CHAPTER 3

ANALYTICAL METHODS

3.1 Materials

Galantamine hydrobromide (Gal) was kindly gifted by SPARC, Vadodara, India; Bovine Serum Albumin (Fraction V, 98% purity) was purchased from Sigma Aldrich, India. Monobasic potassium phosphate, sodium phosphate and HCl were obtained from SD Finechem ltd, Mumbai, India. Acetonitrile, Water and Methanol were of HPLC grade and purchased from Merck Chemicals, India. Methanol, chloroform, Dibutylamine , phosphoric acid (85%), trichloroacetic acid, sodium sulphate and sodium hydroxide were purchased from Spectrochem, India.

Bapineuzumab was purchased from Arihant Traders (India), Bovine serum albumin, 2 - iminothiolane (Traut's reagent), hydrochloride,5,5'-dithio-bis(2-nitro-benzoic acid)(Ellman's reagent) and L – cysteine were procured from Qualigens, India. The polypropylene column for gravity size exclusion chromatography and affinity chromatography was purchased from BioRad, India. Sephadex G25, Sephadex G 100, Sephacryl HR 200 and MabSelect were procured from GE Healthcare, USA. Tris buffer, sodium chloride, glycine, sodium hydroxide were purchased from Sigma Aldrich, India.

3.2 Calibration and interaction study for Galantamine hydrobromide:

To understand the level of interaction between Galantamine hydrobromide and Bovine Serum albumin (BSA), the following UV study was carried out using UV – 1800, Shimadzu, Japan:

3.2.1 Calibration curve for Galantamine hydrobromide:

UV spectrophotometric analysis was performed as per method reported by Patel et al, 2010 (Patel Hitesh et al., 2010). Standard stock solution was prepared by dissolving 5mg of Galantamine hydrobromide in 25 ml of distilled water. Suitable aliquots of the stock solution were pipetted out into 5 ml volumetric flasks and the volume was made upto 5 ml with distilled water to give final concentrations ranging from 40 – 160 μg/ml. The

solutions were mixed using vortex mixer and their absorbances measured at λ_{max} 289 nm using distilled water as blank on Shimadzu 1800 UV-Visible Spectrophotometer and calibration curve was plotted. The above procedure was repeated three times.

3.2.2 UV absorbance of Bovine Serum albumin and Galantamine hydrobromide: (Patel Hitesh et al., 2010)

1 mL of GH solution was mixed with 1 mL BSA (drug : polymer ratio = 1:10).

The UV readings were taken on Shimadzu 1800 UV-Visible Spectrophotometer at λ_{max} 289 nm with distilled water as blank. The above procedure was repeated three times.

3.3 Results and discussion of calibration and interaction study for Galantamine hydrobromide:

Galantamine hydrobromide in distilled water showed a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan showed absorption maximum at 289 nm and this wavelength was chosen as the analytical wavelength (Fig 3.1). Beer's law was obeyed between 40 and 160 μg/mL (Table 3.1). Regression analysis was performed on the experimental data. Regression equation for standard curve was y=0.01060x+0.03903. Correlation coefficient was found to be 0.99892 signifying that a linear relationship existed between absorbance and concentration of the drug. The UV spectrum for Galantamine hydrobromide is shown in Figure 3.1. Parameters indicating linearity for the UV spectrometric method of analysis for Galantamine hydrobromide are shown in Table 3.2

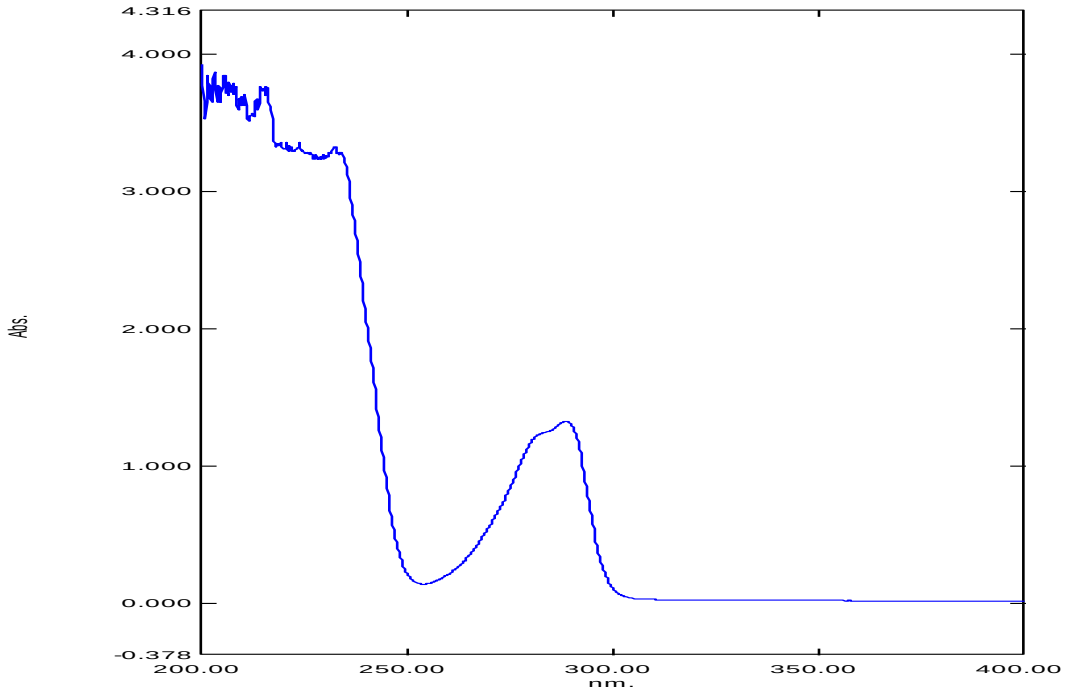

Figure 3.1: UV Spectrum for Galantamine hydrobromide (120 µg/mL**)** in distilled water.

Table No 3.1: Calibration data for Galantamine hydrobromide in Distilled water by UV spectroscopy :

Sr No	**Concentration (µg/mL)**	**Mean Absorbance±SD***
1	20	0.242±0.039
2	40	0.417±0.032
3	80	0.869±0.034
4	120	1.324±0.086
5	160	1.760±0.074

*: Average of three experiments

Table No 3.2: Parameters for estimation of Galantamine hydrobromide in distilled water by UV Spectroscopy:

Parameters	Results
Λ_{max}	289
Linearity range	40-160 µg/mL
Regression Equation	y=0.01060x+0.03903
Correlation Coefficient	0.99892

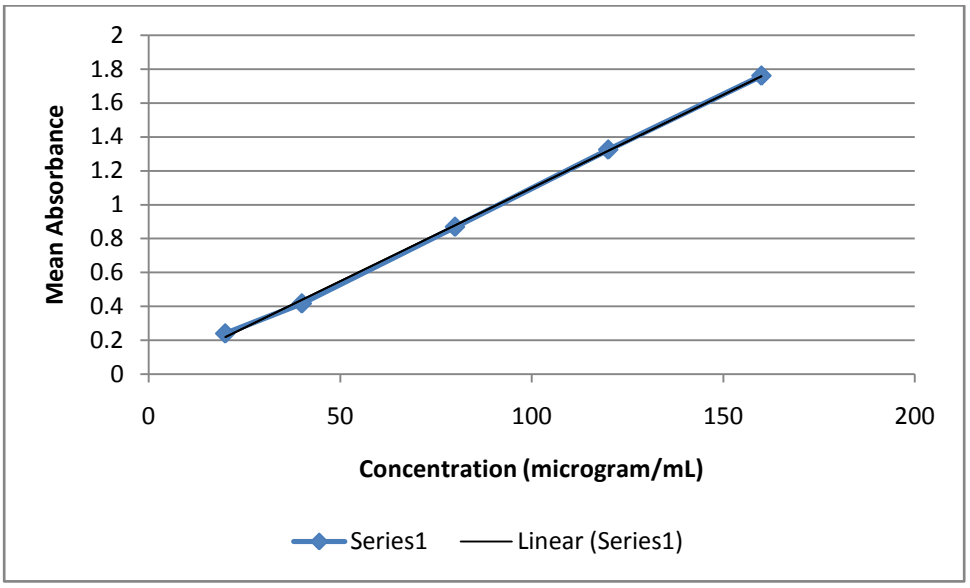

Figure 3.2: Standard plot for Galantamine hydrobromide in distilled water.

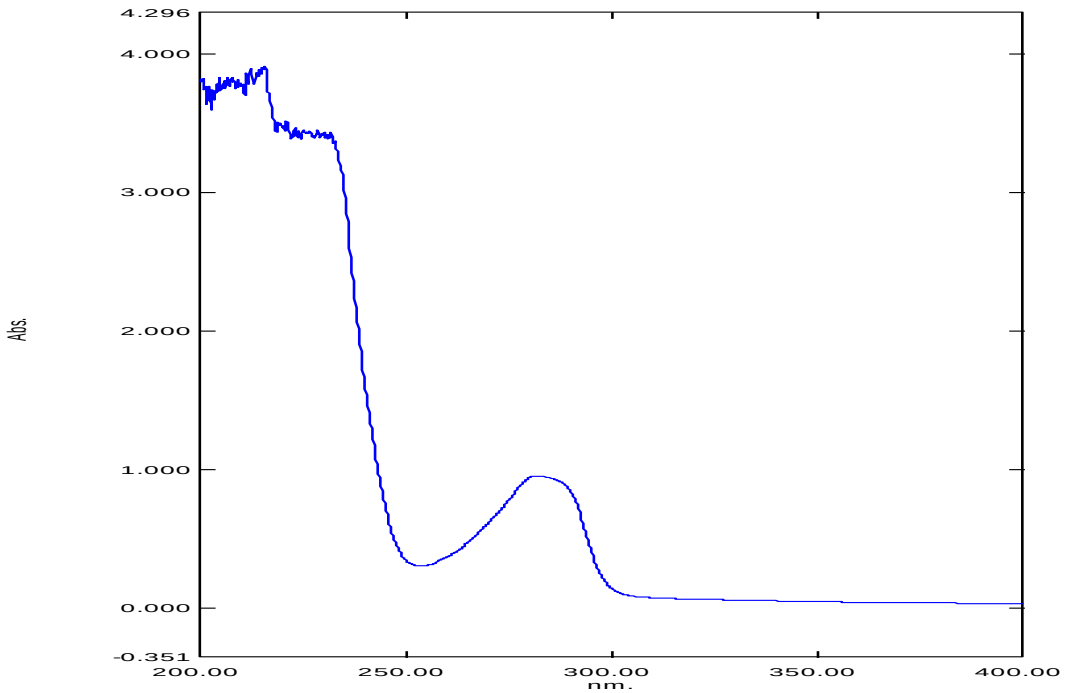

Figure 3.3: UV spectrum of Galantamine hydrobromide in presence of Bovine Serum Albumin (120+1200)

Table No 3.3: Absorbance of Galantamine hydrobromide in presence of Bovine Serum Albumin

Combination (drug+polymer)	**Absorbance at λ = 289 nm of combination* ±SD**	**Absorbance at λ_{max} = 289 nm of Galantamine alone*±SD**	**Difference in Absorbance ±SD**
40+400	0.298 ± 0.021	0.417 ± 0.041	0.119 ± 0.025
80+800	0.653 ± 0.034	0.869 ± 0.027	0.216 ± 0.039
120+1200	0.889 ± 0.022	1.324 ± 0.033	0.435 ± 0.011
160+1600	1.163 ± 0.018	1.760 ± 0.019	0.6 ± 0.022

***:** Experiments were done in triplicate.

The absorbance of Galantmine hydrobromide showed a change indicating a ionic interaction between Galantamine hydrobromide and Bovine Serum Albumin.

Though this interaction is desirable for the proper attachment of Galantamine hydrobromide on Bovine Serum Albumin, it is undesirable for estimation of Galantamine hydrobromide in formulation development and evaluation. Hence during analysis of samples, the blank was prepared in the same way as the sample, except Galantmine hydrobromide was not added to it.

3.4 Estimation of Galantamine hydrobromide by High Performance Liquid Chromatography in plasma: (Tencheva et al., 1987)

3.4.1 Reverse phase Liquid Chromatography conditions:

C_8 column was used. Methanol-water (40:60) mobile phase was modified with $5X10^{-3}$M dibutylamine at pH 7 (adjusted with 85% phosphoric acid) and a flow-rate of 1.2 ml/min were chosen. The presence of dibutylamine significantly improved the chromatographic behaviour of the substances investigated, as it reduced the peak tailing effect (Gill, Alexander, & Moffat, 1982; Nahum & Horváth, 1981). The mobile phase was prepared by mixing 400 mL of methanol, 0.85 mL of dibutylamine and 0.2 mL of 85% phosphoric acid and making to 1000 mL with distilled water. The mixture was degassed before use. All chromatographic investigations were performed at room temperature.

3.4.2 Plasma standards: (Tencheva et al., 1987)

A stock solution was prepared by dissolving 12.82 mg of Galantamine hydrobromide to yield the equivalent of 100 µg/mL Galantamine base. This solution was diluted with blank human plasma obtained from Blood Bank to make standard solutions with final concentrations of 0.5-10 µg/mL.

3.4.3 Sample preparations: (Tencheva et al., 1987)

For 2.00 ml of blood plasma, 2.0 ml of 20% trichloroacetic acid was used as a precipitating agent. The mixture was shaken for 1 min on a vortex mixer. After centrifugation at 2000 g for 10 min, 3.00 ml of the supernatant were transferred in another test-tube, and 0.5 ml of 5 M sodium hydroxide were added to bring the pH of the supernatant to 11.

Then 5.00 ml of chloroform was added. The tube was rotated on a vortex mixer for 30 s and centrifuged at 2000 g for 5 min. A 4.5-ml aliquot of the clear solution was filtered through sodium sulphate. The filter was washed with 1.0 ml of chloroform. The extracts were evaporated at 62-65 °C. The dry residue was dissolved in 0.2 ml of mobile phase, and 100 µ1 were injected into the chromatograph (Jasco with Kya tech Software NAV 1.17.01).

Program parameters were: Flow rate-1.2 mL/min, Detection wavelength - 280 nm, run time – 20 min. The column as equilibrated by passing at least 150 – 200 mL of mobile phase. 20 µL of sample was loaded using syringe through rheodyne injector.

Similar method was utilized to obtain standard plot for Cerbrospinal Fluid of rats.

For Cerebrospinal fluid withdrawal, rat was anaesthetised using ketamine (5mg/kg). Rat was mounted on stereotaxic apparatus (M. C. Dalal agencies, Chennai, India) using earbars. Fur (Hairs) on dorsal part of rat head was cut using scissors and incision (between eyes part vertically at centre). Skin was retracted using artery forcep to right and left side and skull was exposed to make sutures visible (Skin part was rubbed using cotton plug).

Following the procedure of Paxinos and Watson (1986) (Paxinos, 1997) bregma was located.

Using the dental driller, burr hole was made in skull without damaging the brain (to avoid perfused bleeding) using co-ordinated (1.33 mm right lateral, 0.8mm posterior and 1.5 mm ventral). Polyethylene tubing was inserted in hole and CSF was suctioned out and collected.

3.5 Results and discussion for High Performance Liquid Chromatography of Galantamine hydrobromide:

The method for determination of Galantamine hydrobromide in plasma by reverse phase HPLC is reported and hence its validation was not carried out.

This calibration data would help in estimating the drug in plasma and Cerebrospinal Fluid after nasal administration during in vivo studies.

The HPLC chromatogram of Galantamine hydrobromide in plasma is shown in Figure 3.4. The standard plot for Galantamine hydrobromide in plasma is shown in Figure 3.5. The HPLC chromatogram of Galantamine hydrobromide in Cerebrospinal fluid is shown in Figure 3.6. The standard plot for Galantamine hydrobromide in Cerebrospinal fluid is shown in Figure 3.7. Table 3.4 and Table 3.5 shows the calibration data for Galantamine hydrobromide in plasma. Table 3.6 and Table 3.7 shows the calibration data for Galantamine hydrobromide in Cerebrospinal fluid.

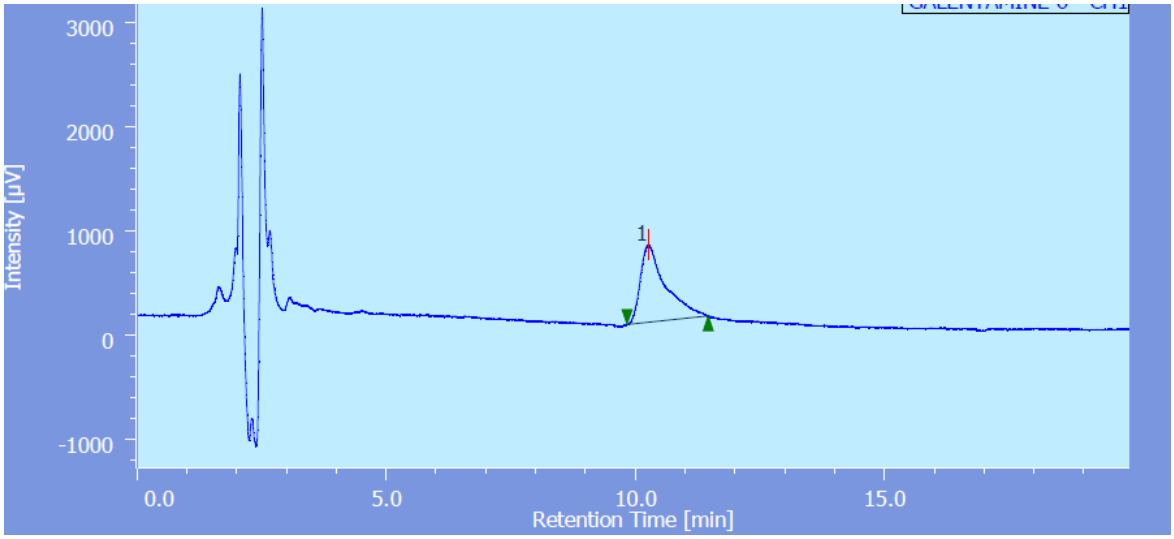

Figure 3.4: Representative chromatogram of Galantamine in plasma at 6 µg/mL standard concentration

Table No 3.4: Calibration data for Galantamine hydrobromide in plasma by HPLC

Sr No	Concentration (µg/mL)	Mean area (µV.sec) ±SD*
1	0.5	2081 ± 8.16
2	1	5593 ± 9.11
3	2	8588 ± 6.77
4	4	12599 ± 15.23
5	6	25933 ± 4.89
6	8	33135 ± 8.27
7	10	41482 ± 5.12

*: Experiments were conducted in triplicate

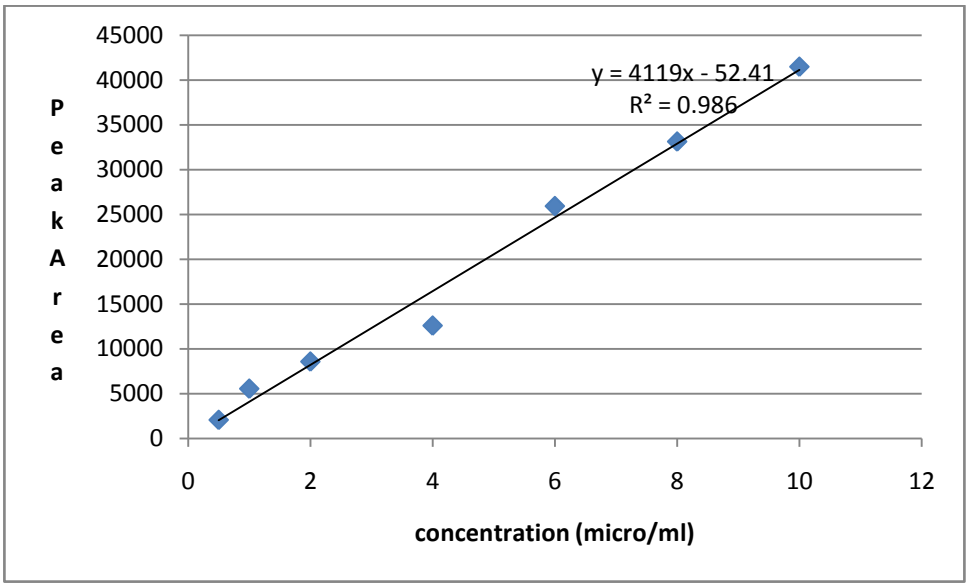

Figure 3.5: Standard plot for Galantamine hydrobromide in plasma

Table No 3.5: Parameters for estimation of Galantamine hydrobromide in plasma by HPLC

Parameters	**Results**
Λ_{max}	280
Linearity range	0.5 – 10 µg/mL
Regression Equation	y = 4119x – 52.41
Correlation Coefficient	0.986

From Table 3.5 it could be seen that, for plasma , the linearity of standard calibration plot was observed from 0.5 – 10 µg/mL. Also the correlation coefficient was near to 1 which indicates that this calibration is linear enough to be used as a standard to estimate drug in plasma during in vivo studies.

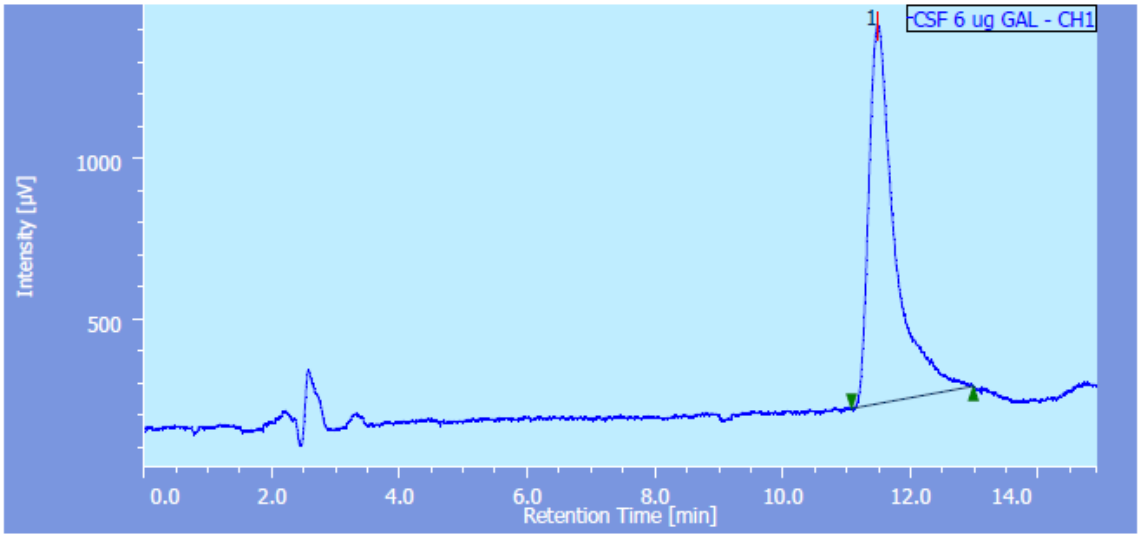

Figure 3.6: Representative chromatogram of Galantamine hydrobromide in Cerebrospinal fluid at 6 µg/mL standard concentration

Table No 3.6 Calibration data for Galantamine hydrobromide in Cerebrospinal fluid by HPLC

Sr No	Concentration (µg/mL)	Mean area (µV.sec) ±SD*
1	0.5	2821 ± 22.14
2	1	7418 ± 31.74
3	2	16802 ± 30.19
4	4	26251 ± 33.68
5	6	33567 ± 28.14
6	8	61586 ± 35.41

*: Experiments were conducted in triplicate

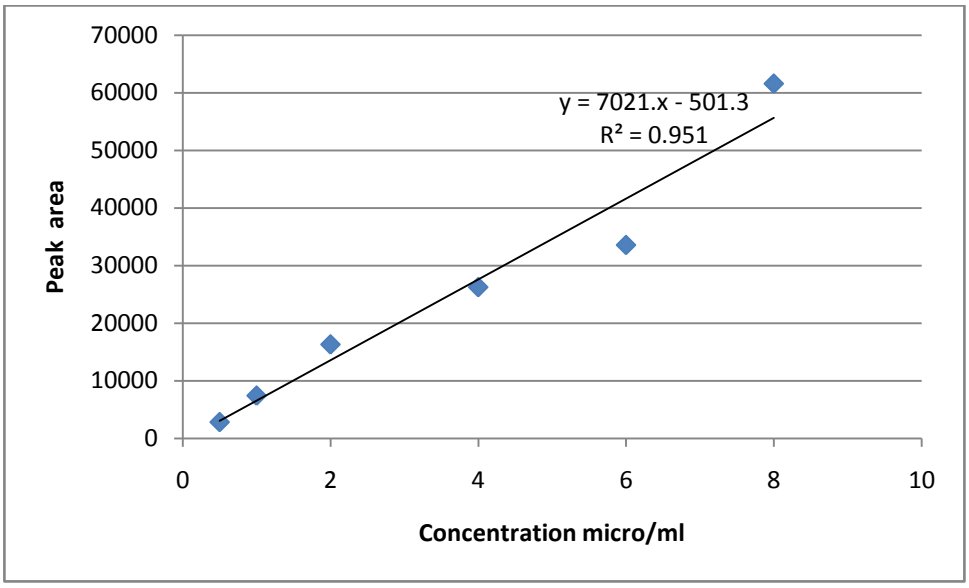

Figure 3.7: Standard plot for Galantamine hydrobromide in Cerebrospinal fluid.

Table No 3.7 Parameters for estimation of Galantamine hydrobromide in Cerebrospinal fluid by HPLC

Parameters	Results
λ_{max}	280
Linearity range	0.5 – 10 µg/mL
Regression Equation	y = 7021x – 501.3
Correlation Coefficient	0.951

3.6 Analytical Method for Bapineuzumab:

The formulation of Bapineuzumab loaded nanoparticles involved modification of the antibody followed by its loading on Bovine Serum Albumin nanoparticles. Therefore the analytical method involved method development for separation of modified antibody from unmodified antibody and calculation of unbound antibody after formulation of loaded nanoparticles.

The Size Exclusion Chromatography is a type of Gel Permeation chromatography mainly utilized for the separation of proteins (Paul-Dauphin et al., 2007). Herein this technique was utilized to separate the modified antibody from unmodified antibody. The modified antibody was loaded on Bovine Serum Albumin nanoparticles by conjugation method.

Size exclusion chromatography (SEC) is a general name for the process of separation of molecules according to their size when a solution flows through a packed bed of porous packing. In general, SEC is a liquid column chromatographic technique in which a sample solution is introduced into a column filled with porous packing and is carried through the column by solvent. Ideally, size separation is achieved by differential pore permeation. The volume of the pore, which is effectively accessible, is greater for a small molecule than for a large one. Therefore, larger molecules have shorter retention times in the pores of the packing than smaller ones and are eluted from the column earlier. (Kostanski, Keller, & Hamielec, 2004).

Initially trials were taken to select the proper resin and column volume of resin to separate modified antibody from unmodified antibody. The details of which are mentioned in the sections to follow.

3.7 General procedure for separation of modified and unmodified antibody:

3.7.1 Sample preparation: The modification of antibody involves the use of imminothiolane (Sylvia Wagner et al., 2010).The solution of antibody was prepared in Tris buffer (pH 7.5) in the concentration of 2 mg/mL. The solution containing antibody and imminothiolane (6.9 mg in 50mL Phosphate buffer, pH 8.0) was loaded on the column in the volume of 500 µL. This 500 µL sample contained 500 µL of 1mg/mL antibody solution, 80 µL of imminothiolane and 500 µL of hydroxyl amine.

3.7.2 Column preparation: A 20 cm X 1 cm Bio Rad polypropylene column was packed with Sephadex G 25 resin upto 15 mL. It was washed by passing sufficient distilled water and then with 50 mM Tris buffer, pH 7.5. Slowly 500 µL of sample solution was added. After 5 minute, 50mM Tris buffer with 150mM NaCl, pH 7.5 was added slowly and elutes was collected from other end of the column. Twenty elutes of 500 µL were collected.

3.7.3 Analysis of elute:

Each elute was analyzed by UV spectrophotometer (Shimadzu 1800) at 280 nm for antibody.

The experiments were performed in triplicate.

Using the procedure mentioned in section 3.7, three resins were evaluated. The resins evaluated were Sephadex G25, Sephadex G 100 and Sephacryl HR 200

3.8 Results and discussion for separating modified from unmodified antibody:

Figure 3.8 shows the elution of antibody after loading the sample solution in Sephadex G 25.

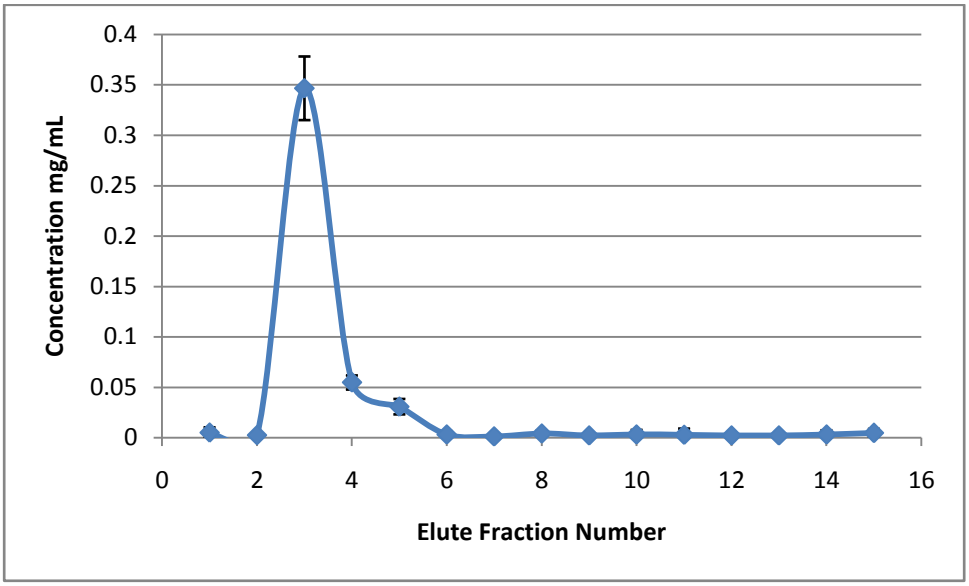

Figure 3.8: Elution of antibody through Sephadex G25 resin.

Only a single peak was observed during the study. This showed that both the modified as well as the ummodified antibody eluted at the same time without getting separated. Therefore Sephadex G 100 which has a wider range of molecular weight (4000–150,000 Da) cut off was used.

Figure 3.9 shows the results after loading the sample solution in Sephadex G 100.

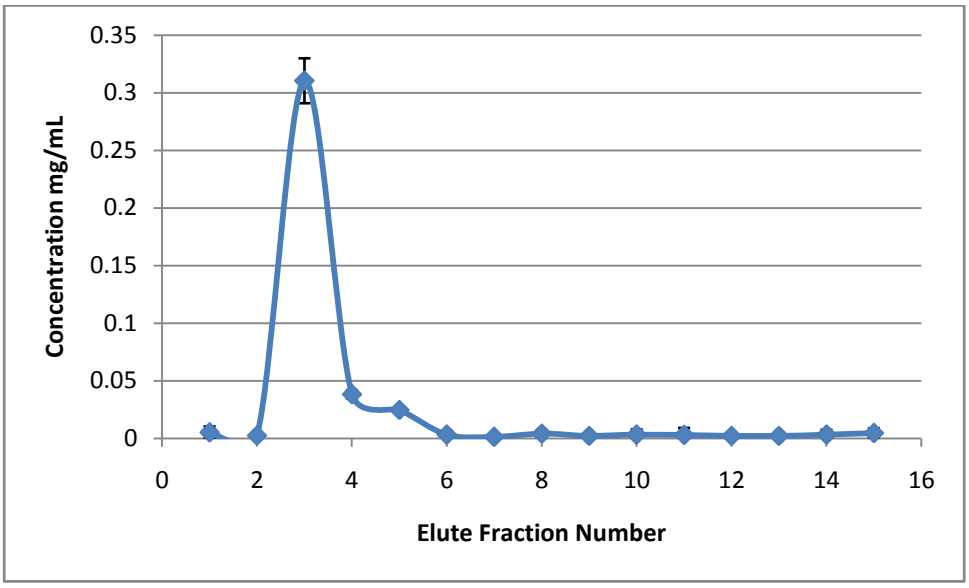

Figure 3.9: Elution of antibody through Sephadex G100 resin.

As seen in Figure 3.9 the resolution of unmodified antibody from modified antibody was not achieved. Therefore Sephadex G 100 was replaced with Sephacryl 200 HR (5 – 250 kDa).

Figure 3.10 shows the results after loading the sample solution in Sephacryl 200 HR.

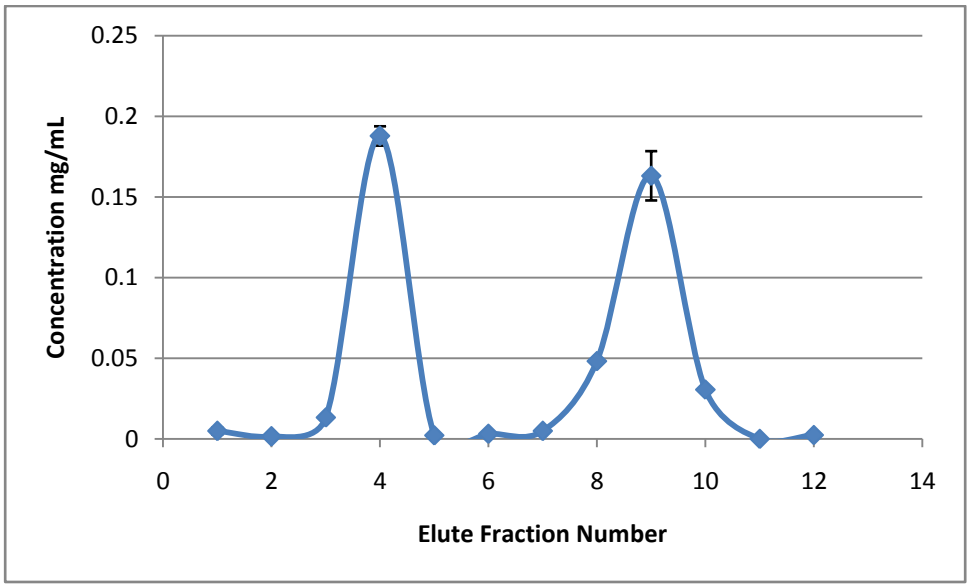

Figure 3.10: Elution of antibody through Sephacryl 200 HR resin.

Figure 3.10 shows two peaks, indicating that the unmodified antibody had resolved from the modified antibody. Modification of antibodies involved

introduction of thiol groups on the antibody, hence an increase in molecular weight. Therefore, modified antibody eluted first followed by the unmodified antibody.

The 50mM Tris buffer with 150mM NaCl, pH 7.5 was used as the elution buffer, as presence of NaCl aids in the elution of the positively charged antibody from the matrix of the gel (Belew, Porath, Fohlman, & Janson, 1978). Additionally, the observed results are in line with the reports stating that Sephacryl 200 HR has better resolving properties than Sephadex G 100. (Belew et al., 1978; G. Morgan & Ramsden, 1978)

To further ascertain the use of Sephacryl 200 HR as the resin to separate modified and unmodified antibody, this method was validated with respect to precision and accuracy in recovery of modified antibody.

3.9 Method validation for separation of modified antibody from unmodified antibody

3.9.1 Accuracy

Accuracy refers to the closeness of an individual observation or mean of the observations to true value (Bolton S & Swarbrick J, 1990). The accuracy is expressed as % bias or % relative error (difference from added concentration) and it takes into account the total error, i.e. systematic and random errors, related to the test result (Hubert et al; 2003). The "true" value is the result which would be observed in absence of error. Accuracy is defined as the percentage of the agreement between the measured value and the true value as follows (Merodio, Campanero, Mirshahi, Mirshahi, & Irache, 2000) .The accuracy was calculated by using following formula:

Accuracy = (True value – Measured value/True value)*100 …….(3.1)

3.9.2 ***Precision***

It refers to the extent of variability of a group of measurements observed under similar conditions. Precision provides an indication of random errors and is generally subdivided into two cases: repeatability and reproducibility, which were determined by calculating RSD

(Relative standard deviation) or CV (Coefficient of variation) of inter-day and intra-day determinations. One of the common ways of expressing the variability, which takes into account its relative magnitude, is the ratio of the standard deviation (SD) to the mean, SD/Mean. This ratio, often expressed as a percentage, is called the *Coefficient of Variation* abbreviated as CV or RSD, the *relative standard deviation*. In biological data, the CV is often between 20 - 50%. The relatively large CV observed in biological experiments is due mostly to "biological variation", the lack of reproducibility in living material(Bolton S & Swarbrick J, 1990).

To calculate these parameters, a total of 12 such runs on column were conducted.

Table No 3.8: Precision and Accuracy for the seperation of modified antibody

Standard concentration (µg/mL)*	**Observed concentration (µg/mL)#**	**Precision (%)[a]**	**Accuracy (%)[b]**
100	99.14 ± 0.25	0.13	99.17
500	498.23 ± 1.28	0.24	99.64
1000	998.19 ± 13.69	0.21	99.81

*: Standard concentration of modified antibody

#: Observed concentration of modified antibody calculated with reference to cysteine.

a: Expressed as relative standard deviation, RSD = (Standard deviation/mean concentration)*100

b: Expressed as (mean observed concentration/actual concentration)*100

To evaluate precision, the mean values and the % RSD values were calculated for each concentration. Table 3.8 shows low % CV values ranging from 0.13 – 0.24 %, which indicated precision of the method. No significant difference between the amounts of antibody added (actual) and observed concentration at all the concentration levels tested was noticed indicating accuracy of the method.

Therefore Sephacryl HR 200 was selected as the resin to separate modified antibody from unmodified antibody.

Sephacryl High Resolution (HR) is a composite gel prepared by covalently cross-linking allyl dextran with N,N'-methylene bisacrylamide to form a hydrophilic matrix of high mechanical strength. The hydrophilic nature of the media minimizes nonspecific adsorption and maximizes recovery. The resin is specifically recommended for purification of antibodies, and has been utilized as reported by Ching, Johanssen. The observed superior performance of Sephacryl HR in comparison to Sephadex grades is reported. This is because of its rigid structure. Also, this resin performs better even if it is loosely packed in a column. This could be beneficial for purifying the antibody at a production level where a larger column is used.(G. Morgan & Ramsden, 1978)

3.10 Estimation of free Bapineuzumab after formulation of nanoparticles

The affinity chromatography by gravity column was utilized to estimate the unloaded modified antibody. A gravity column in a 5 cm X 1 cm Bio Rad polypropylene column packed with MabSelect (GE Healthcare, USA) was used for the purpose. Prior to actual analysis of supernatant obtained after centrifugation of formed nanoparticles, this method was validated by mixing solution of Bovine Serum Albumin and Bapineuzumab and calculating the recovery of Bapineuzumab in the elute.

3.10.1 Sample preparation: 2 mg/mL solution of antibody was prepared in distilled water. 10 mg/mL solution of Bovine Serum Albumin was prepared in distilled water. 250 µL of each solution were mixed together. 500 µL of this solution was then loaded on the packed column. Prior to loading the sample, the solution pH was adjusted to 7 using 2M Tris solution

3.10.2 Column preparation: 5mL resin was packed in the column. It was sufficiently washed with distilled water. The packed resin was equilibrated with 50 mM Tris solution, pH 7.0-7.5. Sample was loaded on the column and the initial flow through was collected from the other side of the column. Column was washed with equilibration buffer (50

mM Tris solution, pH 7.0-7.5) . Elution was carried out with 150mM Glycine buffer, pH 3.5.

After complete elution, column was regenerated by passing 0.1 M NaOH. The column was sequentially washed with equilibration buffer, water and then stored in 20% Ethanol solution for future use.

3.10.3 Elute collection: Twenty elutes (750 µL per elute) were collected. Each elute was analyzed by UV spectrophotometer (Shimadzu 1800) at 280 nm for the content of antibody.

Six such similar experiments were conducted,

3.11 Results and discussions for estimation of free Bapineuzumab

The matrix of MabSelect is a highly cross-linked agarose, produced using a new manufacturing process that gives a very rigid matrix. The matrix of MabSelect allows at least five times higher flow velocities to be used in process scale compared with conventional cross-linked agarose of similar porosity. The recombinant protein A ligand is produced in *Escherichia coli*. Fermentation and subsequent purification of the protein A are done in the absence of mammalian products. The recombinant protein has been specially engineered to favor an oriented coupling that gives an affinity medium with enhanced binding capacity for IgG. The specificity of binding to the Fc region of IgG is similar to that of native protein A, and provides excellent purification in one step. The epoxy-based coupling chemistry ensures low ligand leakage. The high capacity, low ligand leakage and specially developed base matrix make MabSelect ideal for purification of monoclonal antibodies at process scale. (Healthcare, 2008)

Figure 3.11 shows the peaks for antibody eluted through MabSelect resin.

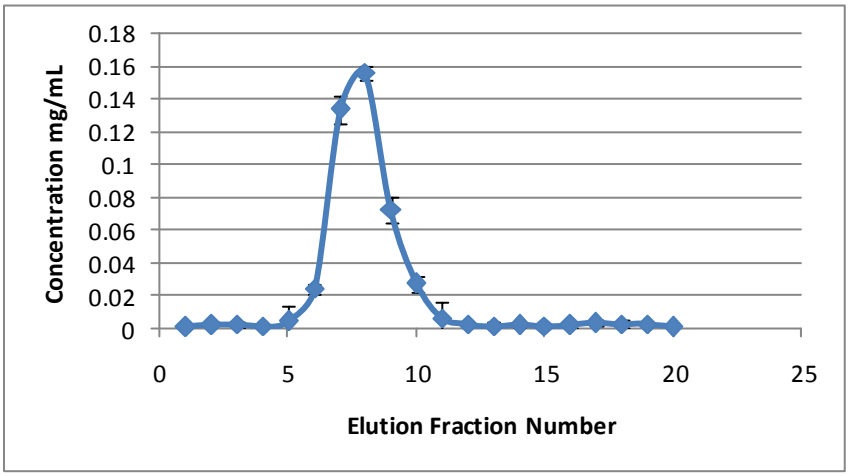

Figure 3.11: Elution of antibody through MabSelect resin.

The antibody was detected in the 5th, 6th, 7th, and 8th fractions out of the 20 elutes collected (Fig 3.11). The recovery efficiency ranged from 99.10 % ± 0.64. Hence MabSelect was selected for estimation of Bapineuzumab in the nanoparticle formulation.

Table No 3.9: Precision and Accuracy for the seperation of modified antibody

Standard concentration (µg/mL)	**Observed concentration (µg/mL)***	**Precision (%)[a]**	**Accuracy (%)[b]**
100	99.14 ± 0.33	0.14	99.14
500	498.22 ± 3.67	0.17	99.64
1000	997.97 ± 11.96	0.16	99.79

*: Results for six experiments.

a: Expressed as relative standard deviation, RSD = (Standard deviation/mean concentration)*100

b: Expressed as (mean observed concentration/actual concentration)*100

To evaluate precision, the mean values and the % RSD values were calculated for each concentration. Table 3.9 shows low % CV values ranging from 0.14 – 0.16 %, which indicated precision of the method. No significant difference between the amounts of antibody added (actual) and observed concentration at all the concentration levels tested was noticed indicating accuracy of the method using MabSelect as the resin.

3.12 References:

- Belew, M., Porath, J., Fohlman, J., & Janson, J.-C. (1978). Adsorption phenomena on sephacryl® S-200 superfine. *Journal of Chromatography A, 147*, 205-212. doi: http://dx.doi.org/10.1016/S0021-9673(00)85132-8
- Bolton S, & Swarbrick J. (1990). Basic definitions and concepts *Pharmaceutical statistics: Practical and clinical applications* (pp. 24). New York: Marcel Dekker.
- Gill, R., Alexander, S. P., & Moffat, A. C. (1982). Comparison of amine modifiers used to reduce peak tailing of 2-phenylethylamine drugs in reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 247*(1), 39-45. doi: http://dx.doi.org/10.1016/S0021-9673(00)84854-2
- Healthcare, G. (2008). MabSelect. *Affinity chromatography.* Retrieved 28, February, 2014, from http://www.gelifesciences.co.jp/catalog/pdf/18114994.pdf
- Kostanski, L. K., Keller, D. M., & Hamielec, A. E. (2004). Size-exclusion chromatography—a review of calibration methodologies. *Journal of Biochemical and Biophysical Methods, 58*(2), 159-186. doi: http://dx.doi.org/10.1016/j.jbbm.2003.10.001
- Merodio, M., Campanero, M. A., Mirshahi, T., Mirshahi, M., & Irache, J. M. (2000). Development of a sensitive method for the determination of ganciclovir by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 870*(1–2), 159-167. doi: http://doi.org/10.1016/S0021-9673(99)01015-8
- Morgan, G., & Ramsden, D. B. (1978). Comparison of the gel chromatographic properties of Sephacryl S-200 Superfine and Sephadex G-150. *Journal of Chromatography A, 161*, 319-323. doi: http://dx.doi.org/10.1016/S0021-9673(01)85244-4
- Nahum, A., & Horváth, C. (1981). Surface silanols in silica-bonded hydrocarbonaceous stationary phases : I. Dual retention mechanism in reversed-phase chromatography. *Journal of Chromatography A, 203*, 53-63. doi: http://dx.doi.org/10.1016/S0021-9673(00)80281-2
- Patel Hitesh, Patel amit, patel vishal, Dave Jayant, & Chhaganbhai, P. (2010). UV - spectrophotometric method development and vaalidation for estimation of Galantamine Hydrobromide in tablet dosage form. *J.Chem.Pharm Res., 2*(2), 44-49.
- Paul-Dauphin, S., Karaca, F., Morgan, T. J., Millan-Agorio, M., Herod, A. A., & Kandiyoti, R. (2007). Probing Size Exclusion Mechanisms of Complex Hydrocarbon Mixtures: The Effect of Altering Eluent Compositions. *Energy & Fuels, 21*(6), 3484-3489. doi: 10.1021/ef700410e
- Paxinos, W. (1997). *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press.
- Tencheva, J., Yamboliev, I., & Zhivkova, Z. (1987). Reversed-phase liquid chromatography for the determination of galanthamine and its metabolites in human plasma and urine. *Journal of Chromatography B: Biomedical Sciences*

and Applications, <HT>421</HT>, 396-400. doi: http://dx.doi.org/10.1016/0378-4347(87)80424-3

- Wagner, S., Rothweiler, F., Anhorn, M. G., Sauer, D., Riemann, I., Weiss, E. C., . . . Langer, K. (2010). Enhanced drug targeting by attachment of an anti αv integrin antibody to doxorubicin loaded human serum albumin nanoparticles. *Biomaterials*, *31*(8), 2388-2398. doi: http://dx.doi.org/10.1016/j.biomaterials.2009.11.093