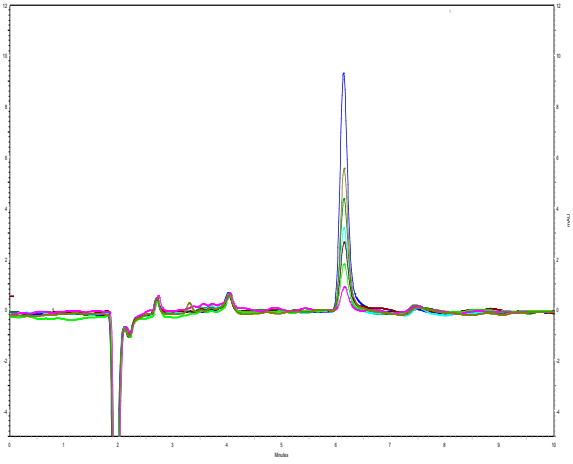
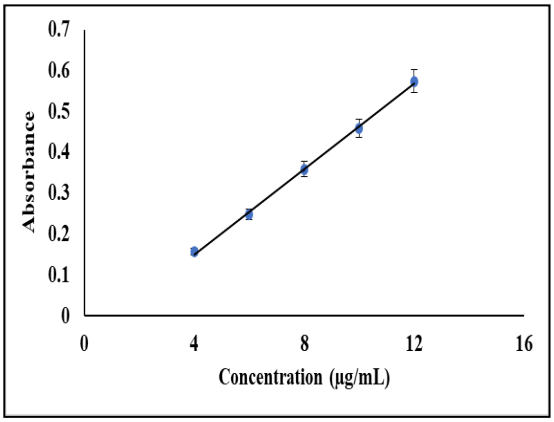


By HPLC

Chapter-3

Analytical techniques

Analysis of drugs



By UV-Visible spectrophotometer

3.1. Equipments:

Digital Analytical Balance (Shimadzu, SCS, Switzerland)

UV Visible spectrophotometer 1900 (Shimadzu, Japan)

HPLC-1220 LC-UV detector (Agilent technology, Germany)

3.2. Analytical techniques:

For estimation of drugs (ATO and RSNa):

Different analytical techniques were used for the quantification of drugs, as follows:

1. UV Visible spectrophotometer (UV-Vis)
2. High Performance Liquid Chromatography with UV detector (HPLC-UV)

3.3. General methods for validation of analytical method:

It is essential to authenticate the performance of an analytical method for acceptable estimation of an analyte. Accuracy, precision, linearity, limit of detection, and limit of quantification are recommended validation parameters [1, 2].

1. Linearity and range:

It is characterized by the ability to elicit tests that are directly or indirectly proportional to the concentration of analyte in samples within a certain range. Analysing separate levels of calibration curve in the given concentration range determined the linearity. The calibration curve of absorbance versus concentration was plotted, and the correlation coefficient and regression line equation were obtained.

2. Precision assay:

It is defined as the degree of agreement between individual test results found when the procedure is applied repeatedly to several samplings of a homogeneous sample. The precision of the test was assessed by repeatability (intraday), intermediate precision (interday), and expressed as % RSD. The % RSD was calculated from the following equation:

$$\%\ Relative\ standard\ deviation\ (\%\ RSD) = \frac{Standard\ deviation}{Average} \times 100$$

3. Accuracy:

It is defined as the extent that the test results obtained by that method come close to the actual value. The following equation was used to determine accuracy:

$$\%\ Accuracy = \frac{Mean\ observed\ concentration}{Actual\ concentration} \times 100$$

4. Limit of detection (LOD):

Under standard experimental conditions, the limit of detection (LOD) is the smallest quantity of analyte in a sample that can be detected but not necessarily quantified.

The following formula was used to calculate LOD:

$$LOD = 3.3 \times \frac{Standard\ deviation}{Slope}$$

5. Limit of quantification (LOQ):

Under standard experimental conditions, the limit of quantification (LOQ) is the lowest quantity of analyte in a sample that can be measured with acceptable precision and accuracy.

The following formula was used to calculate LOQ:

$$LOQ = 10 \times \frac{Standard\ deviation}{Slope}$$

3.4. Analytical technique for Atorvastatin (ATO):

3.4.1. Calibration curve of ATO in methanol by UV Visible spectrophotometer [3]:

- Standard stock preparation:

  100 µg/mL standard stock solution was prepared by dissolving 10 mg of ATO in 100 mL of methanol.

- Procedure for calibration curve in methanol:

  Aliquots of ATO solution (100 µg/mL) ranging from 0.4 to 1.2 ml were transferred into a series of 10 ml volumetric flask and volume was made up to 10 ml with methanol to obtain a concentration range from 4 µg/mL to 12 µg/mL. The absorbance of sample was measured at 246 nm against reference blank (Methanol) by UV Visible spectrophotometer.

Table-3.1. Calibration data of ATO in methanol

Conc. (µg/mL)	Absorbance	%RSD
4	0.1561 ± 0.0018	1.165
6	0.2482 ± 0.0023	0.957
8	0.3589 ± 0.0007	0.189
10	0.4581 ± 0.0058	1.282
12	0.5737 ± 0.0081	1.412

Mean SD	Slope	LOD (µg/mL)	LOQ (µg/mL)
0.0038	0.0523	0.239	0.726

(n=3, ± S.D.)

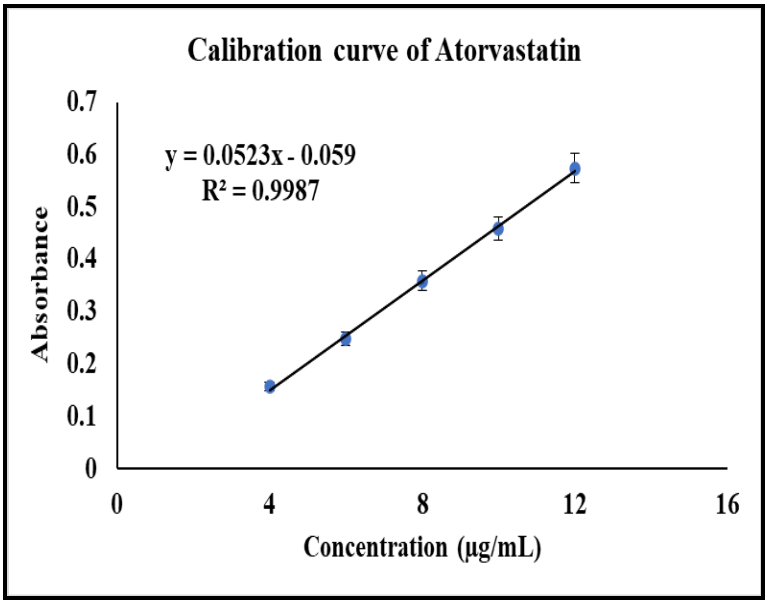


Figure-3.1. Calibration curve of Atorvastatin in methanol

- In methanol, the λ_{max} for atorvastatin was found to be 246 nm. Hence, 246 nm was selected as analytical wavelength. The calibration range is presented in table 3.1, and a standard calibration curve is subsequently developed shown in figure 3.1. The linear relationship between concentration and absorbance was proven by the linear regression analysis's R^2 value of 0.9987.

- Limit of Detection (LOD) and Limit of quantification (LOQ) were found to be 0.239 and 0.726 µg/mL respectively as shown in table-3.1.

3.4.2. Calibration curve in Saline phosphate buffer (pH 7.4):

- Reagent preparation:

  Phosphate buffer pH 7.4 : 2.38 gm of disodium hydrogen phosphate, 0.19 gm of potassium dihydrogen phosphate, and 8.0 gm of sodium chloride were dissolved in sufficient water to produce 1000 ml.

- Standard stock solution preparation:

  10 mg of ATO was dissolved in 100 mL of saline phosphate buffer 7.4 containing 2% v/v propylene glycol, to make a final concentration of 100 µg/mL.

- Procedure for calibration curve:

  Aliquots of ATO solution (100 µg/mL) ranging from 0.4 to 2.4 ml were transferred into a series of 10 ml volumetric flask and volume was made up to 10 ml with saline phosphate buffer 7.4 containing 2% v/v propylene glycol and mixed well to get a range from 4 µg/mL to 24 µg/mL. The absorbance of sample was measured at 244 nm against reference blank (Saline phosphate buffer 7.4 containing 2% v/v propylene glycol) by UV Visible spectrophotometer.

Table-3.2. Calibration data of Atorvastatin in phosphate buffer pH 7.4

Conc. (µg/mL)	Absorbance	%RSD
4	0.152 ± 0.0017	1.14
8	0.296 ± 0.0017	0.586
12	0.441 ± 0.0035	0.796
16	0.599 ± 0.0030	0.509
20	0.751 ± 0.0057	0.768
24	0.903 ± 0.0040	0.447

Mean SD	Slope	LOD (µg/mL)	LOQ (µg/mL)
0.0033	0.0377	0.288	0.875

(n=3, ± S.D.)

- Line of the best fit was determined using least squares mathematical regression analysis. The correlation coefficient value (R2) was calculated to determine the nearness of the data was to the fitted line.

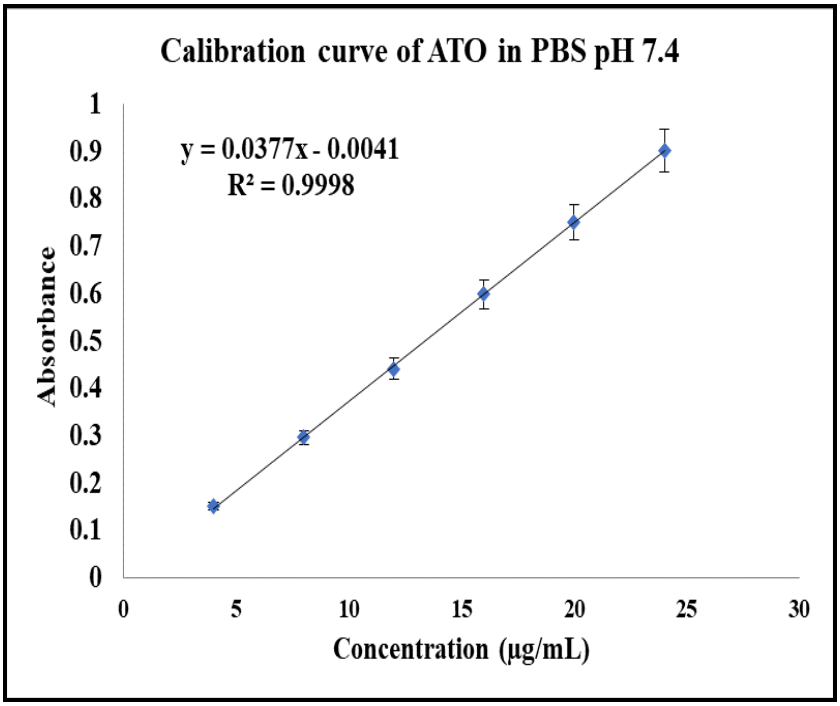


Figure-3.2. Calibration of Atorvastatin in phosphate buffer pH 7.4

- The λ_{max} for ATO in phosphate buffer 7.4 (containing 2 % v/v propylene glycol) was found to be 244 nm. Hence, 244 nm was selected as analytical wavelength. Standard calibration curve was then drawn as shown in figure-3.2 using the range studied as show in table-3.2. The linear regression analysis gave a coefficient of correlation (R2) of 0.9998, indicating a linear relationship between ATO concentration in a saline phosphate buffer at pH 7.4 and absorbance.

- The LOD and LOQ were found to be 0.288 and 0.875 µg/mL respectively as shown in table-3.3. These results indicated the method's sensitivity for accurate drug quantification in samples.

- The accuracy and precision data are presented in table-3.3. The results showed that the proposed method is precise and accurate.

Table- 3.3. Accuracy and precision for ATO in PBS pH 7.4

Level (%)	Standard Concentration (µg/mL)		Precision		Accuracy (%)
	Actual	Observed	Intraday	Interday	
80	14.4	14.20 ± 0.334	1.67	1.72	98.60
100	16.0	15.90 ± 0.225	1.73	1.09	99.37
120	17.6	17.85 ± 0.234	1.16	1.30	101.45

(n=3, ± S.D.)

3.4.3. Calibration curve in Phosphate buffer (pH 5.5):

- Reagent preparation:

  Phosphate buffer pH 5.5:

  Solution A: Accurately weighed 13.61 gm of Potassium dihydrogen phosphate and dissolved in double distilled water and volume was made upto 1000 ml with double distilled water.

  Solution B: Accurately weighed 35.81 gm of Disodium hydrogen phosphate and dissolved in double distilled water and volume was made upto 1000 ml with double distilled water.

  Phosphate buffer pH 5.5: Mixed, 96.4 ml of Solution A and 3.6 ml of solution B.

- Standard stock solution preparation:

  10 mg of ATO was dissolved in phosphate buffer pH 5.5 containing 2% v/v propylene glycol and volume was made up to 100 ml to make final concentration of 100 µg/mL.

- Procedure for calibration curve:

  Aliquots of ATO solution (100 µg/mL) ranging from 0.4 to 2.4 mL were transferred into a series of 10 ml volumetric flask and volume was made up to 10 ml with phosphate buffer solution (PBS) pH 5.5 containing 2% v/v propylene

glycol and mixed well to get a range from 4 µg/mL to 24 µg/mL. The absorbance of sample was measured at 244 nm against reference blank (phosphate buffer 5.5 containing 2% v/v propylene glycol) by UV Visible spectrophotometer.

Table-3.4. Calibration of Atorvastatin in phosphate buffer pH 5.5

Conc. (µg/mL)	Absorbance	%RSD
4	0.154 ± 0.0010	1.120
8	0.333 ± 0.0041	1.240
12	0.510 ± 0.0080	1.580
16	0.691 ± 0.0088	1.280
20	0.885 ± 0.0015	0.172
24	0.958 ± 0.0058	0.605

Mean SD	Slope	LOD (µg/mL)	LOQ (µg/mL)
0.0050	0.0428	0.385	1.16

(n=3, ± S.D.)

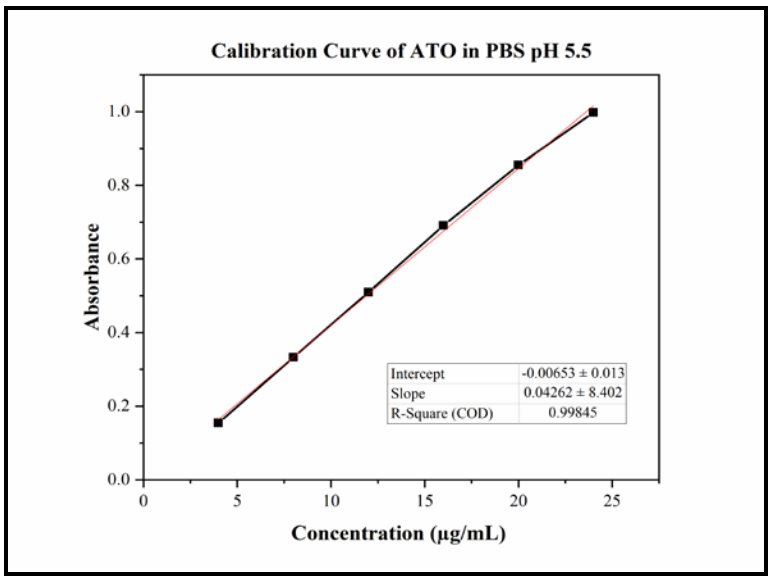


Figure-3.3. Calibration curve of ATO in PBS pH 5.5

- Standard calibration curve was then drawn as shown in figure-3.3 using the range studied as shown in table-3.4. The linear regression analysis gave a coefficient of correlation (R2) of 0.9985, indicating a linear relationship between ATO concentration in a saline phosphate buffer at pH 5.5 and absorbance.

- The LOD and LOQ were found to be 0.385 and 1.16 µg/mL respectively as shown in table-3.5. These results indicated the method's sensitivity for accurate drug quantification in samples.

- The accuracy and precision data are presented in table-3.5. The results showed that the proposed method is precise and accurate.

Table- 3.5. Accuracy and precision for ATO in PBS pH 5.5

Level (%)	Standard Concentration (µg/mL)		Precision		Accuracy (%)
	Actual	Observed	Intraday	Interday	
80	14.4	14.33 ± 0.144	0.99	1.59	99.54
100	16.0	15.78 ± 0.131	1.04	1.31	98.60
120	17.6	17.70 ± 0.109	1.36	0.90	100.55

(n=3, ± S.D.)

3.4.4. Quantification of ATO by RP-HPLC [4]:

- Instrument: 1220 LC (Agilent technology, Germany)

- Chromatographic Condition:

  Column: Agilent 5 HC, C_{18} (250 X 4.6mm), 5 µm,
  Flow rate: 1.0 mL/minute,
  Injection volume: 20 µL,
  Run time: 10 minutes,
  Detection Wavelength: 244 nm,
  Temperature: Ambient.

- Mobile Phase (Isocratic):

  0.05 % formic acid: 50 µL of formic acid in 100 mL of double distilled water.

  Mobile Phase A: Double distilled water pH 2.5 ± 0.05 (Adjusted by 0.05 % of formic acid)

Mobile Phase B: 100% Acetonitrile

Mobile Phase Preparation:

Mixed Mobile Phase A and Mobile phase B in ratio of 30:70. The premixed mobile phase was filtered through 0.45 µ PVDF syringe filter.

- Standard stock solution preparation:

  10 mg ATO was dissolved in 100 mL of methanol to make final concentration of 100 µg/mL.

- Procedure for calibration curve:

  Aliquots from ATO stock solution were taken in 10 mL volumetric flask and volume was made up with mobile phase to get a range from 100 ng/mL to 1000 ng/mL. The area for the range of standard solutions were measured.

Table-3.6. Calibration data for ATO by HPLC

Conc. (ng/mL)	Area (mAU)	% RSD
100	119181 ± 1898.75	1.59
200	230026 ± 3954.99	1.73
300	332284 ± 6368.64	1.90
400	461759 ± 7995.35	1.75
500	576913 ± 5260.35	0.90
1000	1081338 ± 8168.45	0.76

Mean SD	Slope	LOD (ng/mL)	LOQ (ng/mL)
5607.75	1071.8	2.41	7.32

(n=3, ± S.D.)

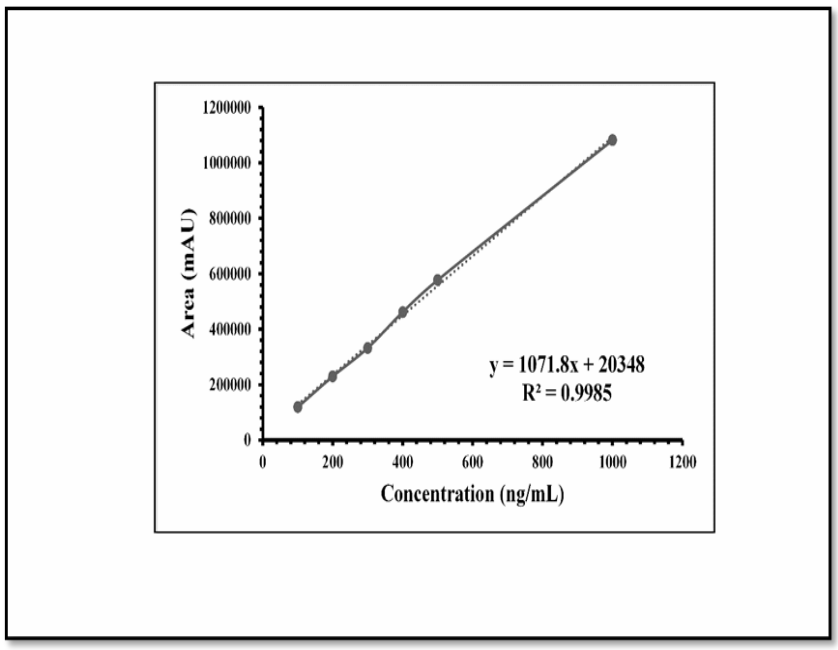


Figure- 3.4. Calibration curve of ATO by RP-HPLC

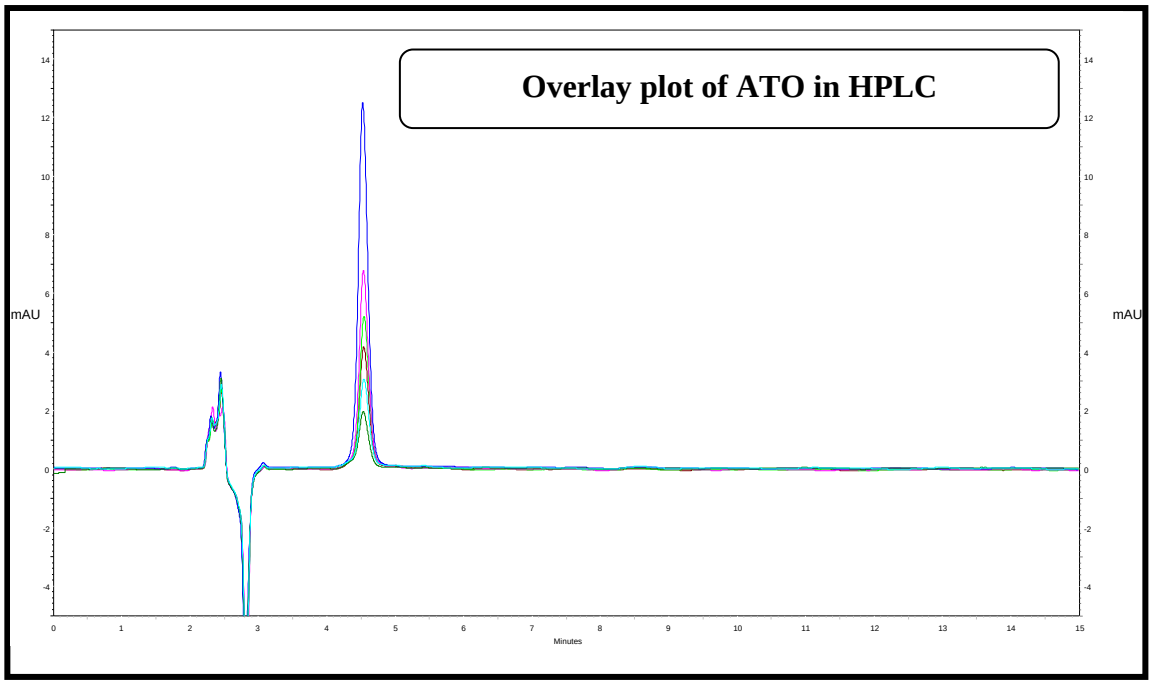


Figure- 3.5. Overlay plot of ATO in RP-HPLC

- The calibration data is shown in table- 3.6 and figure- 3.4. As shown in figure- 3.5, the retention time of ATO was found to be 4.6 ± 0.5 minutes. Regression analysis was used to determine the linearity of the HPLC method for quantifying ATO, and the R^2 value was found to be 0.9985, indicating linear correlation.

- The LOD and LOQ were found to be 2.41 and 7.32 ng/mL respectively. These results indicated the method's sensitivity for accurate drug quantification in samples.

- The accuracy and precision data are presented in table-3.7. The results showed that the proposed method is precise and accurate.

Table- 3.7. Accuracy and precision for ATO by RP-HPLC

Level (%)	**Standard Concentration (ng/mL)**		**Precision**		**Accuracy (%)**
	Actual	**Observed**	**Intraday**	**Interday**	
80	540	535.61 ± 7.14	1.72	1.85	99.19
100	600	605.13 ± 10.01	1.23	1.11	100.85
120	660	656.22 ± 2.75	1.45	0.97	99.43

(n=3, ± S.D.)

3.4.5. Quantification of ATO in plasma by RP-HPLC [4]:

- Instrument: 1220 LC (Agilent technology, Germany)

- Chromatographic Condition:

  Column: Agilent 5 HC, 18 column, 250 X 4.6 mm, 5 µm,
  Flow rate: 1.0 mL/minute,
  Injection volume: 20 µL,
  Run time: 10 minutes,
  Wavelength: 244 nm,
  Temperature ambient.

- Mobile Phase (Isocratic):

  0.05% formic acid: 50 µL of formic acid in 100 mL of double distilled water.

Mobile Phase A: Double distilled water pH 2.5 ± 0.05 (Adjusted by 0.05% of formic acid)
Mobile Phase B: 100% Acetonitrile

Mobile Phase Preparation:

Mixed Mobile Phase A and Mobile phase B in ratio of 30:70. The premix mobile phase was filtered through 0.45 µ PVDF membrane filter.

- Standard stock solution preparation:

  10 mg ATO was dissolved in 100 mL of methanol to make final concentration of 100 µg/mL.

- Preparation of plasma samples:

  Rat plasma was obtained from Pharmacology department, Shri. G H Patel Pharmacy Building, Vadodara. The calibration curve of ATO in plasma was prepared in linearity range of 50 to 1000 ng/mL.

  The samples were prepared by spiking with aliquots from stock solution of 100 µg/mL in 200 µL prepared plasma to get final concentration in the range of 50 to 1000 ng/mL. 0.3 mL of ACN was added for followed by vortex mixed for 2 minutes. After mixing, sample volume was made upto 1 mL with ACN. Then samples were centrifuged at 4000 rpm for about 15 min at room temperature to settle precipitated proteins. The supernatant was separated and filtered through 0.45 µ PVDF syringe filter.

- Method for calibration curve:

  All the plasma samples of ATO were prepared as per above method to get a range from 50 ng/mL to 1000 ng/mL. The area for the range of standard solutions were measured.

- Internal standard: Clinidipine (200 ng/mL)

Table- 3.8. Calibration Data of ATO in Plasma by RP-HPLC

Conc. (ng/ ıL)	Area (mAU)	% RSD
50	50276 ± 1295.51	2.51
100	106423 ± 1932.75	1.78
200	214099 ± 6698.16	3.07
300	334034 ± 10126.96	3.02
400	454442 ± 12176.60	2.66
500	574263 ± 4666.60	0.81
1000	1034253 ± 10854.53	1.06

Mean SD	Slope	LOD (ng/mL)	LOQ (ng/mL)
7976.5	1044.1	3.99	12.10

(n=3, ± S.D.)

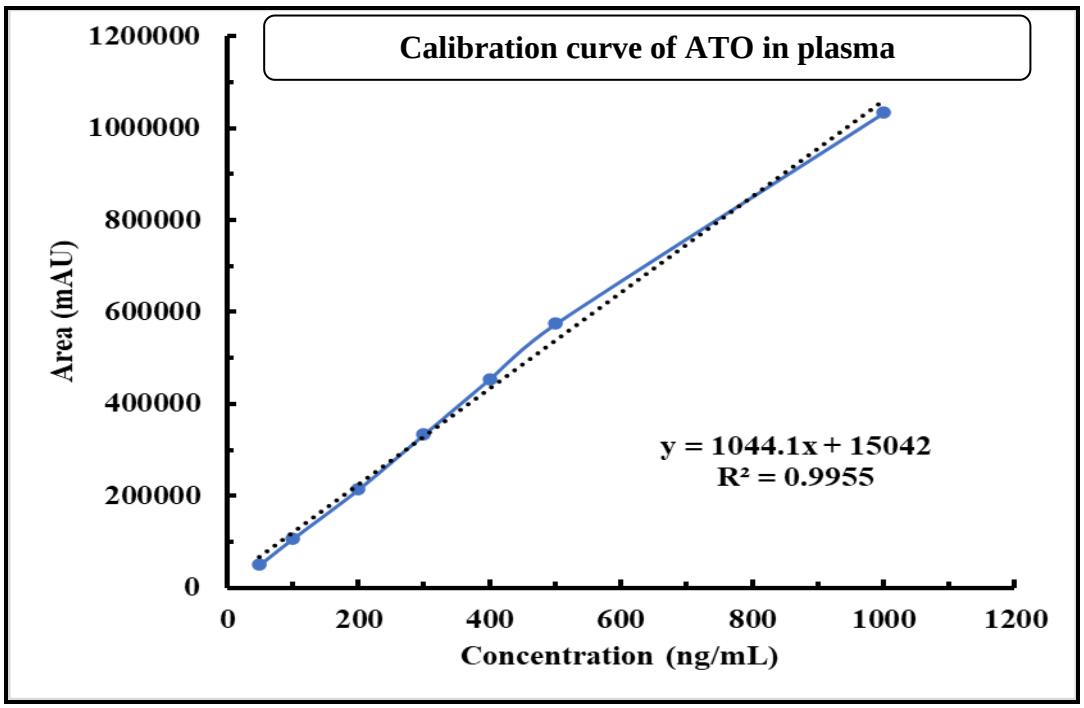


Figure- 3.6. Calibration curve of ATO in plasma by HPLC

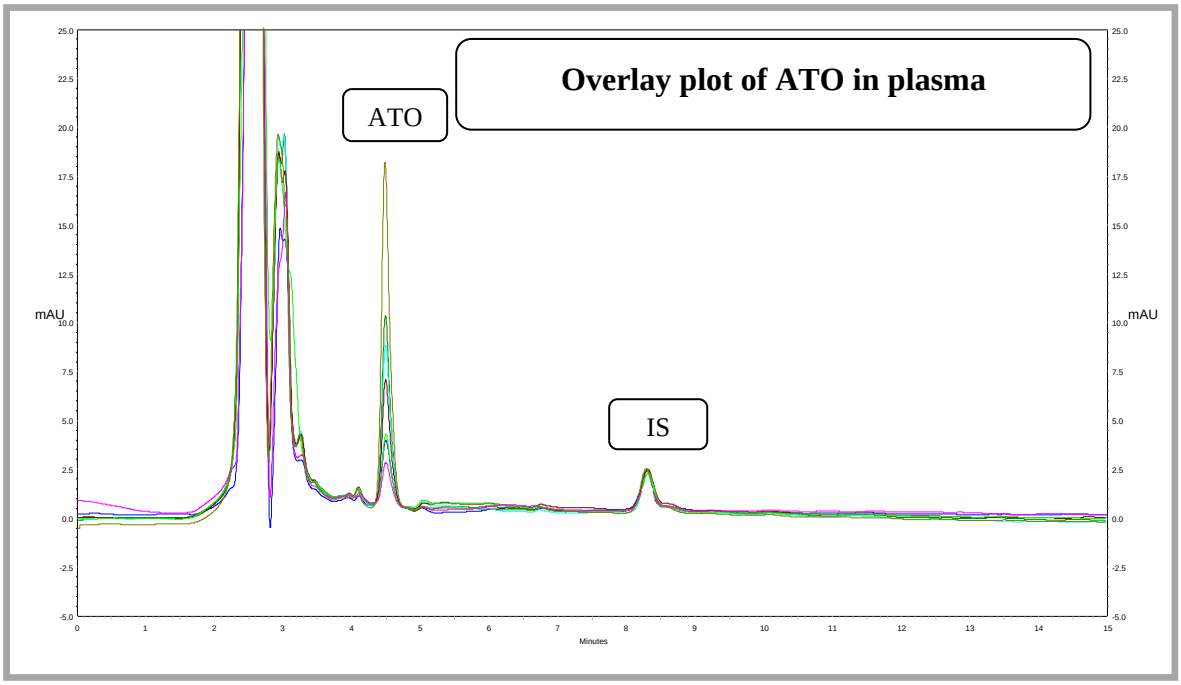


Figure- 3.7. Overlay plot of ATO in plasma by HPLC

- The calibration data is shown in table- 3.8 and figure- 3.6. As shown in figure- 3.7, the retention time of ATO in plasma was found to be 4.5 ± 0.5 minutes and that of internal standard was found to be 8.3± 0.5 minute. Regression analysis was used to determine the linearity of the HPLC method for quantifying ATO, and the R^2 value was found to be 0.9955, indicating linear correlation.

- The LOD and LOQ were found to be 3.99 and 12.10 ng/mL respectively. This indicates sensitivity of method for accurate determination of drug in plasma sample.

- The accuracy and precision data are presented in table-3.9. The results showed that the proposed method is precise and accurate.

Table- 3.9. Accuracy and precision for ATO in plasma by RP-HPLC

Level (%)	Standard Concentration (ng/mL)		Precision		Accuracy (%)
	Actual	Observed	Intraday	Interday	
80	540	490.35 ± 9.62	1.69	1.06	90.80
100	600	530.29 ± 6.78	1.44	1.66	88.38
120	660	606.54 ± 11.17	1.26	1.88	91.90

(n=3, ± S.D.)

3.5. Analytical technique for Risedronate sodium:

3.5.1. Calibration curve of Risedronate sodium in distilled water [5]:

- Standard preparation:

  Weighed accurately about 5 mg of Risedronate sodium (RSNa) and dissolved in 50 ml of distilled water to produce 100 µg/mL standard solution.

- Procedure for calibration curve in distilled water:

  Aliquots of RSNa solution (100 µg/mL) ranging from 0.5 to 5 ml were transferred into a series of 10 ml volumetric flask and volume was made up to 10 ml with distilled water and mixed well to get a range from 5 µg/mL to 50 µg/mL. The absorbance of sample was measured at 262 nm against reference blank (Distilled water) by UV Visible spectrophotometer.

- Standard calibration curve was then drawn as shown in figure-3.8 using the linearity studied as shown in table-3.10. The linear relationship between concentration and absorbance was indicated by the linear regression analysis's R^2 value of 0.9989.

Table- 3.10. Calibration data of Risedronate in distilled water

Conc. (µg/mL)	Absorbance	% RSD
5	0.104 ± 0.0020	1.935
10	0.123 ± 0.0026	1.439
20	0.373 ± 0.0032	0.863
30	0.554 ± 0.0059	1.073
40	0.759 ± 0.0078	1.038
50	0.913 ± 0.0102	1.126

Mean SD	Slope	LOD (µg/mL)	LOQ (µg/mL)
0.0052	0.0183	0.938	2.842

(n=3, ± S.D.)

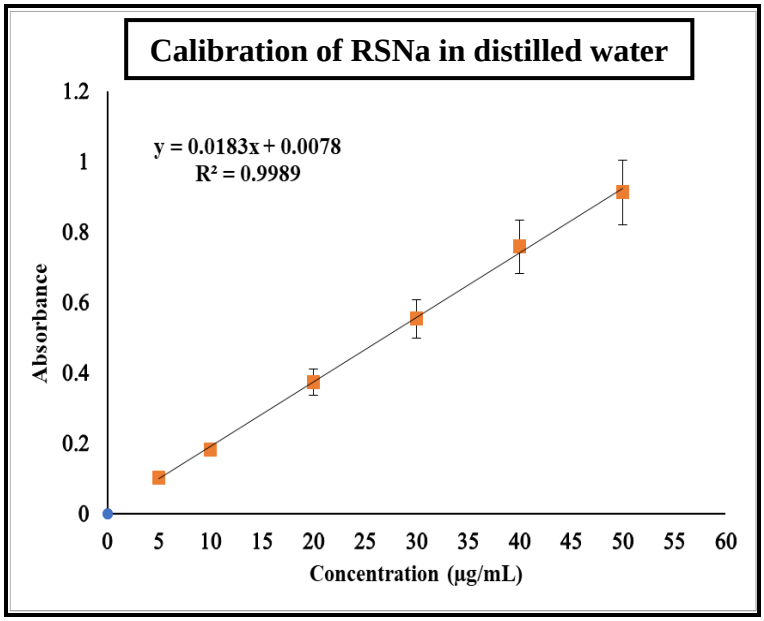


Figure-3.8. Calibration curve of Risedronate sodium in distilled water

- The LOD and LOQ were found to be 0.955 and 2.896 µg/mL respectively as shown in table-3.10. These confirmed the method's sensitivity for the precise quantification of the drug in the sample.

3.5.2. Quantification of RSNa by RP-HPLC [6]:

*Note: This method was used for in vitro drug release study and ex vivo skin permeation studies.

- Instrument: 1220 LC (Agilent technology, Germany)
- Chromatographic Condition:

  Column: Hypersil BDS, C_{18} column, (250 X 4.6 mm), i.d. 5 µm (Make: Thermo scientific)
  Flow rate: 1.0 mL/minute,
  Injection volume: 20 µL,
  Run time: 10 minutes,
  Wavelength: 262 nm,
  Temperature: 35°C.

- Mobile Phase (Isocratic):

  Mobile Phase A: Aqueous solution of buffer contained 11 mM sodium phosphate, 5 mM EDTA-2Na and 5 mM tetrabutylammonium hydroxide dissolved in double distilled water.
  Mobile Phase B: 100% Methanol.

  Mobile Phase Preparation:
  Mixed well Mobile Phase A and Mobile phase B in ratio of 88:12 and was adjusted to pH 6.75 by 1% NaOH and then filtered through 0.45 µ PVDF syringe filter.

- Procedure for calibration curve:

  Aliquots from RSNa stock solution were taken in 10 mL volumetric flask and volume was made up with mobile phase to get a range from 0.5 µg/mL to 6.0 µg/mL. The area for the range of standard solutions were measured.

Table-3.11. Calibration Data of RSNa by HPLC

Conc. (µg/mL)	**Area (mAU)**	**% RSD**
0.5	97018.33 ± 1500.47	1.54
1.0	242993.70 ± 4499.88	1.85
1.5	366801.30 ± 3332.81	0.90
2.0	463793.00 ± 5068.34	1.09
2.5	588131.70 ± 10179.91	1.76
3.0	680500.00 ± 5573.73	0.87
6.0	1214966.00 ± 9814.17	0.80

Mean SD	**Slope**	**LOD (µg/mL)**	**LOQ (µg/mL)**
5709.91	199222	0.094	0.287

(n=3, ± S.D.)

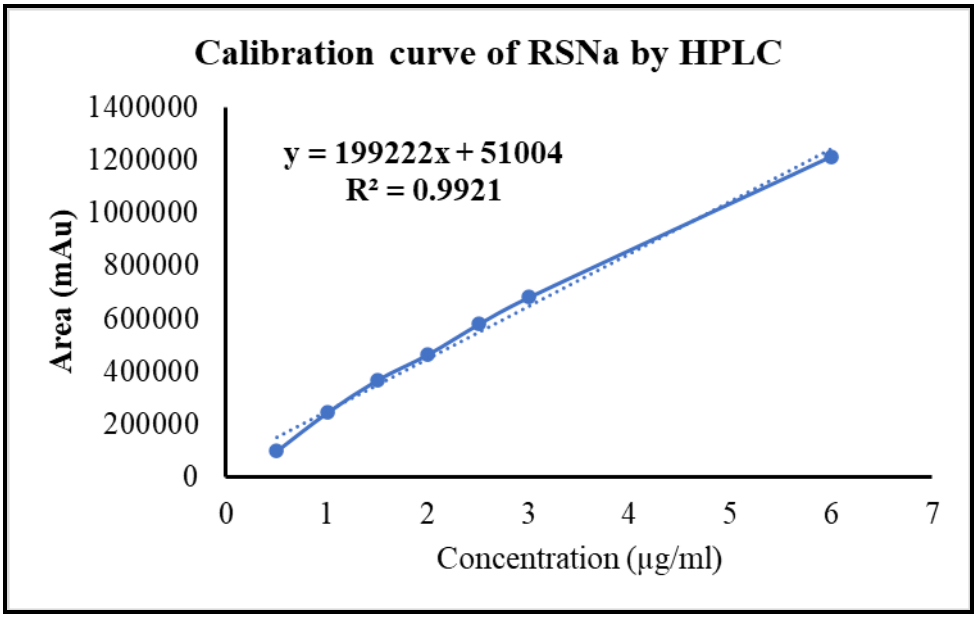


Figure- 3.9. Calibration curve of RSNa by RP-HPLC

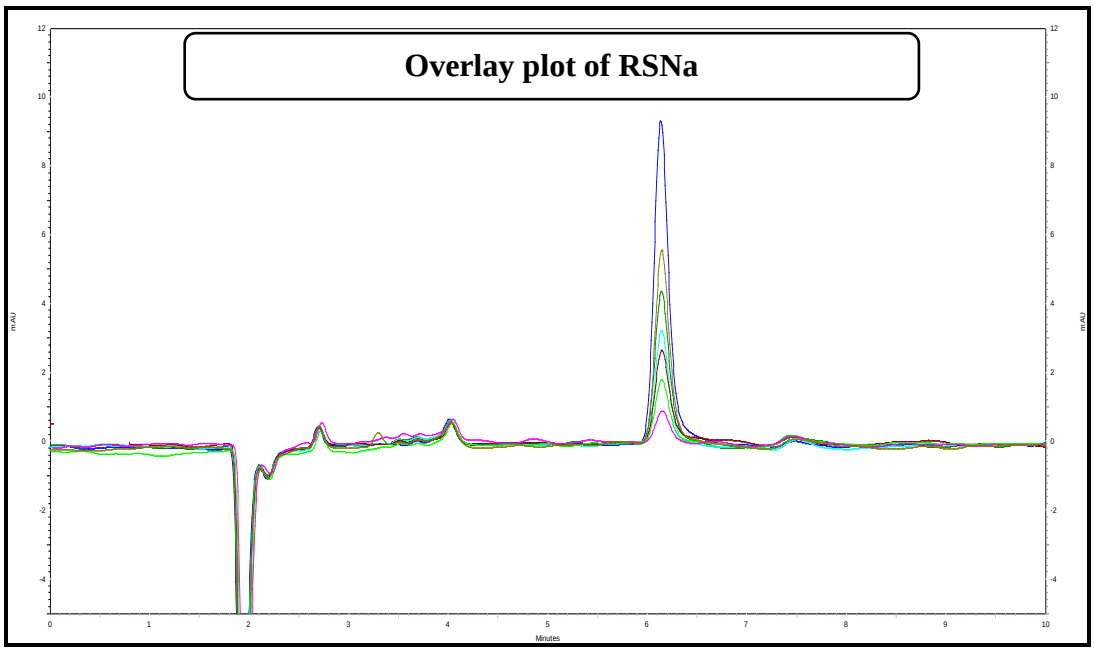


Figure- 3.10. Overlay plot of RSNa by RP-HPLC

- The calibration data is shown in table- 3.9. As per shown in figure- 3.10, the retention time of RSNa in samples was found to be 6.14 ± 0.5 minute. Regression analysis was used to determine the linearity of the HPLC method for quantifying ATO, and the R^2 value was found to be 0.9921, indicating linear correlation. The LOD and LOQ were found to be 0.094 and 0.287 µg/mL respectively. These confirmed the method's sensitivity for the precise quantification of the drug in the sample.

- The accuracy and precision data are presented in table-3.12. The results showed that the proposed method is precise and accurate.

Table- 3.12. Accuracy and precision for RSNa by HPLC

Level (%)	Standard Concentration (ng/mL)		Precision		Accuracy (%)
	Actual	Observed	Intraday	Interday	
80	3.6	3.55 ± 0.03	1.71	1.90	98.73
100	4.0	3.92 ± 0.04	1.47	1.42	98.04
120	4.4	4.33 ± 0.06	1.52	1.35	98.51

(n=3, ± S.D.)

3.5.3. Quantification of RSNa in plasma by RP-HPLC:

- Preparation of plasma samples:

The samples were prepared by spiking with aliquots from stock solution of 100 µg/mL to get concentration in the range of 400 to 1200 ng/mL in 200 µL of plasma. After dilution with approximately 1.4 mL of ultrapure water, the sample was deproteinized by the slow addition (during Vortexing) of 450 µl of 10% (w/v) trichloroacetic acid (TCA), then centrifuged for 15 min at 1500 × g at room temperature. The supernatant was transferred to another tube. A 50 µL aliquot of 1.25 M $CaCl_2$, followed by 57 µL of 30% (w/v) NaOH (freshly prepared) were added to induce the formation of an obvious white precipitate. After thoroughly mixing of the sample with a vortex mixer, the sample was centrifuged for 10 min at 4500 × g. The supernatant was discarded and the pellet was dissolved in 400 µL of 1 M HCl and water heated at 90 °C for 30 min to hydrolyse any pyrophosphate which containing in the plasma. An additional 25 µl of 1.25 M $CaCl_2$, and 90 µl of 30% NaOH were added. This was a critical step to achieve a high extraction recovery of risedronate in plasma. The sample was centrifuged for 10 min at 4500 × g. The pellet was dissolved by addition of 50 µL of 1 M HCl followed by dilution with 2 mL of deionized water. After addition of 45 µL of 30% NaOH, a slight white precipitate formed, and the sample was centrifuged at

4500 × g for 10 min. The last precipitate was dissolved in 80 µL of 0.025 M EDTA-2Na, added 920 µL of mobile phase, vortexed for 10 s, filtered, and subjected to HPLC analysis [6].

- Instrument: 1220 LC (Agilent technology, Germany)

- Chromatographic Condition:

  Column: Hypersil BDS, C_{18} column, (250 X 4.6 mm), i.d 5 µm (Make: Thermo scientific)
  Flow rate: 1.0 mL/minute,
  Injection volume: 50 µL,
  Run time: 10 minutes,
  Wavelength: 262 nm,
  Temperature: 35°C.

- Mobile Phase (Gradient):

- Buffer preparation: Aqueous solution of buffer contained 11 mM sodium phosphate, 5 mM EDTA-2Na and 5 mM tetrabutylammonium hydroxide dissolved in double distilled water and adjusted to pH 6.75 by 1% NaOH.

  Organic Phase: 100% Methanol.

  Mobile Phase A: 80:20∷Buffer: Methanol
  Mobile Phase B: 20:80∷ Methanol: Buffer.

- Gradient programme:

Time (Minutes)	Mobile phase-A (%)	Mobile phase-B (%)
0.00	20	80
3.50	50	50
7.00	50	50
9.00	40	60
10.00	40	60
12.00	20	80

- Internal standard: Ascorbic acid (100 ng)

- Method for calibration curve:

  Aliquots from RSNa stock solution were taken in 10 mL volumetric flask and volume was made up with mobile phase to get a range from 400 ng/mL to 1200 ng/mL. The area for the range of standard solutions were measured.

Table- 3.13. Calibration data of RSNa in plasma by HPLC

Conc. (ng/mL)	Area (mAU)	% RSD
400	326100.33 ± 6381.31	1.96
600	459386.00 ± 8236.43	1.79
800	595609.33 ± 10255.35	1.72
1000	756474.33 ± 9532.10	1.26
1200	977420.67 ± 9600.58	0.98

Mean SD	Slope	LOD (ng/mL)	LOQ (ng/mL)
1538.06	799.86	6.37	19.28

(n=3, ± S.D.)

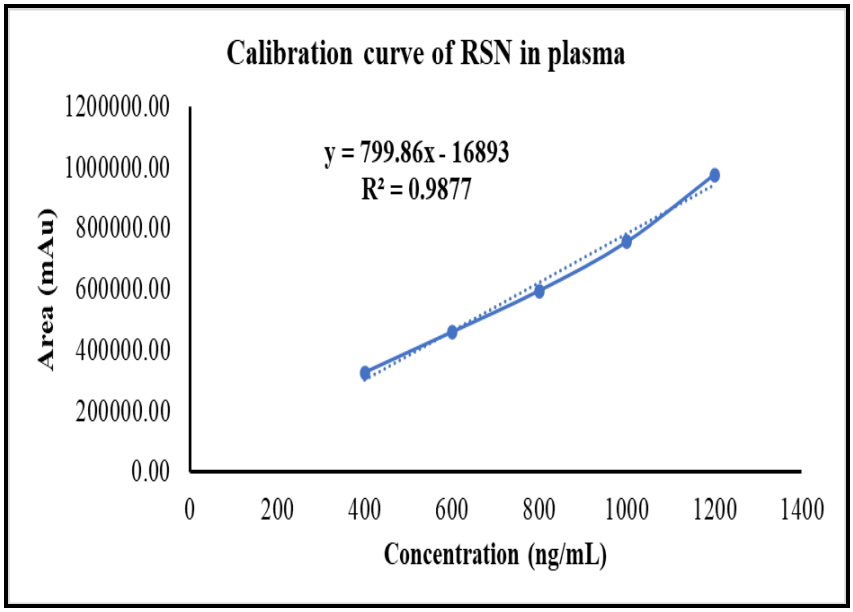


Figure-3.11. Calibration curve of RSNa in plasma

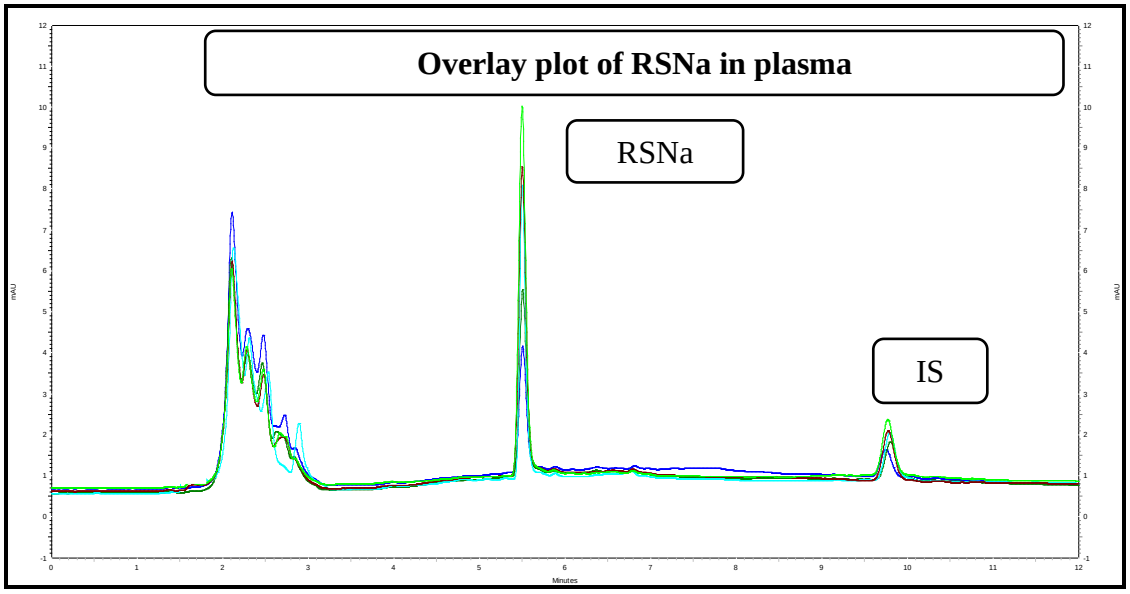


Figure-3.12. Overlay plot of RSNa in plasma by RP-HPLC

- The calibration data is shown in table- 3.11. As per shown in figure- 3.12, the retention time of RSNa in plasma was found to be 5.61 ± 0.5 minute and that of internal standard was found to be 9.9 ± 0.5 minute. Regression analysis was used to determine the linearity of the HPLC method for quantifying ATO, and the R^2 value was found to be 0.9877, indicating linear correlation.

- The LOD and LOQ were found to be 6.37 and 19.28 ng/mL respectively. These confirmed the method's sensitivity for the precise quantification of the drug in the plasma sample.

- The accuracy and precision data are presented in table-3.14. The results showed that the proposed method is precise and accurate.

Table- 3.14. Accuracy and precision for RSNa in plasma by HPLC

Level (%)	Standard Concentration (ng/mL)		Precision		Accuracy (%)
	Actual	Observed	Intraday	Interday	
80	720	659.27 ± 10.61	1.27	1.22	91.56
100	800	88.50 ± 11.69	1.23	1.51	88.50
120	880	791.05 ± 7.31	1.89	1.71	89.89

(n=3, ± S.D.)

Chapter 3 – Analytical Techniques

3.6. References:

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