Chapter 3: Analytical methods

3. ANALYTICAL METHODS

The analytical methods for preparation, optimization and characterization of unconjugated and phycoerythrin-conjugated-anti-mouse-anti-transferrin receptor monoclonal antibody (PE-mAb-Tfr) conjugated PLGA nanoparticles (NPs), microemulsions (ME) and mucoadhesive microemulsions (MME) of nicergoline, hydergine and sibutramine base are employed in this section. For the preparation of sibutramine nanoparticles and microemulsions, sibutramine base was used after extraction from sibutramine hydrochloride monohydrate (SBHM) using chloroform. The extracted base was examined for purity using melting point and DSC long form results and the yield was calculated. The prepared NPs and MEs were characterized for respective parameters like particle size, surface charge in the form of zeta potential, surface morphology by transmission electron microscopy, in- vitro drug release, DSC, ^{1}H-NMR, and conjugation efficiency of PE-mAb-Tfr, etc for NPs, and particle size, surface charge in the form of zeta potential, in- vitro drug release, pH, viscosity, conductivity, etc for MEs. *In vivo* studies in animals include the tissue bio-distribution in the different body organs or tissues and pharmacodynamic studies. The biodistribution studies were carried out after radiolabeling the drug and the formulations with ^{99m}Tc (Chapter 6). The drug delivery to the brain was confirmed by gamma scintigraphy technique. Pharmacodynamic studies were conducted in suitable animal models to investigate the effectiveness of intranasal delivery over conventional routes. The stability studies of unconjugated and conjugated NPs and MEs and MMEs were conducted to determine the physical and chemical instability with respect to particle size, zeta potential, % EE, and the physical changes like caking and discoloration for NPs and particle size, zeta potential, assay, and the physical changes like phase separation and discoloration for ME respectively.

Table 3.1 Materials and equipments

Material	Source
Nicergoline	Gift samples from Ivax Pharmaceuticals s.r.o, Opava – Komarov, Crech Republic.
Hydergine	Gift samples from Ivax Pharmaceuticals s.r.o, Opava – Komarov, Crech Republic.
Sibutramine hydrochloride monohydrate (SBHM)	Gift samples from Matrix Laboratories Ltd., Secunderabad, India.
Water (distilled)	Prepared in laboratory by distillation

PLGA (50:50)	Gift samples from gift sample from Boehringer Ingelheim, Germany
Phycoerythrin conjugated Anti-CD71 anti-mouse monoclonal antibody (PE-mAb-Tfr)	Santa Cruz Biotechnology, Inc. U.S.A.
Potassium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, potassium hydroxide, sodium chloride, sodium hydroxide	S.D. Fine chemicals, Mumbai, India
HPLC grade methanol, acetonitrile.	S.D. Fine chemicals, India.
Nuclepore Polycarbonate membrane 2 µm 25mm	Whatman, USA

Equipments	**Source/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 glasswares	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
Stability oven	Shree Kailash Industries, Vadodara
Spectrofluorophotometer	Shimadzu RF-540, equipped with data recorder, Japan
UV-Visible Spectrophotometer	Shimadzu UV-1601, Japan
Viscometer	Brookfield HADV III+, U.S.A
Conductometer	CM 180 Elico, India
Vacuum Pump F16	Bharat Vacuum pumps, Bangalore
Bath sonicator	DTC 503, Ultra Sonics
Malvern particle size analyser	Malvern zeta sizer NanoZS, U.K.
Transmission electron microscope	Morgagni, Philips, Netherlands
Differential Scanning Calorimeter	Mettler DSC 20, Mettler Toledo, Switzerland

3.1 Nicergoline

Nicergoline (NG) was estimated by UV-Visible spectroscopy by being easy and readily feasible for even number of samples.

3.1.1 Estimation of nicergoline in solution and nanoparticles

3.1.1.1 Estimation of nicergoline in solution

NG shows strong absorbance in UV-Visible region. Hence, the estimation of NG 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 acetonitrile.

Preparation of standard stock solutions of nicergoline in acetonitrile

50mg of NG was accurately weighed using single pan electronic balance and transferred to 50mL volumetric flask. 25mL of acetonitrile AR grade 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 50mL with acetonitrile AR grade to produce 1000µg per mL of NG. 10mL of the above solution was accurately measured by calibrated graduated pipette and transferred to the 100mL volumetric flask. The final volume was made up to 100mL with acetonitrile AR grade to prepare stock solution of 100µg per mL of NG.

Calibration curve of nicergoline in acetonitrile

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10mL of volumetric flasks. The final volume was made up to 10mL with acetonitrile AR grade to give final concentrations of 10, 20, 30, 40, 50µg/mL and analyzed by UV spectrophotometry at 320nm using 2^{nd} derivative spectra. The above procedure was repeated three times. The data was recorded in Table 3.2 along with standard deviation. Figures 3.1 and 3.2 shows the UV spectra and calibration curve respectively of NG in acetonitrile.

Figure 3.1: UV spectrophotometric scan of nicergoline in acetonitrile

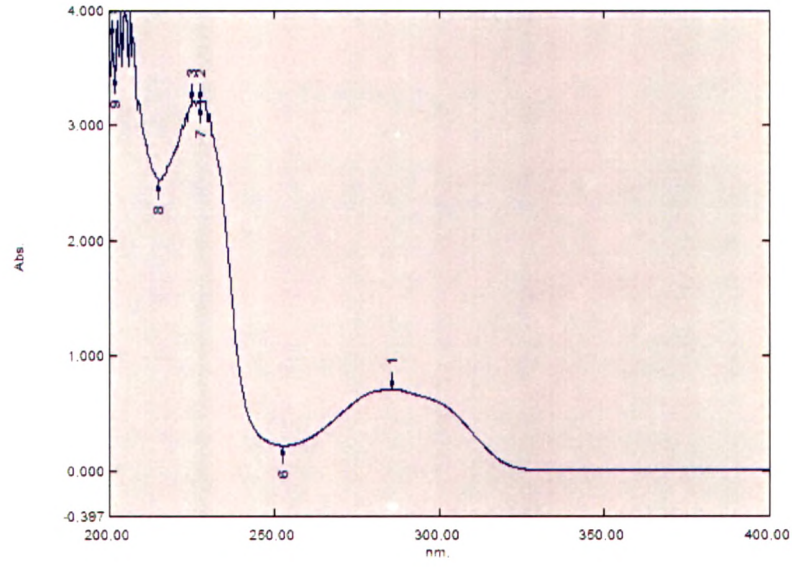
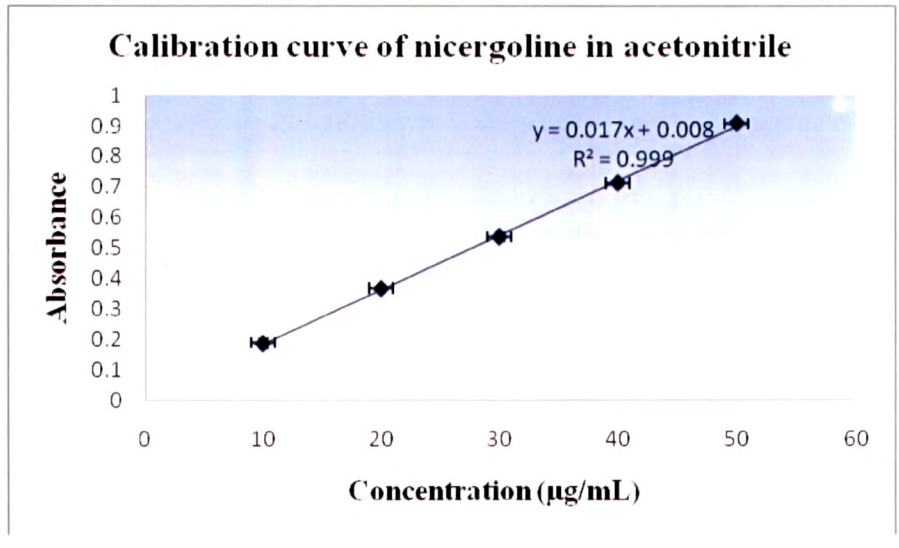


Table 3.2: Calibration of nicergoline in acetonitrile

Concentration (µg/mL)	Mean Absorbance* ± S.D
10	0.189 ± 0.003
20	0.369 ± 0.016
30	0.537 ± 0.008
40	0.713 ± 0.012
50	0.908 ± 0.002

Regression equation** Y=0.017X+0.004; Correlation coefficient=0.999; *n=3

Figure 3.2: Regressed calibration curve of estimation of nicergoline in acetonitrile



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of NG (10µg/mL, 30µg/mL and 50µg/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 nicergoline in acetonitrile

Theoretical Concentration of ETP (µg/mL)	Determined Value (µg/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
10	10.13	0.9133	0.0412	10.13±0.275
30	31.23	2.8409	0.39	31.23±2.6
50	49.92	0.1132	0.0254	49.92±0.17

** At 95% Confidence level; t_{tab} = 4.303 for 2 degrees of freedom*

3.1.1.2 Estimation of nicergoline in nanoparticles

To determine the amount of NG entrapped in the NPs, 2mg of NPs were added to 2mL of acetonitrile and subjected to shaking at room temperature for 4hrs for complete dissolution of PLGA for extraction of the drug from the nanoparticles. The resulting system was centrifuged at 5000rpm for 15mins to remove the precipitated components. The supernatant was further diluted suitably with acetonitrile and estimated at 320 nm using 2nd derivative spectra.

3.1.1.3 Estimation of nicergoline for *in vitro* release

The release studies for NG nanoparticles in 10% methanolic phosphate buffer saline pH 7.4 + 2%w/v polysorbate-80. Nanoparticles equivalent to 1mg drug were suspended in 10mL of release medium in a screw capped tubes, which were placed in a horizontal shaker bath maintained at 37°C and shaken at 60min^{-1}. At specific time intervals following incubation samples were taken out and centrifuged at 15000rpm for 30min. The residue (settled nanoparticles) were collected and dissolved in acetonitrile and analyzed as per the method above. The amount of the drug released was calculated using the following equation:

$$\%\text{Drug released} = 1 - \left(\frac{\text{Amount of drug in nanopartiles settled}}{\text{Amount of drug initially taken}}\right)100$$

3.1.2 Estimation of nicergoline in solution and microemulsion

3.1.2.1 Estimation of nicergoline in solution

The estimation of NG was performed by UV-visible spectrophotometry and a common method for estimation of drug content, assay and *in vitro* release was developed in methanol.

Preparation of standard stock solutions of nicergoline in methanol

50mg of NG was accurately weighed and transferred to 50mL volumetric flask. 25mL of methanol AR grade was accurately measured and transferred to the volumetric flask with the drug allowed to dissolve completely and then the final volume was made up to the mark with methanol AR grade to produce 1000µg per mL of NG. 10mL of the resulting solution was accurately measured using calibrated graduated pipette and transferred to 100mL volumetric flask. The final volume was made up to 100mL with methanol AR grade to prepare stock solution of 100µg per mL of NG.

Calibration curve of nicergoline

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10mL of volumetric flasks. The final volume was made up to 10mL with methanol AR grade to give final concentrations of 10, 20, 30, 40, 50µg/mL and analyzed by UV spectrophotometry at 320nm using 2nd derivative spectra. The above procedure was repeated three times. The data was recorded in Table 3.4 along with standard deviation. Figures 3.3 and 3.4 shows the UV spectra and calibration curve respectively of NG in methanol AR grade.

Figure 3.3: UV spectrophotometric scan of nicergoline in methanol

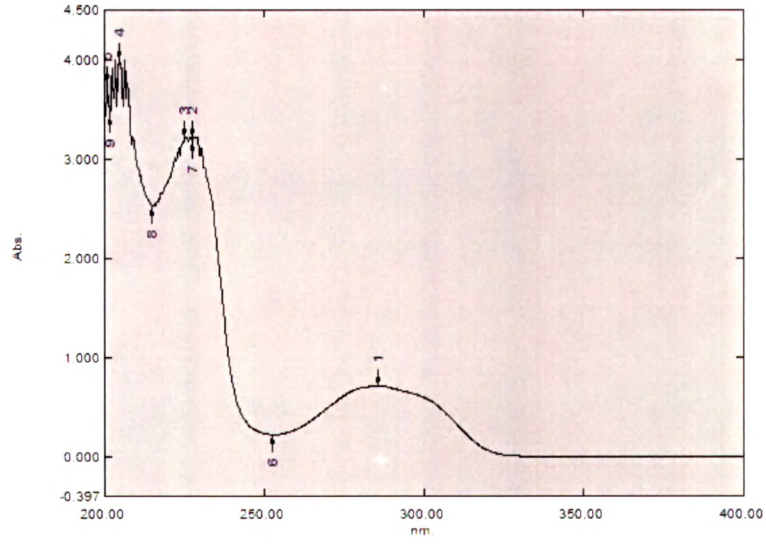
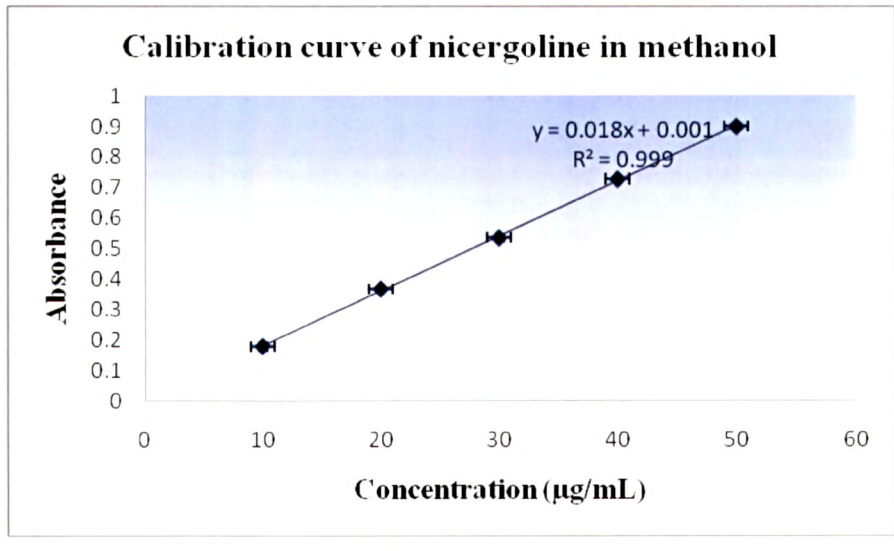


Table 3.4: Calibration of nicergoline in methanol

Concentration (µg/ ml)	Mean Absorbance* ± S.D
10	0.179 ± 0.002
20	0.368 ± 0.013
30	0.536 ± 0.005
40	0.728 ± 0.011
50	0.9 ± 0.007

Regression equation** Y= 0.018X + 0.000; Correlation coefficient = 0.999; *n = 3

Figure 3.4: Regressed calibration curve of estimation of nicergoline in methanol



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of NG (10µg/mL, 30µg/mL and 50µg/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 nicergoline in methanol

Theoretical Concentration of ETP (µg/mL)	Determined Value (µg/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
10	9.91	0.6393	0.0285	9.91±0.19
30	29.87	0.307	0.0412	29.87±0.274
50	51.12	1.5663	0.3551	51.12±2.37

** At 95% Confidence level; t_{tab} = 4.303 for 2 degrees of freedom*

3.1.2.2 Estimation of nicergoline in ME and MME

To determine the amount of NG in ME and MME, 0.2mL of formulation was added to 10mL volumetric flask and the volume was made up to the mark with methanol AR grade. The resultant solution was then sonicated for 3min at ambient temperature, centrifuged and 1mL of the supernatant was transferred to 10mL volumetric flask. The volume was made up to the mark with methanol AR grade and NG estimated at 318 nm using 2nd derivative spectra.

3.1.2.3 Estimation of nicergoline for *in vitro* release

The release studies for NG ME and MME were performed in 10% methanolic phosphate buffer saline pH 6.4 + 2%w/v polysorbate-80. ME and MME equivalent to 2 doses of NG were taken in 1mL of PBS in a dialysis tube of 10kDa molecular weight cut-off, which were immersed in beakers containing diffusion media (50mL) maintained at 37°C on a magnetic stirrer. At specific time intervals samples were withdrawn, diluted suitably with methanol and analyzed by UV spectrophotometer against methanol as blank at 318 nm using 2nd derivative spectra.

In vitro release of NG ME and MME were also performed across excised sheep nasal mucosa. Briefly, 0.2mL of formulation was placed in the donor compartment of Franz diffusion cell along with 1.8mL of phosphate buffer pH 6.4. Recipient compartment

contained 30mL of 10% methanolic phosphate buffer saline pH 6.4 + 2%w/v polysorbate-80. Samples from the receptor compartment were withdrawn at predetermined time intervals, diluted suitably with methanol and analyzed at 318 nm using 2nd derivative spectra against methanol as blank. Each sample removed was replaced by an equal volume of 10% methanolic phosphate buffer saline pH 6.4 + 2%w/v polysorbate-80.

3.2 Sibutramine

Sibutramine base was estimated by UV-Visible spectroscopy by being easy and readily feasible for even number of samples.

3.2.1 Estimation of sibutramine base in solution and nanoparticles

3.2.1.1 Estimation of sibutramine base in solution

The estimation of sibutramine base (SB) was also performed by UV-visible spectrophotometry and a common method for estimation of drug content, entrapment efficiency and *in vitro* release was developed in acetonitrile.

Preparation of standard stock solutions of sibutramine base in acetonitrile

100mg of SB was accurately weighed using single pan electronic balance and transferred to 100mL volumetric flask. 25mL of acetonitrile AR grade was accurately measured and transferred to the volumetric flask, the drug was completely dissolved and the final volume was made up to 100mL with acetonitrile AR grade to produce 1000µg per mL of SB.

Calibration curve of sibutramine base in acetonitrile

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10mL of volumetric flasks. The final volume was made up to 10mL with acetonitrile AR grade to give final concentrations of 200, 300, 400, 500, 600, 700, 800, 900µg/ml and analyzed by UV spectrophotometry at 282.2nm using 2nd derivative spectra. The above procedure was repeated three times. The data was recorded in Table 3.6 along with standard deviation. Figures 3.5, 3.6 show the UV spectra and calibration curve respectively of SB in acetonitrile.

Figure 3.5: UV spectrophotometric scan of sibutramine base in acetonitrile

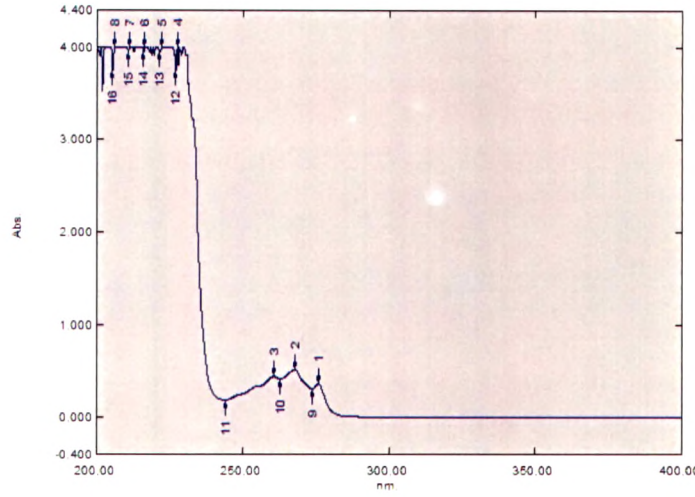
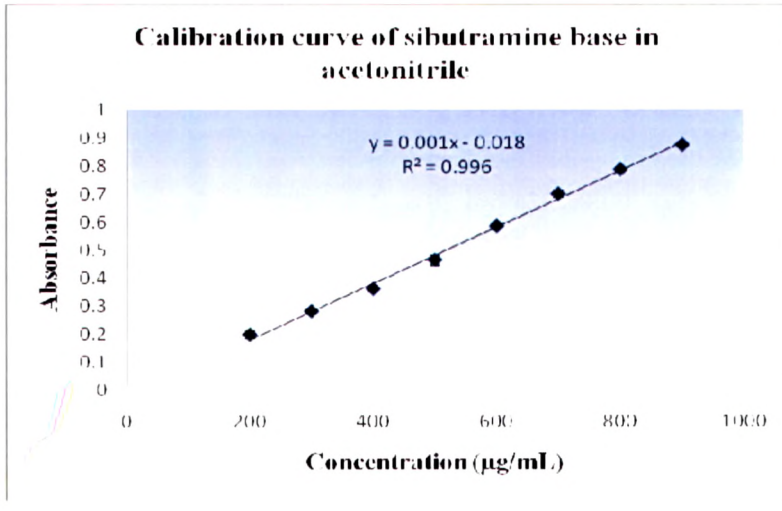


Table 3.6: Calibration of sibutramine base in acetonitrile

Concentration (µg/ ml)	Mean Absorbance* ± S.D
200	0.199 ± 0.002
300	0.282 ± 0.012
400	0.362 ± 0.008
500	0.466 ± 0.011
600	0.587 ± 0.017
700	0.702 ± 0.007
800	0.789 ± 0.013
900	0.876 ± 0.009

Regression equation** Y= 0.001X - 0.009; Correlation coefficient = 0.997; *n = 3

Figure 3.6: Regressed calibration curve of sibutramine base in acetonitrile



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of SB (400µg/mL, 600µg/mL and 800µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in table 3.3.

Table 3.7: Evaluation of accuracy and precision of the estimation method of sibutramine base in acetonitrile

Theoretical Concentration of ETP (µg/mL)	Determined Value (µg/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
400	400.09	0.0159	0.0285	400.09±0.19
600	598.97	0.1215	0.3266	598.97±2.176
800	799.12	0.0778	0.279	799.12±1.859

** At 95% Confidence level; t_{tab} = 4.303 for 2 degrees of freedom*

3.2.1.2 Estimation of sibutramine base in NPs

To determine the amount of SB entrapped in the NPs, 2mg of NPs were added to 2mL of acetonitrile and subjected to shaking at room temperature for 4hrs for complete dissolution of PLGA for extraction of the drug from the nanoparticles. The resulting system was centrifuged at 5000rpm for 15mins to remove the precipitated components. The supernatant was further diluted with acetonitrile and estimated at 282.2nm using 2nd derivative spectra.

3.2.1.3 Estimation of sibutramine base for *in vitro* release

The release studies for SB nanoparticles in 30% ethanolic phosphate buffer saline pH 7.4 + 2%w/w polysorbate-80. Nanoparticles equivalent to 10mg drug were suspended in 10mL of release medium in a screw capped tubes, which were placed in a horizontal shaker bath maintained at 37°C and shaken at 60min^{-1}. At specific time intervals following incubation samples were taken out and centrifuged at 15000rpm for 30min. The residue (settled nanoparticles) were collected and dissolved in acetonitrile and analyzed as per the method above. The amount of the drug released was calculated using the following equation:

$$\%\text{Drug released} = 1 - \left(\frac{\text{Amount of drug in nanopartiles settled}}{\text{Amount of drug initially taken}}\right)100$$

3.2.2 Estimation of sibutramine base in solution and microemulsions

3.2.2.1 Estimation of sibutramine base in solution

The estimation of SB was performed by UV-visible spectrophotometry and a common method for estimation of drug content, assay and *in vitro* release was developed in methanol.

Preparation of standard stock solutions of sibutramine base in methanol

50mg of SB was accurately weighed and transferred to 50mL volumetric flask. 25mL of methanol AR grade was accurately measured and transferred to the volumetric flask with the drug allowed to dissolve completely and then the final volume was made up to the mark with methanol AR grade to produce 1000µg per mL of SB.

Calibration curve of sibutramine base

Suitable aliquots of standard stock solution were accurately measured and transferred to 10mL of volumetric flasks. The final volume was made up to 10mL with methanol AR grade to give final concentrations of 200, 400, 600, 800, 1000 µg/mL and analyzed by UV spectrophotometry at 277nm using 2nd derivative spectra. The above procedure was repeated three times. The data was recorded in Table 3.7 along with standard deviation. Figures 3.7 and 3.8 shows the UV spectra and calibration curve respectively of SB in methanol AR grade.

Figure 3.7: UV spectrophotometric scan of sibutramine base in methanol

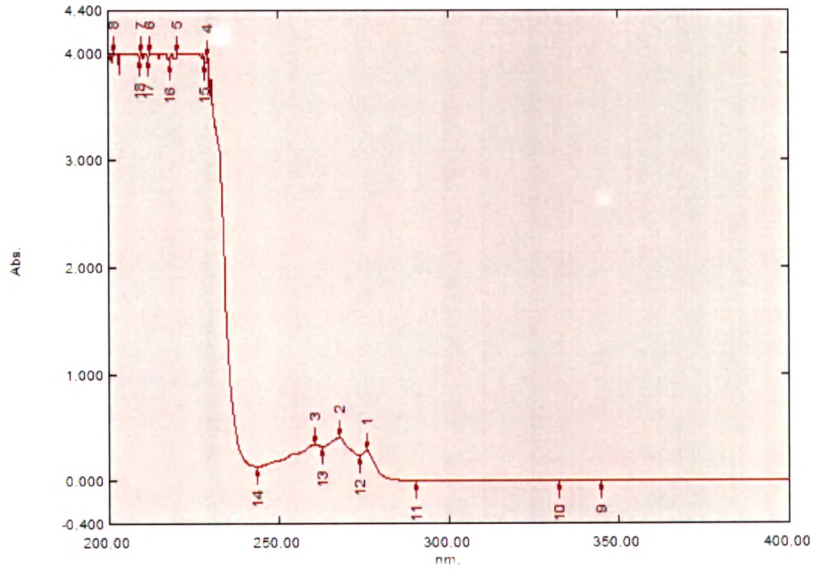
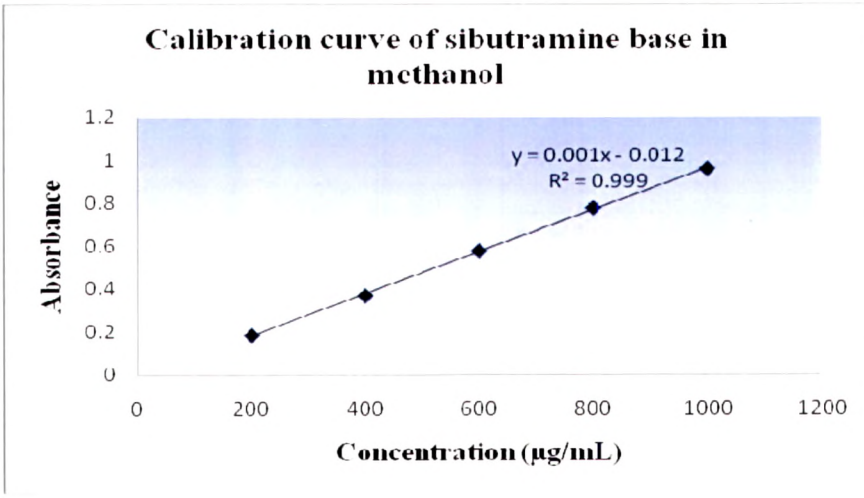


Table 3.8: Calibration of sibutramine base in methanol

Concentration (µg/ ml)	Mean Absorbance* ± S.D
200	0. 185 ± 0.012
400	0.368 ± 0.004
600	0.575 ± 0.012
800	0.774 ± 0.007
1000	0.965 ± 0.015

Regression equation** Y= 0.001X - 0.006; Correlation coefficient = 0.999; *n = 3

Figure 3.8: Regressed calibration curve of sibutramine base in methanol



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of SB (600µg/mL, 800µg/mL and 1000µg/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 sibutramine base in methanol

Theoretical Concentration of ETP (µg/mL)	Determined Value (µg/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
600	605.96	0.6989	1.8898	605.96±12.593
800	793.99	0.5332	1.9057	793.99±12.699
1000	1030.08	0.7092	3.1962	1030.08±21.299

** At 95% Confidence level; t_{tab} = 4.303 for 2 degrees of freedom*

3.2.2.2 Estimation of sibutramine base in ME and MME

To determine the amount of SB in ME and MME, 1 mL of formulation was added to 10 mL volumetric flask and the volume was made up to the mark with methanol AR grade. The resultant solution was then sonicated for 3 min at ambient temperature, centrifuged and one mL of the supernatant was transferred to 10 volumetric flask. The volume was made up to the mark with methanol AR grade and SB estimated at 277nm using 2nd derivative spectra.

3.2.2.3 Estimation of sibutramine base for *in vitro* release

The release studies for SB ME and MME were performed in 30 % ethanolic phosphate buffer saline pH 7.4 + 2 %w/w polysorbate-80. ME and MME equivalent to 5 doses of SB were taken in 1 ml of PBS in a dialysis tube of 10 kDa molecular weight cut-off, which were immersed in beakers containing diffusion media (30 mL) maintained at 37°C on a magnetic stirrer. At specific time intervals samples were withdrawn, diluted suitably with methanol and analyzed by UV spectrophotometer against methanol as blank at 277nm using 2nd derivative spectra.

In vitro release of SB ME and MME were also performed across excised sheep nasal mucosa. Briefly, 1 ml of formulation was placed in the donor compartment of Franz diffusion cell along with 1 ml of phosphate buffer pH 6.4. Recipient compartment contained 30 ml of 30 % ethanolic phosphate buffer saline pH 7.4 + 2 %w/w polysorbate-80. Samples from the receptor compartment were withdrawn at predetermined time intervals, diluted suitably with methanol and analyzed at 277nm using 2nd derivative spectra against methanol as blank. Each sample removed was replaced by an equal volume of 30 % ethanolic phosphate buffer saline pH 7.4 + 2 %w/w polysorbate-80.

3.3 Hydergine

Hydergine (HG) or Ergoloid mesylate was estimated by UV-Visible spectroscopy by being easy and readily feasible for even number of samples.

3.3.1 Estimation of hydergine in solution and nanoparticles

3.3.1.1 Estimation of hydergine in solution

HG was estimated by UV-visible spectrophotometry and a common method for estimation of drug content, entrapment efficiency and *in vitro* release was developed in acetonitrile.

Preparation of standard stock solutions of hydergine in acetonitrile

50 mg of HG was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 25 ml of acetonitrile AR grade 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 acetonitrile AR grade to produce 1000 µg per ml of HG. 10 ml of the above solution was accurately measured by calibrated graduated pipette and transferred to the 100 ml volumetric flask. The final volume was made up to 100 ml with acetonitrile AR grade to prepare stock solution of 100 µg per ml of HG.

Calibration curve of hydergine in acetonitrile

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 acetonitrile AR grade to give final concentrations of 10, 20, 30, 40, 50 µg/ml and analyzed by UV spectrophotometry at 298.5nm using 2nd derivative spectra. The above procedure was repeated three times. The data was recorded in Table 3.10 along with standard deviation. Figures 3.9 and 3.10 shows the UV spectra and calibration curve respectively of HG in acetonitrile.

Figure 3.9: UV spectrophotometric scan of hydergine in acetonitrile

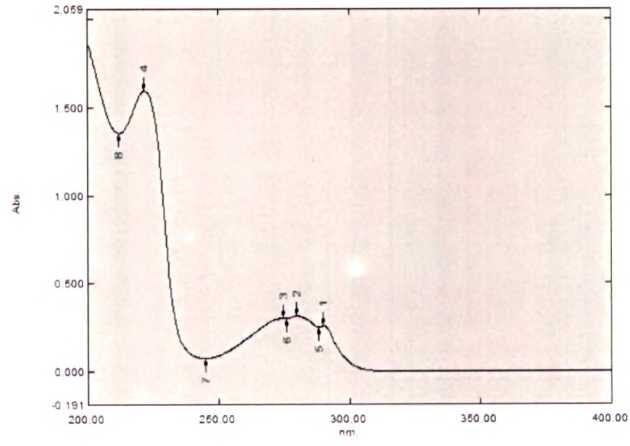
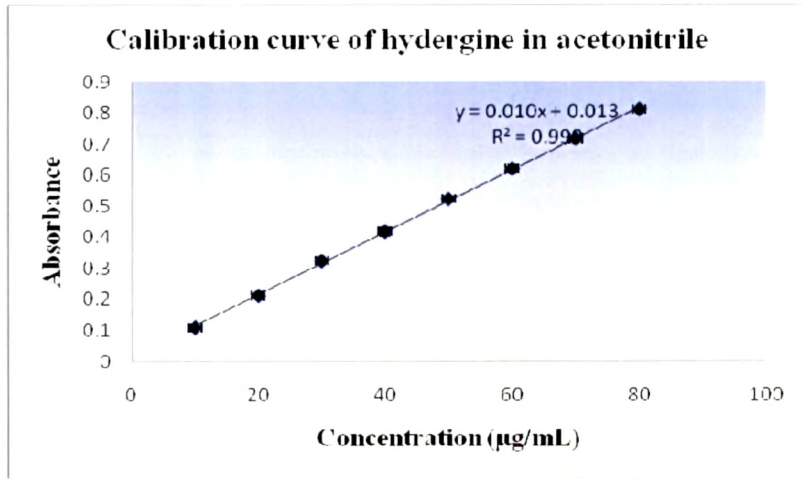


Table 3.10: Calibration of hydergine in acetonitrile

Concentration (µg/ ml)	Mean Absorbance* ± S.D
10	0.108 ± 0.008
20	0.211 ± 0.012
30	0.313 ± 0.006
40	0.418 ± 0.010
50	0.533 ± 0.013
60	0.621 ± 0.008
70	0.718 ± 0.011
80	0.812 ± 0.007

Regression equation** Y= 0.01X + 0.008; Correlation coefficient = 0.999; *n = 3

Figure 3.10: Regressed calibration curve of estimation of hydergine in acetonitrile



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of HG (10µg/mL, 30µg/mL and 50µg/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 hydergine in acetonitrile

Theoretical Concentration of ETP (µg/mL)	Determined Value (µg/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
10	11.08	7.2455	0.3425	11.08±2.282
30	29.89	0.26	0.0349	29.89±0.232
50	51.13	1.58	0.3583	51.13±2.388

** At 95% Confidence level; t_{tab} = 4.303 for 2 degrees of freedom*

3.3.1.2 Estimation of hydergine in NPs

To determine the amount of HG entrapped in the NPs, 2mg of NPs were added to 2 ml of acetonitrile and subjected to shaking at room temperature for 4hrs for complete dissolution of PLGA for extraction of the drug from the nanoparticles. The resulting system was centrifuged at 5000rpm for 15mins to remove the precipitated components. The supernant was further diluted with acetonitrile and estimated at 298.5nm using 2nd derivative spectra.

3.3.1.3 Estimation of hydergine for *in vitro* release

The release studies for HG nanoparticles in 10 % methanolic phosphate buffer saline pH 7.4 + 2%w/v polysorbate-80. Nanoparticles equivalent to 1mg drug were suspended in 10 ml of release medium in a screw capped tubes, which were placed in a horizontal shaker bath maintained at 37°C and shaken at 60min^{-1}. At specific time intervals following incubation samples were taken out and centrifuged at 15000rpm for 30min. The residue (settled nanoparticles) were collected and dissolved in acetonitrile and analyzed as per the method above. The amount of the drug released was calculated using the following equation:

$$\%\text{Drug released} = 1 - \left(\frac{\text{Amount of drug in nanoparticles settled}}{\text{Amount of drug initially taken}}\right)100$$

3.3.2 Estimation of hydergine in solution and microemulsion

3.3.2.1 Estimation of hydergine in solution

The estimation of HG was performed by UV-visible spectrophotometry and a common method for estimation of drug content, assay and *in vitro* release was developed in methanol.

Preparation of standard stock solutions of hydergine in methanol

50 mg of HG was accurately weighed and transferred to 50 ml volumetric flask. 25 ml of methanol AR grade was accurately measured and transferred to the volumetric flask with the drug allowed to dissolve completely and then the final volume was made up to the mark with methanol AR grade to produce 1000 µg per ml of HG. 10 ml of the resulting solution was accurately measured using calibrated graduated pipette and transferred to 100 ml volumetric flask. The final volume was made up to 100 ml with methanol AR grade to prepare stock solution of 100 µg per ml of HG.

Calibration curve of hydergine

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 methanol AR grade to give final concentrations of 10, 20, 30, 40, 50 µg/ml and analyzed by UV spectrophotometry at 292.8nm using 2nd derivative spectra. The above procedure was repeated three times. The data was recorded in Table 3.12 along with standard deviation. Figures 3.11 and 3.12 shows the UV spectra and calibration curve respectively of HG in methanol AR grade.

Figure 3.11: UV spectrophotometric scan of hydergine in methanol

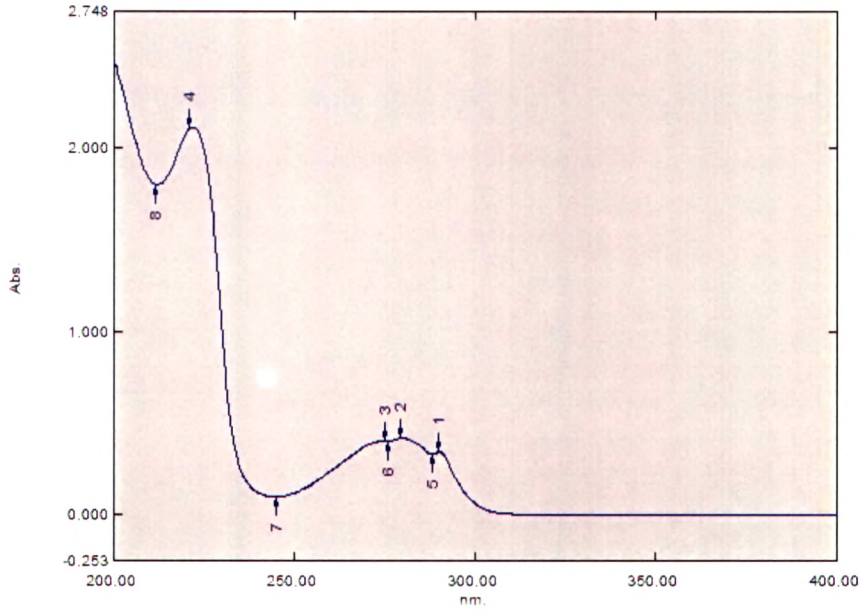
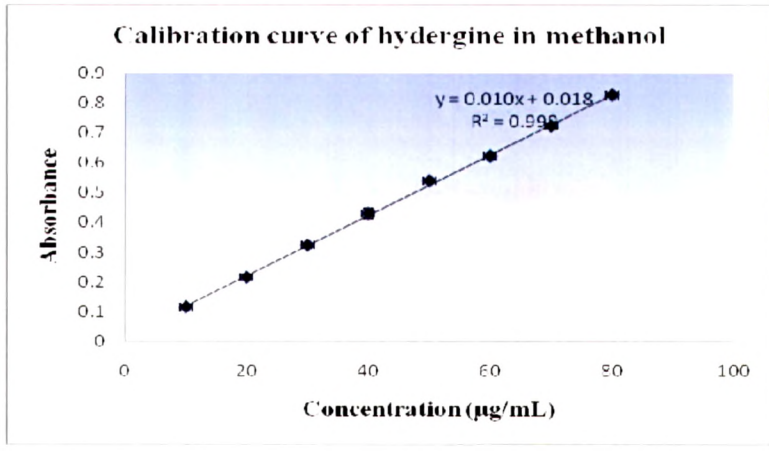


Table 3.12: Calibration of hydergine in methanol

Concentration (µg/ ml)	Mean Absorbance* ± S.D
10	0.116 ± 0.013
20	0.215 ± 0.007
30	0.323 ± 0.011
40	0.429 ± 0.009
50	0.538 ± 0.016
60	0.621 ± 0.008
70	0.722 ± 0.012
80	0.826 ± 0.008

Regression equation** Y= 0.010X + 0.011; Correlation coefficient = 0.999; *n = 3

Figure 3.12: Regressed calibration curve of estimation of hydergine in methanol



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of HG (30µg/mL, 50µg/mL and 70µg/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 hydergine in methanol

Theoretical Concentration of ETP (µg/mL)	Determined Value (µg/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
30	29.85	0.3544	0.0476	29.85± 0.317
50	50.23	0.3245	0.0729	50.23±0.486
70	69.91	0.091	0.0285	69.91±0.19

** At 95% Confidence level; t_{tab} = 4.303 for 2 degrees of freedom*

3.3.2.2 Estimation of hydergine in ME and MME

To determine the amount of HG in ME and MME, 0.2 mL of formulation was added to 10 mL volumetric flask and the volume was made up to the mark with methanol AR grade. The resultant solution was then sonicated for 3 min at ambient temperature, centrifuged and one mL of the supernatant was transferred to 10 volumetric flask. The volume was made up to the mark with methanol AR grade and HG estimated at 292.8 nm using 2nd derivative spectra.

3.3.2.3 Estimation of hydergine for *in vitro* release

The release studies for HG ME and MME were performed in 10 % methanolic phosphate buffer saline pH 6.4 + 2%w/v polysorbate-80. ME and MME equivalent to 2 doses of HG were taken in 1 ml of PBS in a dialysis tube of 10 kDa molecular weight cut-off, which were immersed in beakers containing diffusion media (50 mL) maintained at 37°C on a magnetic stirrer. At specific time intervals samples were withdrawn, diluted suitably with methanol and analyzed by UV spectrophotometer against methanol as blank at 292.8 nm using 2^{nd} derivative spectra.

In vitro release of HG ME and MME were also performed across excised sheep nasal mucosa. Briefly, 0.2 ml of formulation was placed in the donor compartment of Franz diffusion cell along with 1.8 ml of phosphate buffer pH 6.4. Recipient compartment contained 30 ml of 10% methanolic phosphate buffer saline pH 6.4 + 2%w/v polysorbate-80. Samples from the receptor compartment were withdrawn at predetermined time intervals, diluted suitably with methanol and analyzed at 318 nm using 2^{nd} derivative spectra against methanol as blank. Each sample removed was replaced by an equal volume of 10 % methanolic phosphate buffer saline pH 6.4 + 2%w/v polysorbate-80.

3.4 Determination of phycoerythrin-conjugated-anti-mouse-anti-transferrin receptor monoclonal antibody (PE-mAb-Tfr) by spectrofluorometry

The protein PE-mAb-Tfr was estimated by spectrofluorometric method and not by bicinchoninic acid (BCA) based method because of simplicity of the estimation procedure and easy availability of phycoeruythrin conjugated anti-mouse-anti-transferrin receptor monoclonal antibody. The antibody PE-mAb-Tfr was estimated at excitation and emission wavelengths of 488nm and 578 nm respectively against PBS 7.4 as blank.

3.4.1 Estimation of PE-mAb-Tfr in solution

All spectrofluorimetric estimations were performed on a Shimadzu RF-540 spectrofluorometer (Shimadzu Coporation, Japan) equipped with a xenon lamp. The ordinate scale was kept at 7 and the abscissa at 2. The speed of the scanning was kept at

fast. The excitation was set at 488 nm and the emission was scanned in the range of 500-650 nm and determined to be 578 nm.

Preparation of standard stock solutions of PE-mAb-Tfr in PBS pH 7.4

25 µL of PE-mAb-Tfr solution (in PBS pH 7.4) equivalent to 5 µg of antibody was accurately measured using calibrated micropipette and transferred to 10 mL volumetric flask. The final volume was made up to 10 mL with PBS pH 7.4 to prepare stock solution of 500ng per mL of antibody.

Calibration curve of PE-mAb-Tfr in PBS pH 7.4

Suitable aliquots of standard stock solution were accurately measured and transferred to the 5 mL volumetric flasks. The final volume was made up to 5 mL with PBS pH 7.4 to give final concentrations of 20, 30, 40, 50, 60, 70, 80 ng/mL and the relative fluorescence intensity measured setting the $\lambda_{excitation}$ at 488 nm and the corresponding $\lambda_{emission}$ peak intensity was measured at 578 nm (slit widths as mentioned above) using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) against PBS pH 7.4 as blank. The above procedure was repeated three times and the mean relative fluorescence intensity values determined. The data was recorded in Table 3.14 along with standard deviation. Figures 3.13 and 3.14 show the spectrofluorimetric curve and calibration curve respectively of PE-mAb-Tfr in PBS pH 7.4.

Table 3.14: Calibration of PE-mAb-Tfr in PBS pH 7.4

Concentration (ng/mL)	Mean Absorbance* ± S.D
20	9.4 ± 0.004
30	13.2 ± 0.009
40	17.5 ± 0.012
50	21.8 ± 0.017
60	25.9 ± 0.007
70	29.9 ± 0.014
80	35.1 ± 0.013

Regression equation** Y= 0.429X + 0.293; Correlation coefficient = 0.999; *n = 3

Figure 3.13: Spectrofluorimetric curve for λemission for PE-mAb-Tfr

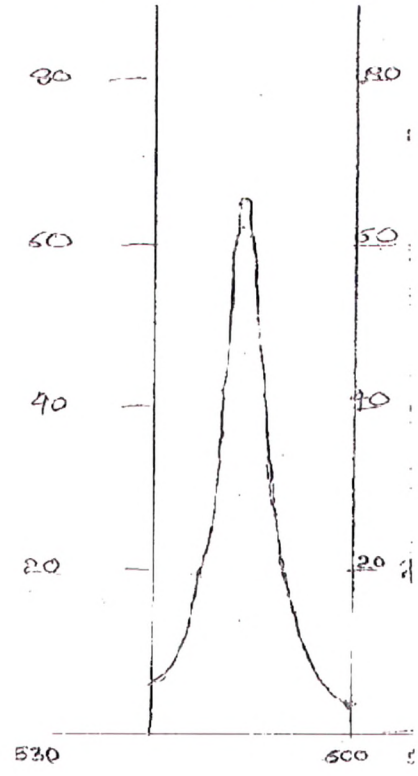
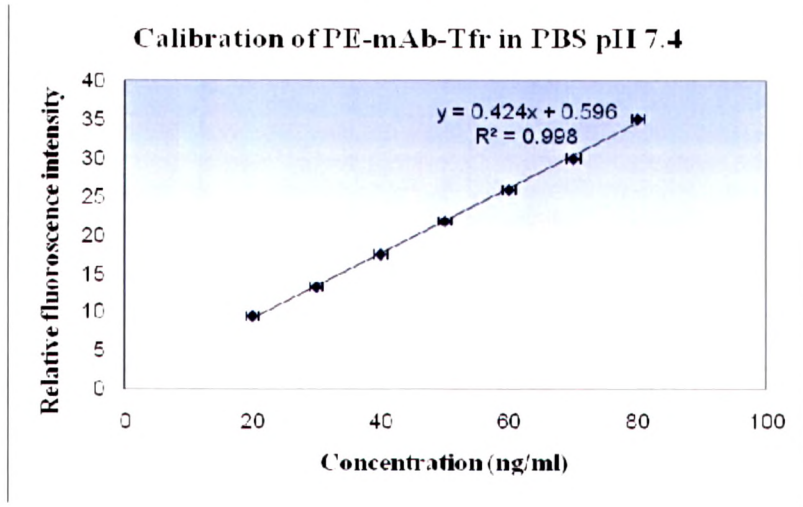


Figure 3.14: Regressed calibration curve for estimation of PE-mAb-Tfr in PBS pH7.4



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of PE-mAb-Tfr (40ng/mL, 60ng/mL and 80ng/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 PE-mAb-Tfr in PBS pH 7.4

Theoretical Concentration of ETP (ng/mL)	Determined Value (ng/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
40	39.81	0.3367	0.0602	39.81±0.402
60	60.11	0.1295	0.0349	60.11±0.232
80	81.06	0.9308	0.3361	81.06±2.24

** At 95% Confidence level; t_{tab} = 4.303 for 2 degrees of freedom*

3.5 Discussion

The drugs NG, SB and HG in nanoparticles, microemulsions and in-vitro release medium were estimated by UV spectrophotometric method. Phycoerythrin-conjugated-anti-mouse-anti-transferrin receptor monoclonal antibody was estimated by spectrofluorometric method.

The calibration of respective drugs NG, SB and HG were developed in acetonitrile and methanol by UV spectrophotometry and were estimated using 2nd derivative spectra in respective solvents. The linearity of NG was found to be in the range of 10-50µg/mL (R^2=0.999) and 10-50µg/mL (R^2=0.999) in acetonitrile and methanol respectively. While for SB the linearity was found to be in the range of 200-900µg/mL (R^2=0.997) and 200-900µg/mL (R^2=0.999) in acetonitrile and methanol respectively, and for HG the linearity was found to be in the range of 10-80µg/mL (R^2=0.999) and 10-80µg/mL (R^2=0.999) in acetonitrile and methanol respectively. The recovery studies for accuracy and precision of the developed method were carried out at various definite concentrations for respective drugs and were found to be more than 90%, indicating the reliability of the method.

To determine the amount of drug entrapped, nanoparticles were dissolved in acetonitrile. The resulting system was centrifuged to remove the precipitated components and the supernatant was diluted suitably with the solvent and subjected to analysis. While, to determine the amount of drugs in ME, a definite quantity was dissolved in methanol and the resulting system was centrifuged to remove the

precipitated components. The supernatant was diluted suitably with the solvent and subjected to analysis.

The *in vitro* release study for NPs was performed using the tube shaking method. At different time intervals, the samples were removed and centrifuged at 15000 rpm and the settled nanoparticles were dissolved in acetonitrile and analysed for the drug remaining in the nanoparticles as the same for entrapment efficiency. The drug released was calculated by taking the difference of the drug taken initially and the drug remaining in the nanoparticles. While for ME, the *in vitro* release study was performed, using the dialysis tube method, and across excised sheep nasal mucosa using Franz diffusion cell. Samples were withdrawn at definite time intervals and the drug analyzed spectrophotometrically using 2^{nd} derivative spectra.

The estimation of PE-mAb-Tfr conjugated to the nanoparticles was carried out by the spectrofluorometric method considering the calibration curve of PE-mAb-Tfr established at 20-80 ng/mL (R^2=0.999) in PBS pH 7.4.

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