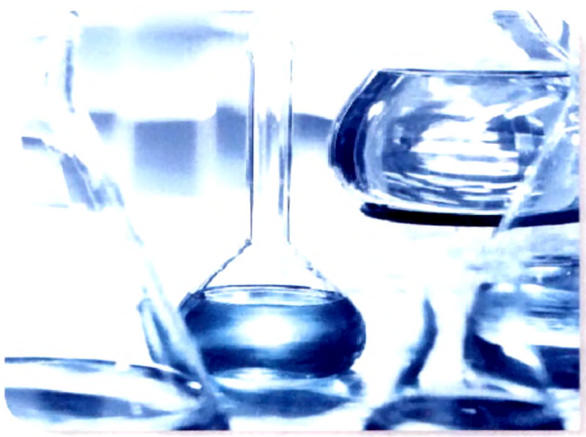
Chapter 3

Analytical Methods



3.1. Introduction

The analytical methods employed in the optimization, preparation and characterization of modified chitosan and unmodified chitosan nanoparticles of both tizanidine HCl and cyclobenzaprine HCl are discussed below. The modified chitosan and unmodified chitosan were characterized by FTIR and ^{1}HNMR (see chapter 4). The nanoparticles were characterized for particle size, zeta potential, surface morphology, *in- vitro* drug release (see chapter 6). The amount of immobilized thiol groups in thiolated chitosan was determined via Ellman's reagent (see chapter 4). RPMI 2650 cells were used to assess the *in vitro* cytotoxicity study of the modified and unmodified chitosan and its formulations (see chapter 8). The stability studies of modified and unmodified chitosan NPs were conducted to determine the particle size, zeta potential, % EE, *in- vitro* drug release and the physical changes like caking and discoloration (see chapter 7). *In vivo* studies in animals include tissue biodistribution in the different body organs or tissues. The biodistribution studies were carried out after radiolabeling of the drug and the nanoparticles formulation with ^{99m}Tc. The distribution of drug in brain was confirmed by gamma scintigraphy technique (see chapter 9).

3.1.1. Materials and Equipments

Table 3.1: List of materials and equipments

Material	Source
Water (distilled)	Prepared in laboratory by distillation
Chitosan low molecular weight and medium molecular weight	Sigma Aldrich, Bangalore, India
Sodium alginate	National chemical, Mumbai, India
Sodium deoxy cholate	Sigma Aldrich, Bangalore, India
Glacial acetic acid, sodium hydroxide, Hydrochloric acid	S.D. Fine chemicals, Mumbai, India
Tizanidine HCl	Gift sample from Endoc Pharma, Rajkot, India
Cyclobenzaprine HCl	Gift sample from Ranbaxy, Gurgaon, India
Sodium iodide and Methyl iodide	Spectrochem, Mumbai, India
Thioglycollic acid and acetonitrile (HPLC grade)	Merck, Mumbai, India
Ellman's reagent	Himedia
Rhodamine B	Himedia
HPLC grade methanol, acetonitrile	Loba Chemicals, India.
Nuclepore Polycarbonate membrane 2 µm 25mm	Whatman, USA

Equipments	Make
Calibrated pipettes of 1.0 ml, 5.0 ml and 10.0 ml, volumetric flasks of 10 ml, 25 ml, 50 ml and 100 ml capacity, Funnels (i.d. 5.0 cm), beakers (250 ml) and other requisite glassware	Schott & Corning (India) Ltd., Mumbai
Analytical balance	AX 120, EL 8300, Shimadzu Corp., Japan
pH meter	Pico^{+} Labindia, Mumbai, India
Cyclomixer, magnetic stirrer	Remi Scientific Equipments, Mumbai
Cooling Centrifuge	3K 30, Sigma Laboratory centrifuge, Osterode, GmBH.
Lyophilizer	DW1, 0-60E, Heto Drywinner, Denmark
UV-Visible Spectrophotometer	Shimadzu UV-1601, Japan
Particle and Zeta size Analyzer	Malvern zeta sizer NanoZS, U.K.
Transmission electron microscopy	Morgagni, Philips, Netherlands
^{1}H-NMR	av300, Bruker, UK
HPLC system	LC 20-AT prominence, Shimadzu Corp., Japan
Stability oven	Shree Kailash Industries, Vadodara
Vacuum Pump F16	Bharat Vacuum pumps, Bangalore
Bath sonicator	INCO, Ambala

3.2. Estimation of Tizanidine HCl and Cyclobenzaprine HCl by U.V. spectroscopy method

3.2.1. Estimation of Tizanidine HCl in distilled water

Tizanidine HCl shows strong absorbance in UV-Visible region. Hence, the estimation of tizanidine HCl was performed by UV-visible spectrophotometry. A common method for estimation of drug content, entrapment efficiency and *in vitro* release was developed. The method was developed in distilled water.

Preparation of standard stock solutions of Tizanidine HCl in distilled water

25 mg of Tizanidine HCl was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 50 ml of distilled water was accurately measured and transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 50 ml with distilled water produce 500 µg/ml of tizanidine HCl.

Calibration curve of Tizanidine HCl

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with distilled water to give final concentrations of 5, 7.5, 10, 12.5, 15, 17.5 µg/ml and

analyzed by UV spectrophotometry at 320nm. The above procedure was repeated three times. The data was recorded in Table 3.2 along with standard deviation. Figures 3.1 show the calibration curve of Tizanidine HCl in distilled water.

Table 3.2: Calibration for Tizanidine HCl in distilled water

Concentration (µg/ ml)	Mean Absorbance* ± S.D
0	0
5	0.264±0.002
7.5	0.363±0.011
10	0.479±0.003
12.5	0.597±0.004
15	0.779±0.001
17.5	0.857±0.004

Regression equation** y = 0.0495x - 0.0001; Correlation coefficient = 0.995

***(Mean ± S.D., *n* = 3)**

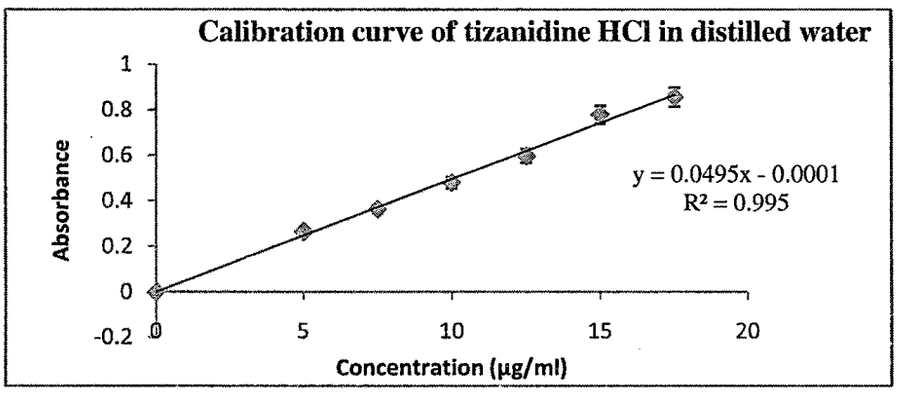


Figure 3.1: Regressed calibration curve for estimation of Tizanidine HCl in distilled water

Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of Tizanidine HCl (5µg/mL, 10µg/mL and 15µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 3.3.

Table 3.3: Evaluation of accuracy and precision of the estimation method of Tizanidine HCl

Concentration Added (µg/ml)	Mean ± SD	Recovery ± RSD	% RSD or *coefficient of variance (CV)*
5	5.1±0.115	102 ± 0.0225	2.25
10	9.9±0.126	99 ± 0.0127	1.27
15	15.1±0.189	100.66 ± 0.0125	1.25

(Mean ± SD, n=3)

3.2.2. Estimation of Tizanidine HCl in Phosphate buffer (pH 5)

Tizanidine HCl shows strong absorbance in UV-Visible region. Hence, the estimation of Tizanidine HCl was performed by UV-visible spectrophotometry. A common method for estimation of drug content, entrapment efficiency and *in vitro* release was developed. The method was developed in phosphate buffer (pH 5).

Preparation of standard stock solutions of Tizanidine HCl in Phosphate buffer (pH 5)

25 mg of Tizanidine HCl was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 50 ml of phosphate buffer (pH 5) was accurately measured and transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 50 ml with phosphate buffer (pH 5) produce 500 µg/ml of Tizanidine HCl.

Calibration curve of Tizanidine HCl

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with phosphate buffer (pH 5) to give final concentrations of 2.5, 5, 7.5, 10, 12.5, 15, 17.5 µg/ml and analyzed by UV spectrophotometry at 320nm. The above procedure was repeated three times. The data was recorded in Table 3.4 along with standard deviation. Figures 3.2 show the calibration curve of Tizanidine HCl in phosphate buffer (pH 5).

Table 3.4: Calibration for Tizanidine HCl in phosphate buffer (pH 5)

Concentration (µg/ ml)	Mean Absorbance* ± S.D
0	0
2.5	0.156±0.004
5	0.234±0.004
7.5	0.348±0.003
10	0.518±0.003
12.5	0.641±0.006
15	0.775±0.004
17.5	0.886±0.004

Regression equation** y= 0.0509x - 0.0003; Correlation coefficient = 0.996

***(Mean ± S.D., n = 3)**

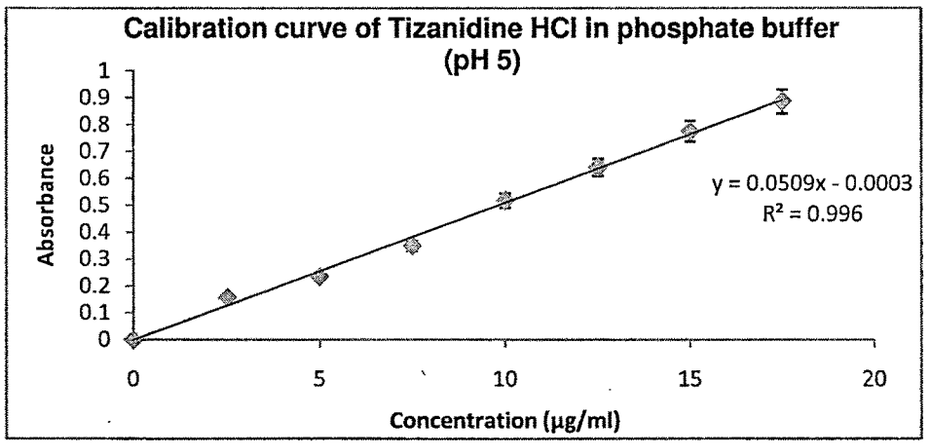


Figure 3.2: Regressed calibration curve for estimation of Tizanidine HCl in phosphate buffer (pH 5)

Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of Tizanidine HCl (2.5µg/mL, 10µg/mL and 15µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 3.5.

Table 3.5: Evaluation of accuracy and precision of the estimation method of Tizanidine HCl

Concentration Added (µg/ml)	Mean ± SD	Recovery ± RSD	% RSD or *coefficient of variance (CV)*
2.5	2.4±0.027	102 ± 0.0112	1.12
10	10.1±0.173	101 ± 0.0171	1.71
15	14.9±0.165	99.33 ± 0.0110	1.10

(Mean ± S.D., n = 3)

3.2.3. Estimation of Cyclobenzaprine HCl in distilled water

Cyclobenzaprine HCl shows strong absorbance in UV-Visible region. Hence, the estimation of Cyclobenzaprine HCl was performed by UV-visible spectrophotometry. A common method for estimation of drug content, entrapment efficiency and *in vitro* release was developed. The method was developed in distilled water.

Preparation of standard stock solutions of Cyclobenzaprine HCl in distilled water

25 mg of Cyclobenzaprine HCl was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 50 ml of distilled water was accurately measured and transferred to the above volumetric flask, the drug was

dissolved properly and then the final volume of the flask was made up to 50 ml with water produce 500 µg/ml of Cyclobenzaprine HCl.

Calibration curve of Cyclobenzaprine HCl

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with distilled water to give final concentrations of 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25µg/ml and analyzed by UV spectrophotometry at 290nm. The above procedure was repeated three times. The data was recorded in Table 3.6 along with standard deviation. Figures 3.3 show the calibration curve of Cyclobenzaprine HCl in distilled water.

Table 3.6: Calibration for Cyclobenzaprine HCl in distilled water

Concentration (µg/ ml)	Mean Absorbance* ± S.D
0	0
5	0.228±0.009
7.5	0.252±0.009
10	0.331±0.019
12.5	0.403±0.007
15	0.477±0.010
17.5	0.565±0.006
20	0.649±0.007
22.5	0.738±0.010
25	0.814±0.002

Regression equation** y = 0.031x + 0.021; Correlation coefficient = 0.993

***(Mean ± S.D., *n* = 3)**

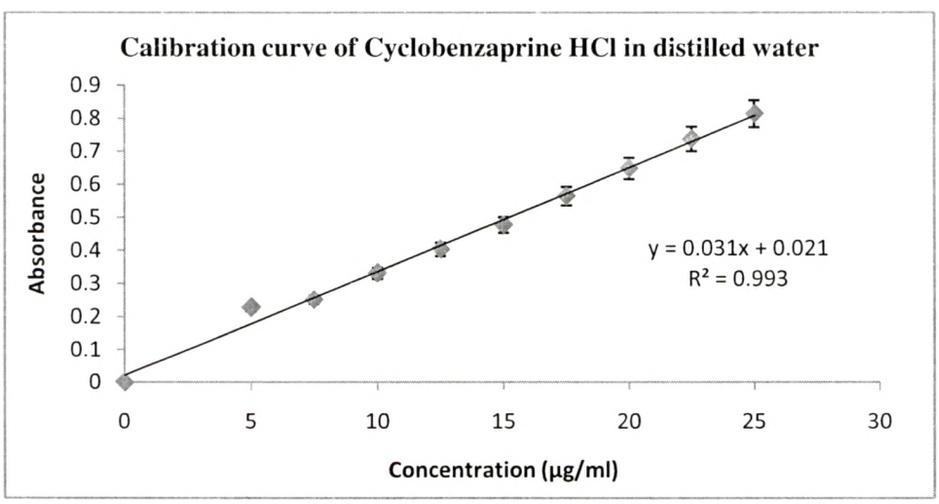


Figure 3.3: Regressed calibration curve for estimation of Cyclobenzaprine HCl in distilled water

Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of Cyclobenzaprine HCl (5µg/mL, 15µg/mL and 25µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 3.7.

Table 3.7: Evaluation of accuracy and precision of the estimation method of Cyclobenzaprine HCl

Concentration Added (µg/ml)	Mean ± SD	Recovery ± RSD	% RSD or *coefficient of variance (CV)*
5	4.9±0.067	98.00 ± 0.0136	1.36
15	14.8±0.169	98.66 ± 0.0114	1.14
25	24.9±0.335	99.60 ± 0.0134	1.34

(Mean ± S.D., *n* = 3)

3.2.4. Estimation of Cyclobenzaprine HCl in phosphate buffer (pH 5)

Cyclobenzaprine HCl shows strong absorbance in UV-Visible region. Hence, the estimation of Cyclobenzaprine HCl was performed by UV-visible spectrophotometry. A common method for estimation of drug content, entrapment efficiency and *in vitro* release was developed. The method was developed in phosphate buffer (pH 5).

Preparation of standard stock solutions of Cyclobenzaprine HCl in phosphate buffer (pH 5)

25 mg of Cyclobenzaprine HCl was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 50 ml of phosphate buffer (pH 5) was accurately measured and transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 50 ml with phosphate buffer (pH 5) produce 500 µg/ml of Cyclobenzaprine HCl.

Calibration curve of Cyclobenzaprine HCl

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with phosphate buffer (pH 5) to give final concentrations of 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25µg/ml and analyzed by UV spectrophotometry at 290nm. The above procedure was repeated three times. The data was recorded in Table 3.8 along with standard deviation. Figures 3.4 show the calibration curve of Cyclobenzaprine HCl in phosphate buffer (pH 5).

Table 3.8: Calibration for Cyclobenzaprine HCl in phosphate buffer (pH 5)

Concentration (µg/ ml)	Mean Absorbance* ± S.D
0	0
5	0.189±0.002
7.5	0.246±0.003
10	0.320±0.001
12.5	0.392±0.002
15	0.494±0.003
17.5	0.589±0.006
20	0.647±0.004
22.5	0.797±0.001
25	0.817±0.003

Regression equation** y = 0.033x - 0.001; Correlation coefficient = 0.993

***(Mean ± S.D., *n* = 3)**

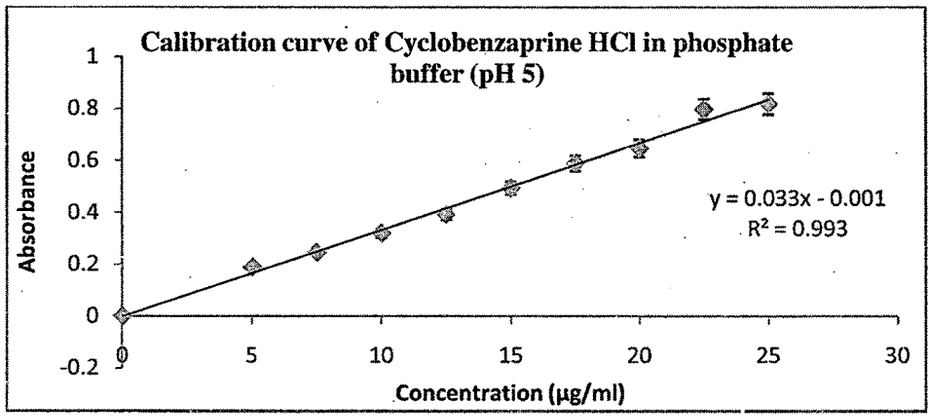


Figure 3.4: Regressed calibration curve for estimation of Cyclobenzaprine HCl in phosphate buffer (pH 5)

Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of Cyclobenzaprine HCl (5µg/mL, 15µg/mL and 25µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 3.9.

Table 3.9: Evaluation of accuracy and precision of the estimation method of Cyclobenzaprine HCl

Concentration Added (µg/ml)	Mean ± SD	Recovery ± RSD	% RSD or *coefficient of variance (CV)*
5	5.1±0.116	98.04 ± 0.0227	2.27
15	15.2±0.201	101.33 ± 0.0132	1.32
25	24.8±0.356	99.20 ± 0.0143	1.43

(Mean ± S.D., *n* = 3)

3.3. Estimation of Tizanidine HCl and Cyclobenzaprine HCl by High Performance Liquid Chromatography (HPLC) method

3.3.1. Estimation of Tizanidine HCl in solution

The estimation of Tizanidine HCl was performed by HPLC method. The drug was estimated using a Shimadzu HPLC system (Shimadzu, Japan). The HPLC system was composed of a pump (LC-20AT prominence, Shimadzu), a sample 20-µl loop injector (Rheodyne 7725) and a UV-visible spectrophotometric detector (SPD-20A prominence, Shimadzu).The separation was carried out on a Phenomenex C_{18} 250 x 4.6 mm HPLC column (Phenomenex) having particle size of 5µm. Mobile phase for Tizanidine HCl consisted of Acetonitrile: Water (80:20), UV detection wavelength was 241nm and mobile phase flow rate 1 ml/min. The retention time of Tizanidine HCl was 5.73 minutes.

Preparation of standard stock solutions of Tizanidine HCl in mobile phase

20 mg of Tizanidine HCl was accurately weighed using single pan electronic balance and transferred to 100 ml volumetric flask. 100 ml of mobile phase was accurately measured and transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 100 ml with mobile to produce 200 µg/ml of Tizanidine HCl.

0.5 ml of the above solution was accurately measured by micropipette and transferred to the 10 ml volumetric flask. The final volume was made up to 10 ml with mobile phase to prepare stock solution of 10 µg/ml of Tizanidine HCl.

Preparation of calibration curve of Tizanidine HCl:

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with HPLC mobile phase to give final concentrations of 0.25, 0.5, 1, 2, 4, 6, 8 and 10 µg/ml and analyzed in above mentioned HPLC system. The above procedure was repeated three times. The data was recorded in Table 3.10 along with standard deviation. Figures 3.5 and Figure 3.6 show the HPLC chromatogram and calibration curve for Tizanidine HCl in mobile phase respectively.

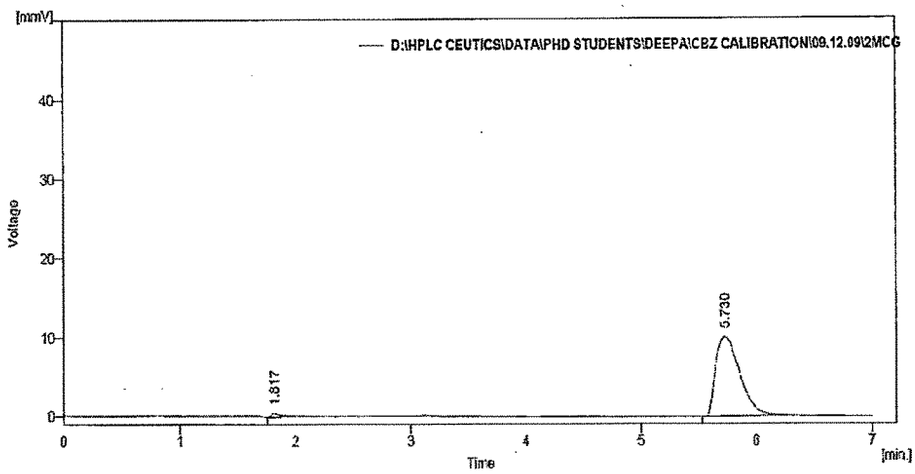


Figure 3.5: HPLC Chromatogram for Tizanidine HCl

Table 3.10: Calibration for Tizanidine HCl

Concentration (µg/ml)	Mean area(mAU)* ± S.D
0	0
0.25	29.06±0.005
0.5	46.77±0.009
1	76.087±0.007
2	146.141±0.005
4	242.349±0.003
6	368.952±0.008
8	472.535±0.006
10	570.222±0.010

Regression equation** Y= 56.52x + 17.51; Correlation coefficient R^2= 0.997

***(Mean ± S.D., *n* = 3)**

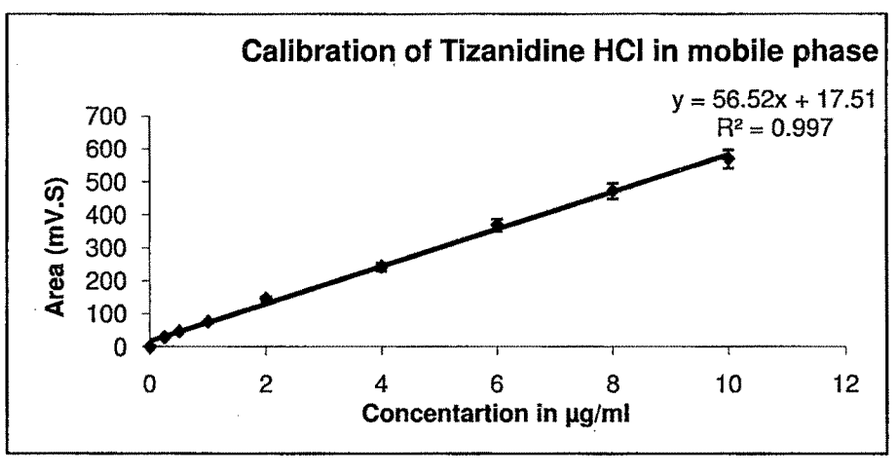


Figure: 3.6: Regressed calibration curve for estimation of Tizanidine HCl

Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of Tizanidine HCl (0.25µg/mL, 4µg/mL and 10µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 3.11.

Table 3.11: Evaluation of accuracy and precision of the estimation method of Tizanidine HCl

Concentration Added (µg/ml)	Mean ± SD	Recovery ± RSD	% RSD or *coefficient of variance (CV)*
0.250	0.256±0.004	102.4 ± 0.0156	1.56
4	3.9±0.094	97.5 ± 0.0241	2.41
10	10.2±0.181	102 ±0.0177	1.77

(Mean ± S.D., *n* = 3)

3.3.2. Estimation of Cyclobenzaprine HCl in solution

The estimation of Cyclobenzaprine HCl was performed by HPLC method. The drug was estimated using a Shimadzu HPLC system (Shimadzu, Japan). The HPLC system was composed of a pump (LC-20AT prominence, Shimadzu) , a sample 20-µl loop injector (Rheodyne 7725) and a UV-visible spectrophotometric detector (SPD-20A prominence, Shimadzu).The separation was carried out on a Phenomenax C_{18} 250 x 4.6 mm HPLC column (Phenomenax) having particle size of 5µm. Mobile phase for Cyclobenzaprine HCl consisted of Acetonitrile: Methanol: Water (50:30:20), UV detection wavelength was 290nm and mobile phase flow rate 1 ml/min. The retention time of cyclobenzaprine HCl was 4.49 minutes.

Preparation of standard stock solutions of Cyclobenzaprine HCl in mobile phase

20 mg of Cyclobenzaprine HCl was accurately weighed using single pan electronic balance and transferred to 100 ml volumetric flask. 100 ml of mobile phase was accurately measured and transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 100 ml with mobile to produce 200 µg/ml of Cyclobenzaprine HCl.

0.5 ml of the above solution was accurately measured by micropipette and transferred to the 10 ml volumetric flask. The final volume was made up to 10 ml with mobile phase to prepare stock solution of 10 µg/ml of Cyclobenzaprine HCl.

Preparation of calibration curve of cyclobenzaprine HCl:

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with HPLC mobile phase to give final concentrations of 0.25, 0.5, 1, 2, 4, 6, 8, 10, 15 and 20 µg/ml and analyzed in above mentioned HPLC system. The above procedure was repeated three times. The data was recorded in Table 3.12 along with standard deviation. Figures 3.7 and Figure 3.8 show the HPLC chromatogram and calibration curve respectively for Cyclobenzaprine HCl in mobile phase.

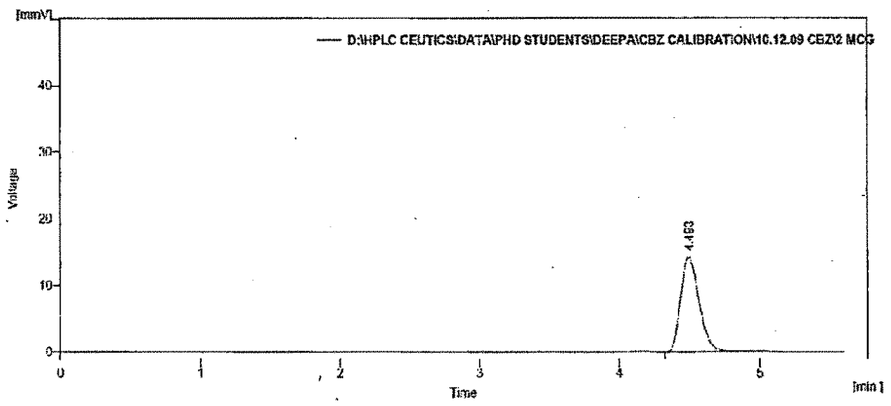


Figure 3.7: HPLC Chromatogram for Cyclobenzaprine HCl

Table 3.12: Calibration for Cyclobenzaprine HCl

Concentration (µg/ml)	Mean area(mAU)* ± S.D
0	0
0.25	27.82±0.007
0.5	44.5±0.009
1	75.631±0.004
2	128.797±0.010
4	216.117±0.033
6	314.166±0.015
8	390.665±0.019
10	498.04±0.022
15	620.864±0.013
20	899.019±0.009

Regression equation** Y= 43.05x + 31.07; Correlation coefficient R^2= 0.991

***(Mean ± S.D., n = 3)**

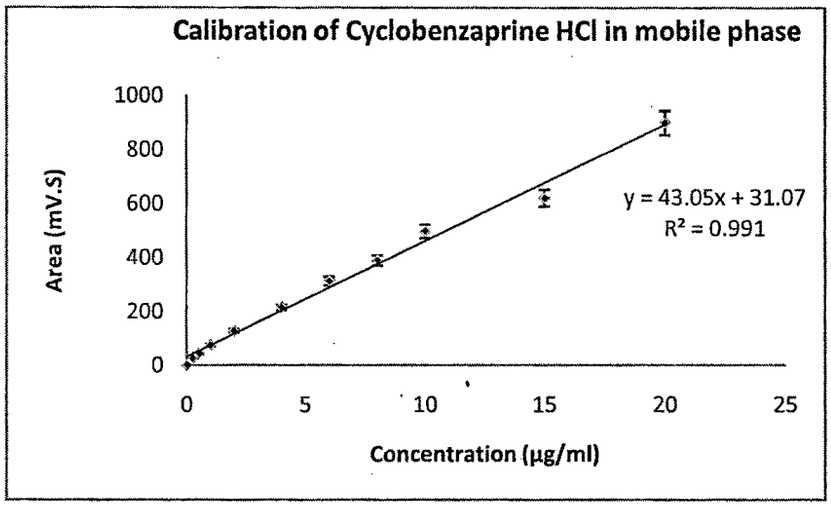


Figure 3.8: Regressed calibration curve for estimation of Cyclobenzaprine HCl

Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of Cyclobenzaprine HCl (0.25µg/mL, 6µg/mL and 15µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 3.13.

Table 3.13: Evaluation of accuracy and precision of the estimation method of Cyclobenzaprine HCl

Concentration Added (µg/ml)	Mean ± SD	Recovery ± RSD	% RSD or *coefficient of variance (CV)*
0.250	0.244±0.003	97.60 ± 0.0122	1.22
6	5.9±0.145	98.33 ± 0.0245	2.45
15	15.1±0.207	100.66 ± 0.0137	1.37

(Mean ± S.D., $n = 3$)

3.4. Estimation of Rhodamine B by U.V. method

3.4.1. Estimation of Rhodamine B in distilled water

Rhodamine B shows strong absorbance in UV-Visible region. Hence, the estimation of Rhodamine B was performed by UV-visible spectrophotometry. The common method for qualitative estimation of Rhodamine B penetration across the nasal mucosa. The method was developed in distilled water.

Preparation of standard stock solutions of Rhodamine B in distilled water

25 mg of Rhodamine B was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 50 ml of distilled water was accurately

measured and transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 50 ml with distilled water produce stock 500 µg/ml of Rhodamine B.

Calibration curve of Rhodamine B

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with distilled water to give final concentrations of 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 µg/ml and analyzed by UV-visible spectrophotometry at 553nm. The above procedure was repeated three times. The data was recorded in Table 3.14 along with standard deviation. Figures 3.9 show the calibration curve of Rhodamine B in distilled water.

Table 3.14: Calibration for Rhodamine B in distilled water

Concentration (µg/ ml)	Mean Absorbance* ± S.D
0	0
1	0.229 ±0.002
1.5	0.305 ±0.001
2	0.401 ±0.001
2.5	0.510 ±0.003
3	0.588 ±0.002
3.5	0.692 ±0.001
4	0.723 ±0.002
4.5	0.861 ±0.001
5	0.946 ±0.002

Regression equation** y = 0.184x + 0.028; Correlation coefficient = 0.995

***(Mean ± S.D., *n* = 3)**

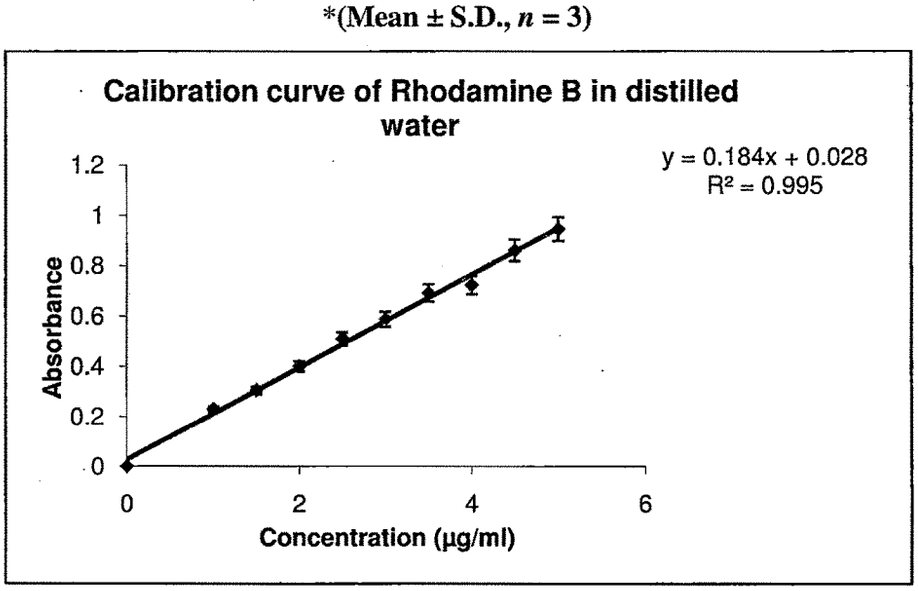


Figure 3.9: Regressed calibration curve for estimation of Rhodamine B in distilled water

Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of Rhodamine B (1 µg/mL, 3µg/mL and 5µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in Table 3.15.

Table 3.15: Evaluation of accuracy and precision of the estimation method of Rhodamine B

Concentration Added (µg/ml)	Mean ± SD	Recovery ± RSD	% RSD or *coefficient of variance (CV)*
1	1.01±0.021	101.00± 0.0207	2.07
3	2.9±0.062	96.66 ± 0.0213	2.13
5	4.9±0.116	98.00 ± 0.0236	2.36

(Mean ± S.D., *n* = 3)

3.5. Estimation of Tizanidine HCl and Cyclobenzaprine HCl in NPs

To determine the amount of tizanidine HCl and cyclobenzaprine HCl, the drug loaded nanoparticles suspension transferred into a 50 ml volumetric flask, and then diluted with 1% (v/v) acetic acid solution and sonicated for 30 minutes then filtrated through 0.22 µm microporous membrane. The total drug loading into the NPs formulation was measured using U.V spectroscopy. The entrapment efficiency was determined upon separation of NPs from the aqueous suspension containing non-entrapped drug by centrifugation at 18000 rpm 4 °C for 30 minutes. The amount of free drug in the supernatant was measured by U.V spectroscopy (Xiaomei et al., 2008) at 320 nm and 290 nm for tizanidine HCl and cyclobenzaprine HCl respectively. Drug entrapment efficiency (%EE) in the drug loaded NPs was calculated according to the equations below:

$$\% \text{ EE} = \frac{\text{Total amount of drug loading - free drug in supernant}}{\text{Total amount of drug loading}} \times 100$$

3.6. Estimation of Tizanidine HCl and Cyclobenzaprine HCl for *in-vitro* release

In-vitro release was also performed using Franz diffusion cell for the determination of drug release from the drug loaded nanoparticles using phosphate buffer (pH 5). Nanoparticles suspension was placed on nasal mucosa holding between the donor compartment and acceptor compartment. The entire system was incubated at 37°C

with stirring. At specific time intervals samples were taken out and the amount of the drug released was measured by HPLC.

3.7. Determination of Particle Size, Polydispersity and Zeta potential

The particle size was measured with Malvern zetasizer NanoZS. The instrument is based on the principle of dynamic light scattering (DLS), also sometimes referred to as photon correlation spectroscopy (PCS) or quasi elastic light scattering. DLS is a technique of measuring the size of particles typically in the sub-micron region and is usually applied to the measurement of particle suspended within a liquid. The technique measures particle diffusion due to Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them. The parameter calculated is defined as the translational diffusion coefficient. The particle size is then calculated from the translational diffusion coefficient using the Strokes-Einstein equation. Malvern zetasizer NanoZS was used to measure the zeta potential of the particles based on the electrophoresis and electrical conductivity of the formed nanoparticles dispersion. The electrophoretic mobility (μm/s) of the particles was converted to the zeta potential by in-built software based on Helmholtz- Smoluchowski equation.

A 2.0 mg sample of nanoparticles was suspended in distilled water, and the particle size, polydispersity index and zeta potential were measured using the principle of laser light scattering with zeta sizer (Nano-ZS, Malvern Instruments, UK).

3.8. Morphological characterization

The morphology of the nanoparticles was analyzed using TEM (Transmission Electron Microscopy). Aqueous nanoparticles suspension was negatively stained with phosphotungstic acid (2% PTA), Samples were then observed with Morgagni, Philips, Eindhoven, Netherlands. TEM micrograph showed that the drug loaded chitosan and thiolated chitosan and trimethyl chitosan NPs were uniform particle size in nano range.

3.9. Results and discussion

Tizanidine HCl and Cyclobenzaprine HCl estimation in nanoparticles was done using UV-visible spectrophotometry and drug in the *in-vitro* release medium was estimated by HPLC method for both the drug. The calibration curve of Tizanidine HCl by U.V visible spectrophotometry was established in both distilled water and phosphate buffer (pH 5). The linearity of Tizanidine HCl was found to be 5 to 17.5 μg/ml (R^2 =0.995)

and 2.5 to 17.5 µg/ml (R^2 =0.996) in distilled water and Phosphate buffer (pH 5) respectively. The calibration curve of Cyclobenzaprine HCl by U.V visible spectrophotometry was established in both distilled water and phosphate buffer (pH 5). The linearity of Cyclobenzaprine HCl was found to be 5 to 25 µg/ml (R^2 =0.993) and 5 to 25 µg/ml (R^2 =0.993) in distilled water and Phosphate buffer pH 5 respectively. Drug entrapment in nanoparticles for both the drugs was measured by the U.V visible spectrophotometry in supernant and nanoparticles both.

The calibration curve of Tizanidine HCl and Cyclobenzaprine HCl was also established in Acetonitrile: Water (80:20) and Acetonitrile: Methanol: Water (50:30:20) system by HPLC at 241nm and 290nm respectively. The linearity of Tizanidine HCl and Cyclobenzaprine HCl was found to be 0.25 to 10µg/ml (R^2=0.997) and 0.25 to 20µg/ml (R^2=0.991). The *in-vitro* release study was performed using Franz diffusion cell. At different time intervals, the samples were removed and diluted with mobile phase and analyzed for the drug content in diffusion medium.

The estimation of Rhodamine B was carried out using U.V visible spectrophotometry at 553 nm. The calibration curve was established at 1 to 5 µg/ml (R^2=0.995).

3.10. Conclusions

Developed analytical methods of tizanidine HCl and cyclobenzaprine HCl in nanoparticles formulations and in *in-vitro* release medium showed good linearity, accuracy and precision. So these methods were further used for other studies.

3.11. References

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