results were found tobe within limits.

Acceptance criteria

The mean % recovery of the Aspirin and Atorvastatin each spike level should be not less than 98.0 % and not more than 102.0 %.

Specificity

A) Aspirin and Atorvastatin identification:

Solutions of Standard and Sample were prepared as per test procedure and injected into the HPLC system.

Acceptance criteria

Chromatogram of standard and sample should be identical with near Retention time.

B) Placebo interference:

A study to establish the interference of placebo was conducted. A sample of placebo was injected into the HPLC system as per the test procedure.

Acceptance criteria

Chromatogram of placebo should not show any peak at the retention time of analyte peak. There is no interference due to placebo at the retention time of analyte. Hence the method is specific.

C) Blank interference:

A study to establish the interference of blank was conducted. Diluent was injected into HPLC system as per the test procedure.

Acceptance criteria

Chromatogram of blank should not show any peak at the retention time of analyte peak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

Ruggedness

The simultaneous estimation of Aspirin and Atorvastatin was performed by different analysts on different days.

Acceptance criteria

% Relative standard deviation of peak areas and R_t should not be more than 2.0 %.

Robustness

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like flow rate and mobile phase composition, temperature variations which may differ but the responses were still within the specified limits of the assay[14].

a) Effect of variation of flow rate:

A study was conducted to determine the effect of variation in flow rate. The flow rate was varied at 0.6 ml/min to 1.0 ml/min. Standard solution 20 ppm Aspirin and 10 ppm Atorvastatin were prepared and analysed using the varied flow rates along with method flow rate. The results are summarized on evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.The method is robust only in less flow condition. The effect of variation of flow rate was evaluated.

b) Effect of variation of mobile phase composition:

A study was conducted to determine the effect of variation in mobile phase ratio by changing the ratio of mobile phase. The Organic composition in the Mobile phase was varied from 30 % to 70 %. Standard solution 20 µg/ml of Aspirin and 10µg/ml Atorvastatin were prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.Standard solution was prepared and injected into the HPLC system and the Chromatograms which were recorded.

Limit of Detection

For Aspirin (Preparation of 10 µg/ml solution):

10mg of Aspirin was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added , sonicated to dissolve it completely and volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution). Further 0.1ml of the above stock solution was pipetted out into a 10ml volumetric flask and dilute up to the mark with diluent to give the concentration of 10 µg/ml.

Preparation of 0.15% solution at Specification level (0.004 µg/ml solution):

Further 1ml of the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 10 µg/ml. 0.1 ml of 10 µg/ml solution was pipette into a 10 ml of volumetric flask and diluted up to the mark with diluent give the concentration of 0.004 µg/ml [15].

For Atorvastatin (Preparation of 10 µg/ml solution):

10mg of Atorvastatin working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicate to dissolve it completely and the volume was made up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution). Further 0.1 ml of the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 10 µg/ml.

Preparation of 0.22% solution At Specification level (0.006 µg/ml solution):

Further 1ml of the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 10 µg/ml. 0.22 ml of 1µg/ml solution was pipette into a 10 ml of volumetric flask and diluted up to the mark with diluent to give the concentration of 0.006 µg/ml[16].

LOD sample: From the 10 µg/ml of Aspirin and 10 µg/ml of Atorvastatin of the indiduval stock solution we prepared 0.004 µg/ml and 0.006µg/ml injected into the system.

The LOD is determined by the formula

LOD = **S**/N

Where**,**

N = Average Baseline Noise obtained from Blank

S = Signal Obtained from LOD solution (0.25% of target assay concentration).

Acceptance Criteria: S/N Ratio value shall be not more than 3 for LOD solution.

Limit of Quantification (LOQ)

For Aspirin (Preparation of 10 µg/ml solution):

10 mg of Aspirin was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicate to dissolve it completely and make volume up to the mark with the same solvent to give the concentration of 1000 µg/ml. (Stock solution).

Further 0.1ml of the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 10 µg/ml [17].

Preparation of 0.05% solution At Specification level (0.015 µg/ml solution):

Further 1ml of the above stock solution was pipette into a 10ml volumetric flask and dilute up to the mark with diluent to give the concentration of 1 µg/ml. 0.05 ml of 10 µg/ml solution was pipetted into a 10 ml of volumetric flask and diluted up to the mark with diluentto give the concentration of 0.015 µg/ml.

For Atorvastatin (Preparation of 10 µg/ml solution):

10mg of Atorvastatin was accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and make volume up to the mark with the same solvent to give the concentration of 1000 µg/ml[18]. (Stock solution). Further 0.1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 10 µg/ml.

Preparation of 0.06% solution At Specification level (0.02 µg/ml solution): Further 1ml of the above stock solution was pipetted into a 10ml volumetric flask and diluted up to the mark with diluent to give the concentration of 10 µg/ml**.** 0.06 ml of 10 µg/ml solution was pipetted into a 10 ml of volumetric flask and diluted up to the mark with diluent to give the concentration of 0.02 µg/ml.

3. Results and Discussion

Method development:

Linearity

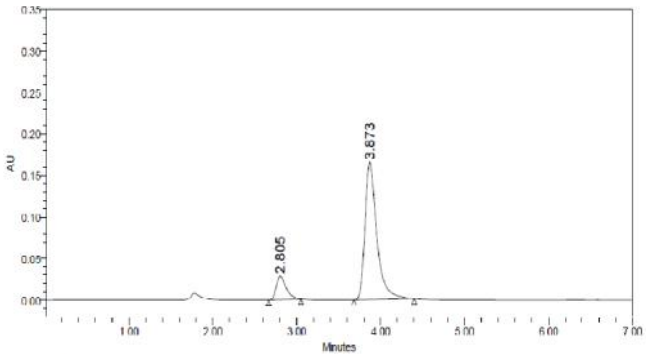


Figure 3: Chromatogram for Linearity level 1

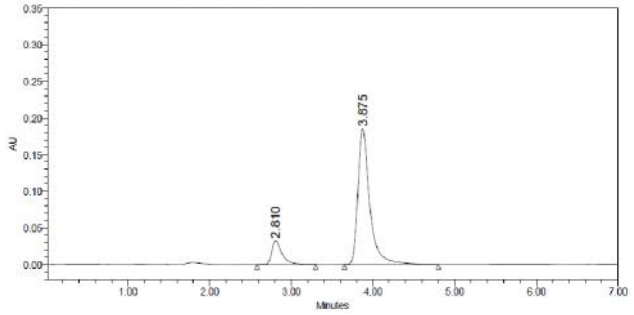


Figure 4: Chromatogram for Linearity level 2

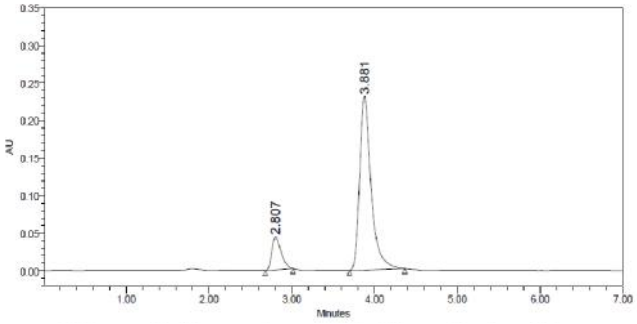


Figure 5: Chromatogram for Linearity level 3

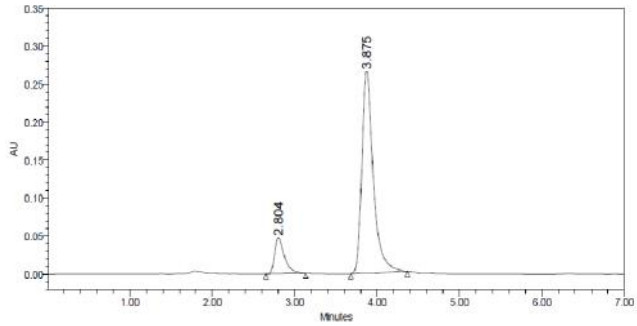


Figure 6: Chromatogram for Linearity level 4

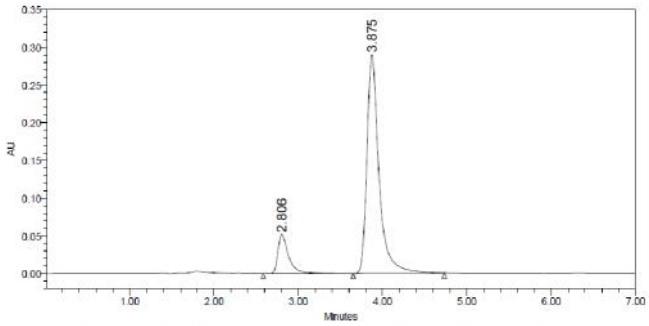


Figure 7: Chromatogram for Linearity level 5

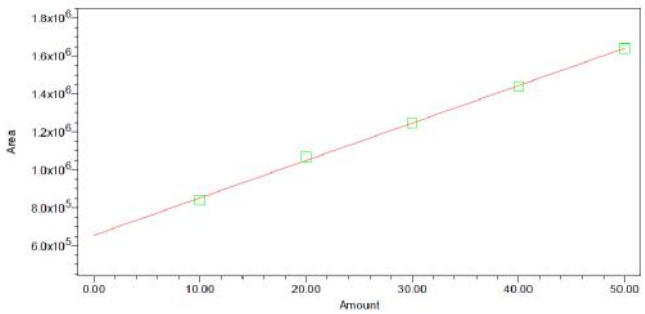


Figure 8: Calibration curves of Aspirin

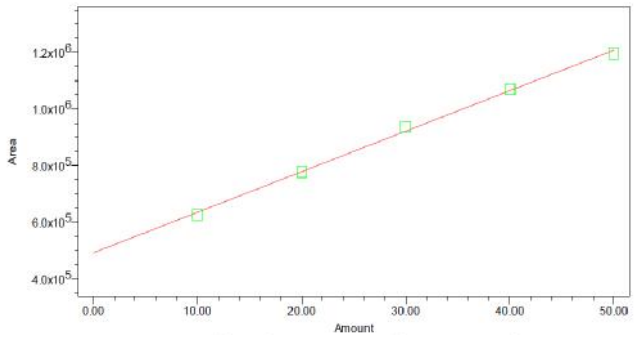
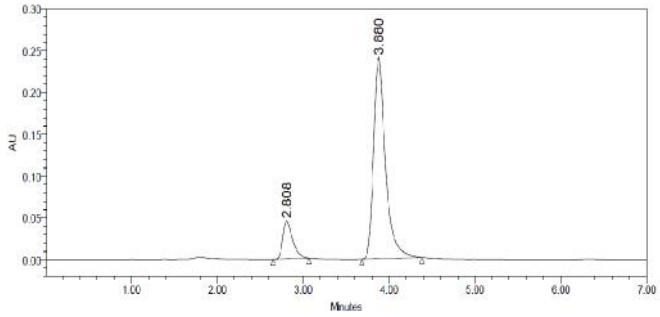
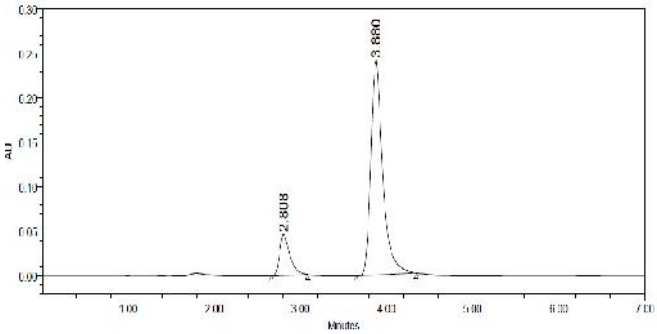


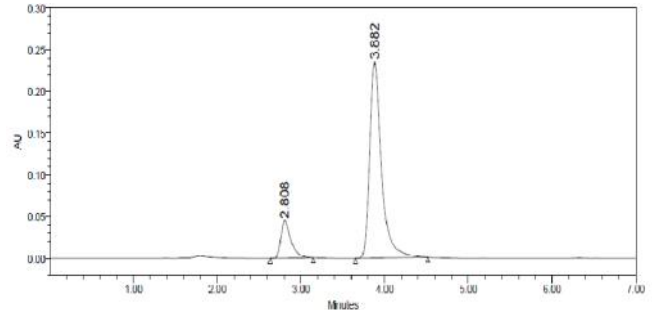
Figure 9: Calibration curve of Atorvastatin

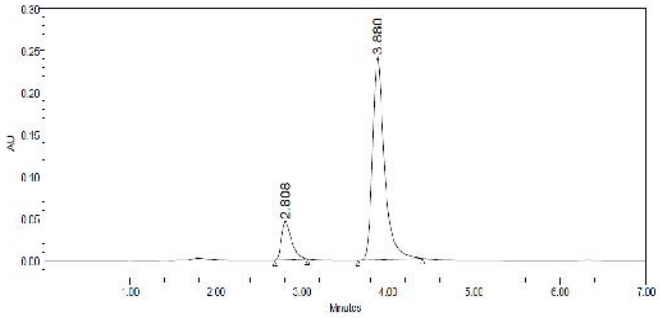
Precision

A. Repeatability:









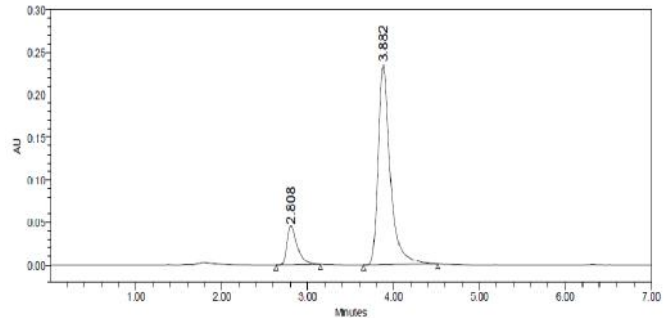


Figure 10: Sample Chromatograms for Repeatability

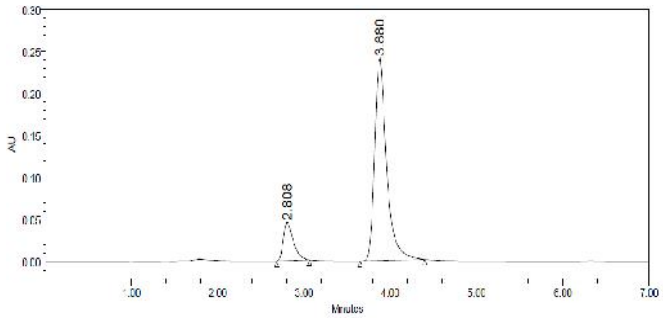
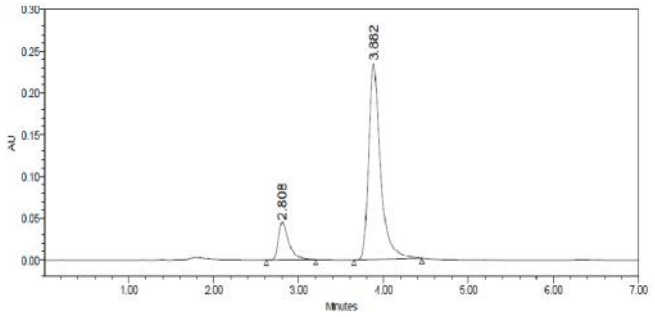
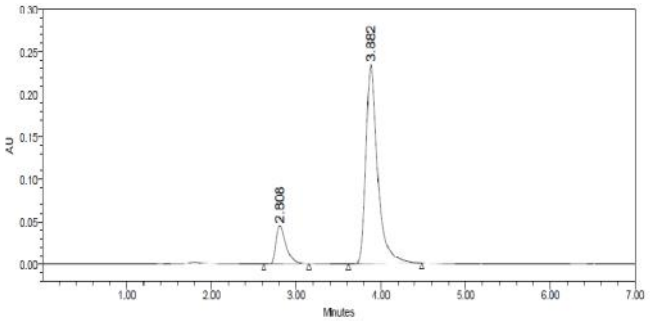


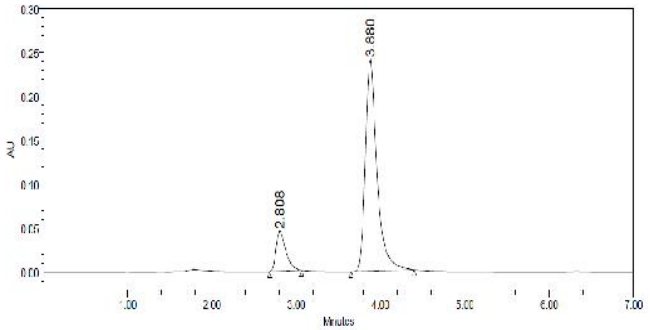
Figure 11: Chromatograms for Intermediate precision

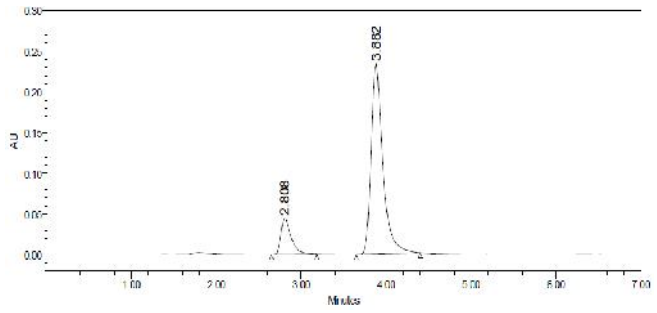
Ruggedness

B) Intermediate precision (Analyst to Analyst variability):









Accuracy

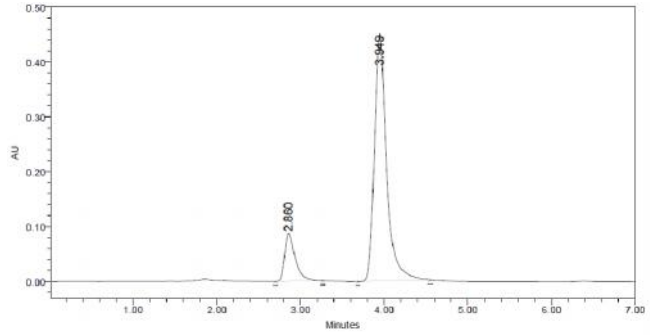
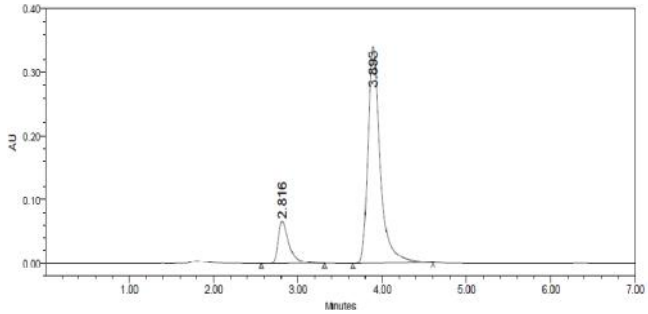
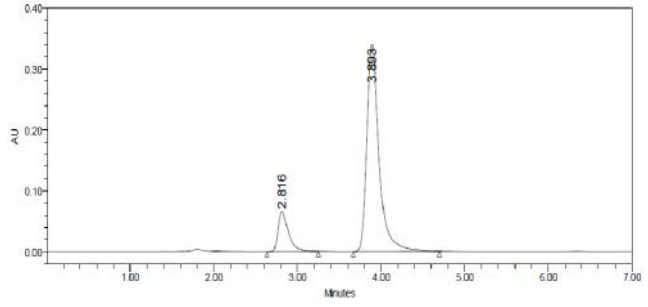


Figure 12: Standard Chromatogram for Accuracy





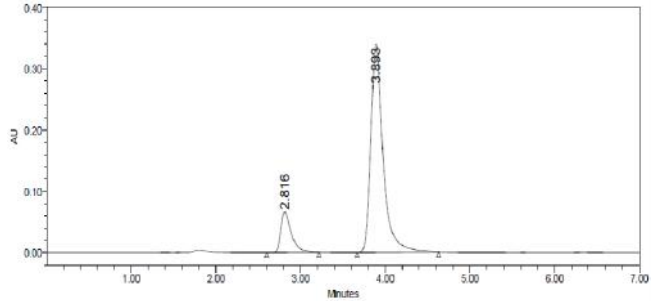


Figure 13: Chromatograms for Accuracy 50%

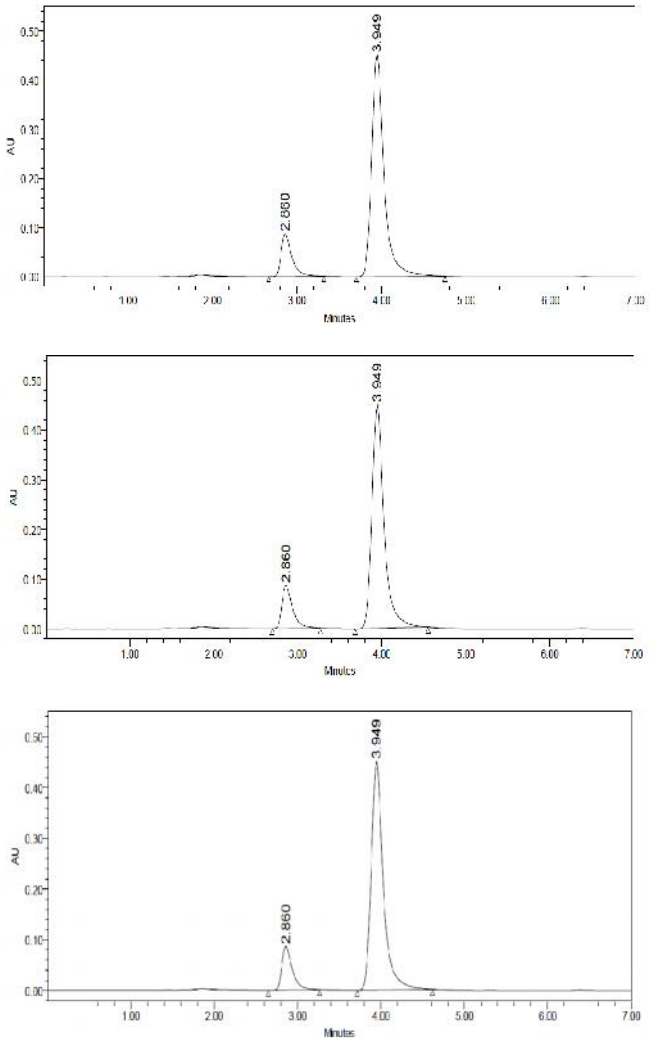


Figure 14: Chromatograms for Accuracy 100%

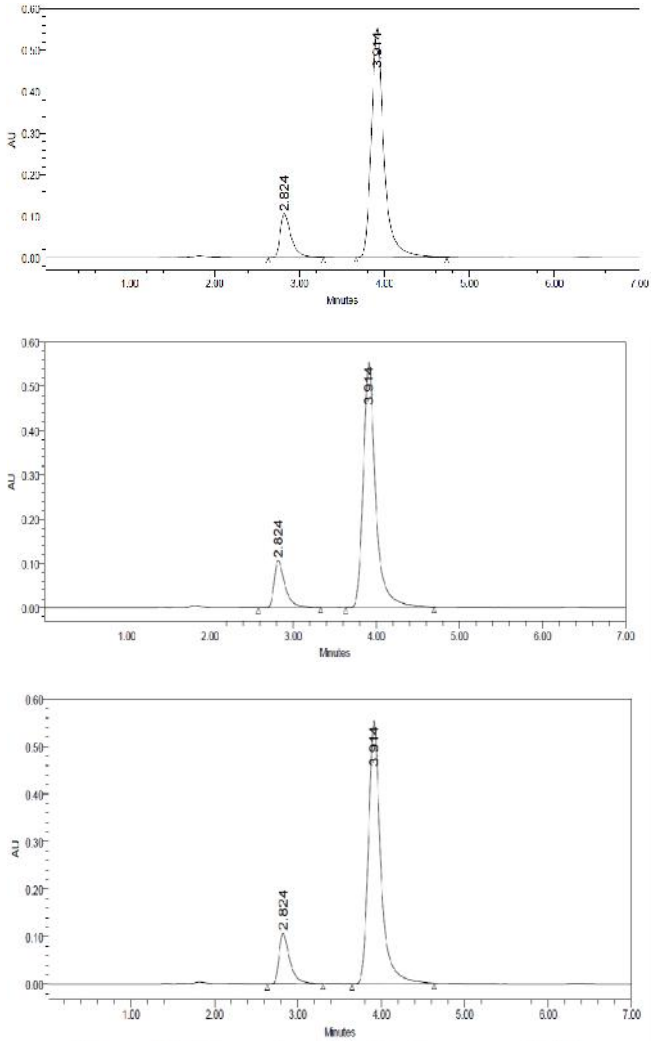


Figure 15: Chromatograms for Accuracy 150%

Table no.1 List of Standard and Sample details

S.No	Name	Batch no.	Manufaturer/ supplier
1.	Aspirin working standard	-	KP labs pvt. Ltd
2.	Atorvastatin working standard	-	KP labs pvt. Ltd

Table 2: List of Equipment/Instrument details

S.No	Instrument name	Model
1.	HPLC system	WATERS, software: Empower, 2695 separation module. 2996 PDA detector.
2.	Semi micro balance	Sartorius ME235P
3.	P^H Meter	Lab India ph. meter
4.	Sonicator	Ultrasonic cleaner power sonic 420
5.	UV/VIS spectrophotometer	LABINDIA UV
6.	Constant temperature water bath	Thermolab GMP

Table 3: List of Chemicals and Reagents

S.No	Name	Manufacturer	Grade
1.	Potassium dihydrogenorthophosphate	Merck	GR
2.	Sodium perchlorate	Merck	GR
3.	Perchloric acid	Merck	GR
4.	Ortho phosphoric acid	Merck	GR
5.	Methanol	Merck	HPLC

6.	Acetontrile	Merck	HPLC
7.	Water	Milli-pore	Milli-Q
8.	0.45 µm Nylon filter	Axivia	S0761009
9.	0.45µm PVDF filter	Rankem	D004A07

Table 4: Linearity results for Aspirin

S.No	Linearity Level	Concentration	Area
1	I	20 ppm	839286
2	II	40 ppm	1067774
3	III	60 ppm	1246474
4	IV	80 ppm	1439994
5	V	100 ppm	1639065
Correlation Coefficient			0.99932

Table 5: Linearity results for Atorvastatin

S.No	Linearity Level	Concentration	Area
1	I	10 ppm	626221
2	II	15 ppm	778750
3	III	20 ppm	931447
4	IV	25 ppm	1070162
5	V	30 ppm	1196060
Correlation Coefficient			0.99916

Table 6: Calibration parameters for Aspirin and Atorvastatin

Parameter	Results for Aspirin	Results for Atorvastatin
Slope	19718	14311
Intercept	65498	49120
Correlation co-efficient	0 .9993	0.99916

Table 7 A: Sample Chromatogram values for Repeatability of Aspirin

Injection No	Peak Area	R_t
1	1247256	2.808
2	1248579	2.807
3	1243273	2.804
4	1243262	2.806
5	1249574	2.805
Avg	1246389	
SD	2965.62	
% RSD	0.23793	

Table 7 B: Sample Chromatogram values for Repeatability of Atorvastatin

Injection No	Peak Area	R_t
1	935035	3.880
2	929353	3.882
3	930459	3.881
4	932389	3.878
5	922057	3.882
Avg	929858.6	
SD	4865.16	
% RSD	0.5232	

Table 8 A: Sample Chromatogram values for intermediate Precision of Aspirin

Injection No	Peak Area	R_t
1	1231404	2.808
2	1233196	2.806
3	1231008	2.805
4	1238575	2.807

5	1232407	2.804
Mean	1233318	**Mean**
SD	3061.06	
%RSD	0.2481	

Table 8 B: Sample Chromatogram values for intermediate Precision of Atorvastatin

Injection No	Peak Area	R_t
1	912412	3.882
2	913062	3.880
3	909642	3.801
4	916881	3.882
5	914005	3.880
Mean	913200.4	
SD	2621.886	
% RSD	0.287	

Table 9: Chromatogram Values for Accuracy of Atorvastatin

Sample No	Spike Level	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery
1	50 %	5	4.9	98%	100%
		5	5.1	102%	
		5	5	100%	
2	100 %	10	9.88	98.8%	99.13%
		10	9.91	99.1%	
		10	9.95	99.5%	
3	150 %	15	14.89	99.2%	99.69%
		15	14.86	99.0%	
		15	14.82	99.79%	

***Acceptance Criteria:** The % Recovery for each level should be between 98.0 to 102.0%

4. Conclusion

A simple, Accurate, precise method was developed for the simultaneous estimation of the Aspirin and Triamterene in Tablet dosage form. Chromatogram was run through Inertsil -Agilent C18, 4.6 mm, 5µm. Mobile phase containing Methanol and Phosphater buffer in the ratio of 70:30 was pumped through column at a flow rate of 0.8ml/min. Optimized wavelength for Aspirine and Atorvastatin was 254nm. Retention time of Aspirine and Atorvastatin calcium were found to be 2.972min and 3.548 min. %RSD of the Aspirine and Atorvastatin calcium were and found to be 0.24 and 0.28 respectively. %assay was obtained for Aspirin and Atorvastatin within the limits. LOD, LOQ values are obtained from Aspirin and Atorvastatin within the limits respectively.

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