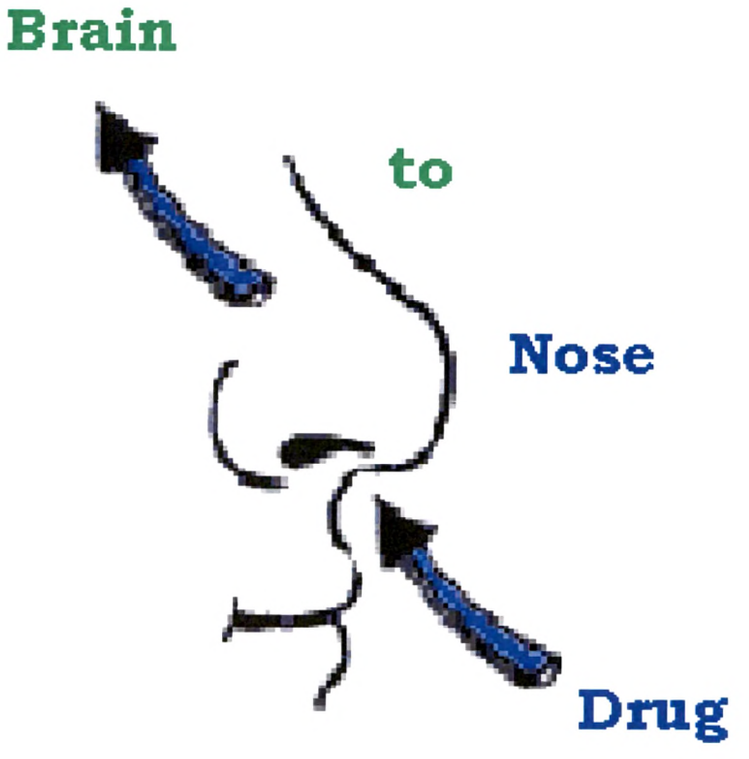

CHAPTER 3

ANALYTICAL METHODS

3.1 Estimation of Tacrine Free Base (Drug Substance)

Tacrine free base was obtained by neutralization of tacrine HCl solution with 10%w/v NaOH solution. Tacrine free base was estimated using UV-Visible double beam spectrophotometric method.

3.1.1 Methodology:

Reagents

Methanol analytical reagent grade was used to prepare the primary stock solution and subsequent dilutions for the estimation of Tacrine.

Preparation of Primary stock solution

Tacrine was weighed (approx. 10 mg) and transferred to 100 mL volumetric flask. About 70 mL of the methanol was added to volumetric flask. The solution was sonicated for 2 min at ambient temperature. The final dilution was made to 100 mL (i.e. 100 µg/mL) using methanol. 10 mL of diluted solution of tacrine was taken in 50 mL clean and dry volumetric flask and volume was made up to 50 mL (i.e. 20 µg/mL) using methanol. The primary stock solution was stored at 2°C to 8°C till assayed.

Preparation of Test solution

Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 µg/mL.

Determination of UV Absorbance Maxima of Tacrine: Tacrine test solution of concentration 10 µg/mL was scanned for determination of absorbance maxima (λ_{max}) on a UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan). The scanning was carried out in a range of 200-400 nm.

Calibration Curve of Tacrine: The calibration curve of tacrine was prepared in methanol. Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 µg/mL. Six different sets of primary stock solutions were prepared and final dilution was made as mentioned above

using methanol. The absorbance of samples was measured at λ_{max} 326 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width). Methanol was used as a blank. The results are recorded in Table 3.1. Calibration curve is obtained by plotting mean absorbance vs. concentration (Figure 3.1).

Table 3.1 Calibration curve of tacrine free base in methanol at 326 nm

Sr. No.	Concentration (µg/mL)	Absorbance ± SD (n=6)
1	0	0.000 ± 0.000
2	2	0.125 ± 0.013
3	4	0.256 ± 0.012
4	6	0.382 ± 0.013
5	8	0.517 ± 0.014
6	10	0.639 ± 0.013
7	12	0.767 ± 0.016
8	14	0.893 ± 0.035
9	16	1.015 ± 0.022
10	18	1.156 ± 0.012
11	20	1.259 ± 0.040

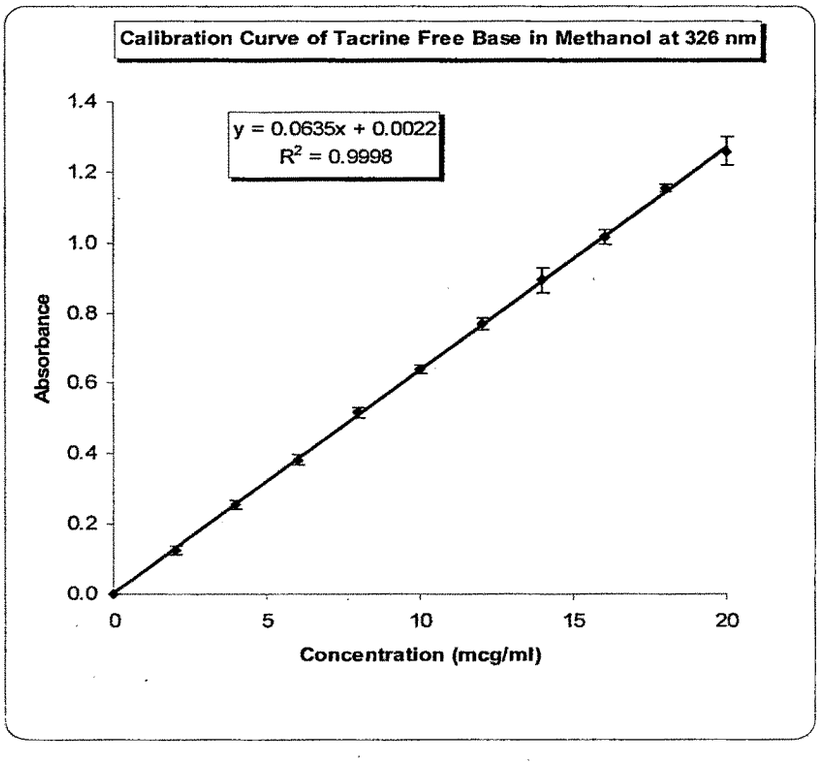

Figure 3.1 Calibration curve of tacrine free base in methanol at 326 nm

3.1.2 Method Validation:

3.1.2.1 Linearity:

The linearity of an analytical method is its ability to elicit, test results that are directly, or by well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. The linearity of the assay was determined by diluting the primary stock solution using methanol to obtain final concentrations in the range of 2 – 20 µg/mL. Six different sets of primary stock solutions were prepared and final dilution was made using methanol. The absorbance of samples was measured on three consecutive days at λ_{max} 326 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width). Methanol was used as a blank. Calibration curves were obtained by plotting mean absorbance vs. concentration. Linear least-square regression analyses of the calibration graphs were performed and the values are noted in Table 3.2

Table 3.2 Calibration curves of tacrine free base in methanol at 326 nm on different days.

Day	Number of Runs (n)	Slope	Intercept	Linear Least Square Regression (r^2)
1	6	0.0635	0.0022	0.9998
2	6	0.0636	0.0021	0.9998
3	6	0.0637	0.0021	0.9997

3.1.2.2 Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value (The United States Pharmacopoeia 27 NF 22, 2004). The intra-day and inter-day accuracies were determined by replicate analysis of the solutions of known concentrations of tacrine at three quality control concentration (low – LQC, medium – MQC, and high – HQC) levels. The observed concentrations of the drug were then back calculated (from absorbance) using the equation of standard calibration curve and compared with the actual concentrations. The % relative error was calculated using the formula,

$$\%\ \mathrm{Re}\ lative\ \ error\ = \frac{Observed\ \ value\ - True\ \ value}{True\ \ value} \times 100 \quad \textbf{(Equation 3.1)}$$

Intra-day Accuracy of the Assay: Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2 (LQC), 10 (MQC) and 20 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples were measured at λ_{max} 326 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width) three times on the same day. The solutions were prepared freshly on each time. Methanol was used as a blank. The % relative error was calculated and the results are recorded in Table 3.3.

Inter-day Accuracy of the Assay: Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2 (LQC), 10 (MQC) and 20 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples were measured at λ_{max} 326 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width) on three consecutive days. The solutions were prepared freshly on each day. Methanol was used as a blank. The % relative error was calculated and the results are recorded in Table 3.4.

3.1.2.3 Precision:

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of homogenous sample (The United States Pharmacopoeia 27 NF 22, 2004). The precision of an analytical method is usually expressed as the Standard Deviation (SD) or Relative Standard Deviation (RSD). The standard deviation is calculated from following formula given in equation below,

$$SD = \sqrt{\sum (X_i - X)^2 / (N-1)} \quad \textbf{(Equation 3.2)}$$

Where X_i is an individual measurement in a set

X is the arithmetic mean of the set and

N is the total number of replicated measurement taken in the set

Precision between different samples can be compared with RSD as follows:

$$\%RSD = \frac{SD}{Mean} \times 100 \quad \textbf{(Equation 3.3)}$$

The intra- and inter day precisions of the assay were calculated by replicate analysis of the solutions of known concentrations of tacrine at three quality control concentration (LQC, MQC, and HQC) levels. The observed concentrations of the drug were then back calculated (from absorbance) using the equation of standard calibration curve. The variations between the observed concentrations were determined by calculating the % RSD using equation 3.3.

Intra-day Precision of the Assay: Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2 (LQC), 10 (MQC) and 20 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples were measured at λ_{max} 326 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width) three times on the same day. The solutions were prepared freshly on each time. Methanol was used as a blank. The % relative error was calculated and the results are recorded in Table 3.3.

Inter-day Precision of the Assay: Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2 (LQC), 10 (MQC) and 20 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples were measured at λ_{max} 326 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width) on three consecutive days. The solutions were prepared freshly on each day. Methanol was used as a blank. The % relative error was calculated and the results are recorded in Table 3.4.

Table 3.3 Intra day accuracy and precision for tacrine determination.

Run#	Tacrine Concentration					
	Low QC, 2 µg/mL		Medium QC, 10 µg/mL		High QC, 20 µg/mL	
	Observed concentration	% Relative Error	Observed concentration	% Relative Error	Observed concentration	% Relative Error
Set I						
Run #1	1.98	-0.94	10.12	1.23	20.30	1.48
Run #2	2.04	2.20	10.25	2.49	19.89	-0.57
Run #3	1.97	-1.73	9.86	-1.45	19.54	-2.30
Run #4	2.03	1.42	10.11	1.07	19.60	-1.98
Run #5	2.04	2.20	10.17	1.70	19.78	-1.12
Run #6	1.97	-1.73	9.89	-1.13	19.67	-1.67
Mean	**2.00**		**10.07**		**19.79**	
SD	**0.038**		**0.159**		**0.275**	
Precision as % RSD	**1.91**		**1.58**		**1.39**	
Accuracy (%)	**100.24**		**100.65**		**98.97**	
Set II						
Run #1	2.04	2.20	10.25	2.49	20.39	1.95
Run #2	2.04	2.20	10.17	1.70	19.70	-1.51
Run #3	1.98	-0.94	10.22	2.17	19.51	-2.46
Run #4	1.97	-1.73	9.84	-1.61	19.81	-0.96
Run #5	2.03	1.42	10.19	1.86	20.30	1.48
Run #6	2.01	0.63	9.92	-0.82	19.48	-2.61
Mean	**2.01**		**10.10**		**19.86**	
SD	**0.033**		**0.173**		**0.393**	
Precision as % RSD	**1.64**		**1.71**		**1.98**	
Accuracy (%)	**100.63**		**100.97**		**99.32**	
Set III						
Run #1	2.04	2.20	10.08	0.76	20.22	1.09
Run #2	2.03	1.42	10.28	2.80	19.62	-1.91
Run #3	1.95	-2.52	9.87	-1.29	19.76	-1.20
Run #4	2.00	-0.16	10.23	2.33	19.49	-2.54
Run #5	2.01	0.63	9.81	-1.92	20.23	1.17
Run #6	2.06	2.99	10.08	0.76	19.71	-1.43
Mean	**2.02**		**10.06**		**19.84**	
SD	**0.039**		**0.189**		**0.313**	
Precision as % RSD	**1.94**		**1.88**		**1.58**	
Accuracy (%)	**100.76**		**100.57**		**99.20**	

Table 3.4 Inter day accuracy and precision for tacrine determination.

Run#	Tacrine Concentration					
	Low QC, 2 µg/mL		Medium QC, 10 µg/mL		High QC, 20 µg/mL	
	Observed concentration	% Relative Error	Observed concentration	% Relative Error	Observed concentration	% Relative Error
Day 1						
Run #1	2.06	2.99	10.22	2.17	20.52	2.58
Run #2	2.03	1.42	10.15	1.54	19.57	-2.14
Run #3	2.00	-0.16	10.23	2.33	19.70	-1.51
Run #4	1.98	-0.94	9.79	-2.08	19.60	-1.98
Run #5	2.01	0.63	10.28	2.80	20.26	1.32
Run #6	2.03	1.42	9.95	-0.50	19.76	-1.20
Mean	**2.02**		**10.10**		**19.90**	
SD	**0.028**		**0.192**		**0.392**	
Precision as % RSD	**1.37**		**1.90**		**1.97**	
Accuracy (%)	**100.89**		**101.04**		**99.52**	
Day 2						
Run #1	1.97	-1.73	10.17	1.70	20.25	1.24
Run #2	2.04	2.20	10.22	2.17	19.54	-2.30
Run #3	1.95	-2.52	10.17	1.70	19.82	-0.88
Run #4	2.00	-0.16	9.86	-1.45	19.70	-1.51
Run #5	2.01	0.63	10.12	1.23	20.36	1.80
Run #6	2.03	1.42	9.89	-1.13	19.79	-1.04
Mean	**2.00**		**10.07**		**19.91**	
SD	**0.036**		**0.158**		**0.322**	
Precision as % RSD	**1.82**		**1.57**		**1.62**	
Accuracy (%)	**99.97**		**100.70**		**99.55**	
Day 3						
Run #1	2.00	-0.16	9.92	-0.82	20.47	2.35
Run #2	2.03	1.42	10.17	1.70	19.46	-2.69
Run #3	2.06	2.99	10.19	1.86	19.95	-0.25
Run #4	2.01	0.63	9.76	-2.39	19.60	-1.98
Run #5	1.98	-0.94	9.90	-0.98	20.23	1.17
Run #6	2.06	2.99	10.06	0.60	20.03	0.14
Mean	**2.02**		**10.00**		**19.96**	
SD	**0.033**		**0.168**		**0.378**	
Precision as % RSD	**1.61**		**1.68**		**1.89**	
Accuracy (%)	**101.15**		**99.99**		**99.79**	

3.1.2.4 Robustness and Ruggedness:

Robustness and ruggedness of the method was evaluated by changing solvents, analyzing samples using different spectrophotometer and different analyst. Unknown concentrations (MQC) were back-calculated from the linearity curve (mean of n=6).

3.1.2.5 Limit of Detection and Limit of Quantification: The Limit of Detection (LoD) is a quantitative parameter. It is the lowest concentration of the analyte in a sample that can be detected with acceptable precision and accuracy under stated experimental conditions, but not necessarily quantities as an exact value (The United States Pharmacopoeia 27 NF 22, 2004). It is expressed as the concentration of analyte in the sample. The limit is usually expressed in terms of µg/mL, ng/mL, pg/mL, etc. LoD values are always specific for a particular set of experimental conditions. Anything that changes the sensitivity of a method, including instrument, sample preparation etc will change detection limits.

Limit of Quantification (LoQ) is the lowest concentration of analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. The value of LoQ is almost 10 times higher than that of the blank.

Six random readings (absorbance) for analytical blank signal after "Auto Zero" were as follows 0.002, 0.001, 0.001, 0.001, 0.002 and 0.001.

LoD and LoQ were determined using the following equation.

$$LoD(or)LoQ = \frac{k.S_B}{S}$$ **(Equation 3.4)**

Where,

k = a constant (3 for LoD and 10 for LoQ)

S_B = the standard deviation of the analytical blank signal

S = the slope of the concentration/response graph

3.1.3 Interference of the excipients used:

Certain excipients may interfere with the estimation of drug(s). Hence, Interference of the excipients used in the formulation has been evaluated at highest concentration and the results are summarized in Table 3.5.

Table 3.5 Interference of excipients observed during estimation of tacrine by UV spectrophotometric method

Sr. No	Name of Excipient	Quantity Taken (% w/w)	Observation
1	Propylene glycol I.P.	60	No interference observed
2	Labrafil M 1944 CS®	10 – 20	No interference observed
3	Labrafac CC®	10 – 20	No interference observed
4	Cremophor RH 40®	20 – 50	No interference observed
5	Cremophor EL®	20 – 50	No interference observed
6	Corn oil	10 – 20	No interference observed
7	Sunflower oil	10 – 20	No interference observed
8	Isopropyl Myristate	10 – 20	No interference observed
9	Transcutol P®	10 – 30	No interference observed

3.2 Estimation of Tacrine (Formulation/ Diffusion Medium)

Estimation of tacrine in formulation and biological fluid/tissues has been reported by many scientists (Hsieh et al. 1983; Park et al. 1986; Forsyth et al. 1988; Aparico et al. 1998; Vargas et al. 1998; Jaskari et al. 2000; Bollo et al. 2000; Yang et al. 2001; Jiang et al. 2003). Estimation of tacrine in formulation and diffusion medium has been carried out using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) as mentioned above in section 3.1 for estimation of drug substance.

3.2.1 Reagents:

Methanol analytical reagent grade was used to prepare the primary stock solution and subsequent dilutions for the estimation of Tacrine.

3.2.2 Estimation of Tacrine (Formulation):

Tacrine formulation (solution, microemulsion, and mucoadhesive microemulsion -0.10 mL) was taken in a 10 mL volumetric flask. The formulation was diluted up to 10 mL using methanol (AR grade) and sonicated for 2 min at ambient temperature. The diluted solution (0.50 mL) was transferred in to 10 mL volumetric flask and volume was made up to 10 mL using methanol (AR grade) and analyzed using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) as mentioned above in section 3.1 for estimation of drug substance. The concentrations of the active ingredient (tacrine) were then back calculated (from absorbance) using the equation of standard calibration curve.

3.2.3 Estimation of Tacrine (Diffusion Medium):

Tacrine containing diffusion medium (0.20 mL) was taken in a 10 mL volumetric flask. Then it was diluted up to 10 mL using methanol (AR grade) and sonicated for 2 min at ambient temperature. The diluted solutions were analyzed using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) as mentioned above in section 3.1 for estimation of drug substance. The concentrations of tacrine were then back calculated (from absorbance) using the equation of standard calibration curve.

3.2.4 Estimation of Tacrine (Drug Retention at Stress and Accelerated Conditions):

Tacrine formulation (0.10 mL) was taken in a 10 mL volumetric flask. The formulation was diluted up to 10 mL using methanol (AR grade) and sonicated for 2 min at ambient temperature. The diluted solution (0.50 mL) was transferred in to 10 mL volumetric flask and volume was made up to 10 mL using methanol (AR grade). The estimation of tacrine was performed as previously mentioned under estimation of tacrine free base in formulation in this chapter (section 3.2.2).

3.3 Estimation of Donepezil Free Base (Drug Substance)

Donepezil free base was obtained by neutralization of donepezil HCl solution with 10%w/v NaOH solution. Donepezil free base was estimated using UV-Visible double beam spectrophotometric method.

3.3.1 Methodology:

Reagents

Methanol analytical reagent grade was used to prepare the primary stock solution and subsequent dilutions for the estimation of donepezil.

Preparation of Primary stock solution

Donepezil was weighed (approx. 10 mg) and transferred to 100 mL volumetric flask. About 70 mL of the methanol was added to volumetric flask. The solution was sonicated for 2 min at ambient temperature. The final dilution was made to 100 mL (i.e. 100 µg/mL) using methanol. The primary stock solution was stored at 2°C to 8°C till assayed.

Preparation of Test solution

Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg/mL.

Determination of UV Absorbance Maxima of Donepezil: Donepezil test solution of concentration 25 µg/mL was scanned for determination of absorbance maxima (λ_{max}) on a UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan). The scanning was carried out in a range of 200-400 nm.

Calibration Curve of Donepezil: The calibration curve of donepezil was prepared in methanol. Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg/mL. Six different sets of primary stock solutions were prepared and final dilution was made as mentioned above using methanol. The absorbance of samples was measured at λ_{max} 313 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width). Methanol was used as a blank. The results are recorded in Table 3.6. Calibration curve is obtained by plotting mean absorbance vs. concentration (Figure 3.2).

Table 3.6 Calibration curve of donepezil free base in methanol at 313 nm

Sr. No.	Concentration (µg/mL)	Absorbance ± SD (n=6)
1	0	0.000 ± 0.000
2	5	0.134 ± 0.004
3	10	0.264 ± 0.004
4	15	0.403 ± 0.008
5	20	0.544 ± 0.006
6	25	0.665 ± 0.005
7	30	0.782 ± 0.005
8	35	0.919 ± 0.005
9	40	1.058 ± 0.007
10	45	1.168 ± 0.005
11	50	1.316 ± 0.006

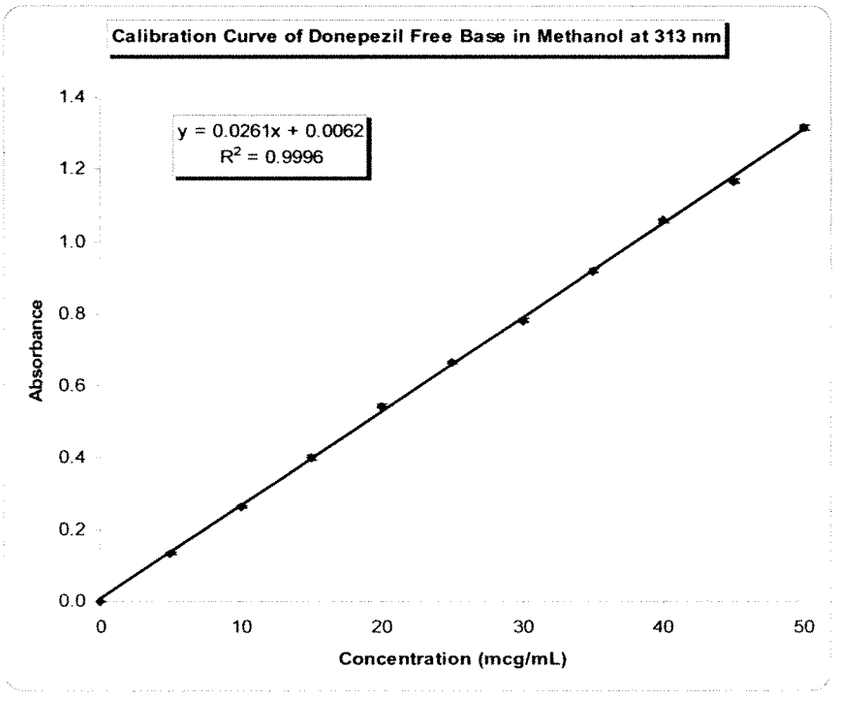

Figure 3.2 Calibration curve of donepezil free base in methanol at 313 nm

3.3.2 Method Validation:

3.3.2.1 Linearity:

The linearity of an analytical method is its ability to elicit, test results that are directly, or by well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. The linearity of the assay was determined by diluting the primary stock solution using methanol to obtain final concentrations in the range of 5 – 50 µg/mL. Six different sets of primary stock solutions were prepared and final dilution was made using methanol. The absorbance of samples was measured on three consecutive days at λ_{max} 313 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width). Methanol was used as a blank. Calibration curves were obtained by plotting mean absorbance vs. concentration. Linear least-square regression analyses of the calibration graphs were performed and the values are noted in Table 3.7.

Table 3.7 Calibration curves of donepezil free base in methanol at 313 nm on different days.

Day	Number of Runs (n)	Slope	Intercept	Linear Least Square Regression (r^2)
1	6	0.0261	0.0062	0.9996
2	6	0.0261	0.068	0.9997
3	6	0.0261	0.066	0.9996

3.3.2.2 Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The intra-day and inter-day accuracies were determined by replicate analysis of the solutions of known concentrations of donepezil at three quality control concentration (low – LQC, medium – MQC, and high – HQC) levels. The observed concentrations of the drug were then back calculated (from absorbance) using the equation of standard calibration curve and compared with the actual concentrations. The % relative error was calculated using equation 3.1

Intra-day Accuracy of the Assay: Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 5 (LQC), 25 (MQC) and 50 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at λ_{max} 313 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601) having ultraviolet rays as light source (1 mm width) three times on the same day. The solutions were prepared freshly on each time. Methanol was used as a blank. The % relative error was calculated and the results are recorded in Table 3.8.

Inter-day Accuracy of the Assay: Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 5 (LQC), 25 (MQC) and 50 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at λ_{max} 313 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width) on three consecutive days. The solutions were prepared freshly on each day. Methanol was used as a blank. The % relative error was calculated and the results are recorded in Table 3.9.

3.3.2.3 Precision:

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical method is usually expressed as the SD (equation 3.2) or RSD (equation 3.3). The intra- and inter day precisions of the assay were calculated by replicate analysis of the solutions of known concentrations of donepezil at three quality control concentration (LQC, MQC, and HQC) levels. The observed concentrations of the drug were then back calculated (from absorbance) using the equation of standard calibration curve. The variations between the observed concentrations were determined by calculating the % RSD using equation 3.3.

Intra-day Precision of the Assay: Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 5 (LQC), 25 (MQC) and 50 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at λ_{max} 313 nm using UV-

Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width) three times on the same day. The solutions were prepared freshly on each time. Methanol was used as a blank. The % relative error was calculated and the results are recorded in Table 3.8.

Inter-day Precision of the Assay: Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 5 (LQC), 25 (MQC) and 50 µg/mL (HQC). Six different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at λ_{max} 313 nm using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source (1 mm width) on three consecutive days. The solutions were prepared freshly on each day. Methanol was used as a blank. The % relative error was calculated and the results are recorded in Table 3.9.

Table 3.8 Intra day accuracy and precision for donepezil determination.

Run#	Donepezil Concentration					
	Low QC, 5 µg/mL		Medium QC, 25 µg/mL		High QC, 50 µg/mL	
	Observed concentration	% Relative Error	Observed concentration	% Relative Error	Observed concentration	% Relative Error
Set I						
Run #1	4.90	-2.07	25.55	2.19	50.80	1.59
Run #2	5.01	0.23	25.24	0.97	50.64	1.29
Run #3	4.93	-1.30	24.55	-1.79	50.45	0.90
Run #4	5.09	1.76	25.51	2.04	49.72	-0.55
Run #5	4.97	-0.54	25.36	1.43	50.30	0.60
Run #6	5.13	2.53	25.24	0.97	50.34	0.67
Mean	**5.01**		**25.24**		**50.38**	
SD	**0.089**		**0.362**		**0.371**	
Precision as % RSD	**1.77**		**1.43**		**0.74**	
Accuracy (%)	**100.10**		**100.97**		**100.75**	
Set II						
Run #1	4.93	-1.30	25.24	0.97	49.84	-0.32
Run #2	4.86	-2.84	25.59	2.34	50.45	0.90
Run #3	5.01	0.23	25.47	1.89	50.76	1.52
Run #4	4.90	-2.07	25.32	1.27	50.30	0.60
Run #5	5.05	1.00	24.70	-1.18	50.61	1.21
Run #6	5.01	0.23	25.36	1.43	49.65	-0.70
Mean	**4.96**		**25.28**		**50.27**	
SD	**0.075**		**0.307**		**0.438**	
Precision as % RSD	**1.52**		**1.21**		**0.87**	
Accuracy (%)	**99.21**		**101.12**		**100.53**	
Set III						
Run #1	4.86	-2.84	25.51	2.04	49.61	-0.78
Run #2	5.01	0.23	25.05	0.20	50.64	1.29
Run #3	4.93	-1.30	25.39	1.58	50.61	1.21
Run #4	4.90	-2.07	25.47	1.89	50.87	1.75
Run #5	5.09	1.76	25.36	1.43	50.15	0.29
Run #6	5.05	1.00	24.74	-1.03	49.76	-0.48
Mean	**4.97**		**25.25**		**50.27**	
SD	**0.091**		**0.298**		**0.515**	
Precision as % RSD	**1.82**		**1.18**		**1.02**	
Accuracy (%)	**99.46**		**101.02**		**100.55**	

Table 3.9 Inter day accuracy and precision for donepezil determination.

Run#	Donepezil Concentration					
	Low QC, 5 µg/mL		Medium QC, 25 µg/mL		High QC, 50 µg/mL	
	Observed concentration	% Relative Error	Observed concentration	% Relative Error	Observed concentration	% Relative Error
Day 1						
Run #1	4.93	-1.30	25.55	2.19	49.46	-1.09
Run #2	4.90	-2.07	24.63	-1.49	50.53	1.06
Run #3	4.86	-2.84	24.70	-1.18	50.80	1.59
Run #4	5.01	0.23	25.39	1.58	50.61	1.21
Run #5	5.13	2.53	25.55	2.19	50.30	0.60
Run #6	4.97	-0.54	25.36	1.43	49.76	-0.48
Mean	**4.97**		**25.20**		**50.24**	
SD	**0.095**		**0.419**		**0.524**	
Precision as % RSD	**1.92**		**1.66**		**1.04**	
Accuracy (%)	**99.34**		**100.79**		**100.48**	
Day 2						
Run #1	4.90	-2.07	25.24	0.97	49.72	-0.55
Run #2	4.93	-1.30	25.32	1.27	50.49	0.98
Run #3	4.97	-0.54	25.36	1.43	50.91	1.82
Run #4	4.93	-1.30	25.62	2.50	50.53	1.06
Run #5	5.05	1.00	24.74	-1.03	50.18	0.37
Run #6	5.13	2.53	24.63	-1.49	49.15	-1.70
Mean	**4.99**		**25.15**		**50.16**	
SD	**0.086**		**0.385**		**0.636**	
Precision as % RSD	**1.73**		**1.53**		**1.27**	
Accuracy (%)	**99.72**		**100.61**		**100.33**	
Day 3						
Run #1	5.09	1.76	25.51	2.04	49.42	-1.16
Run #2	4.97	-0.54	25.36	1.43	51.03	2.05
Run #3	4.90	-2.07	25.39	1.58	50.15	0.29
Run #4	4.93	-1.30	25.28	1.12	50.45	0.90
Run #5	5.05	1.00	24.63	-1.49	50.61	1.21
Run #6	4.86	-2.84	24.51	-1.95	49.65	-0.70
Mean	**4.97**		**25.11**		**50.22**	
SD	**0.089**		**0.428**		**0.605**	
Precision as % RSD	**1.79**		**1.71**		**1.20**	
Accuracy (%)	**99.34**		**100.45**		**100.43**	

3.3.2.4 Robustness and Ruggedness:

Robustness and ruggedness of the method was evaluated by changing solvents, analyzing samples using different spectrophotometer and different analyst. Unknown concentrations (MQC) were back-calculated from the linearity curve (mean of n=6).

3.3.2.5 Limit of Detection and Limit of Quantification: The Limit of Detection (LoD) is a quantitative parameter. It is the lowest concentration of the analyte in a sample that can be detected with acceptable precision and accuracy under stated experimental conditions, but not necessarily quantities as an exact value (USP 27, NF 22). It is expressed as the concentration of analyte in the sample. The limit is usually expressed in terms of µg/mL, ng/mL, pg/mL, etc. LoD values are always specific for a particular set of experimental conditions. Anything that changes the sensitivity of a method, including instrument, sample preparation etc will change detection limits.

Limit of Quantification (LoQ) is the lowest concentration of analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. The value of LoQ is almost 10 times higher than that of the blank.

Six random readings (absorbance) for analytical blank signal after "Auto Zero" were as follows 0.002, 0.001, 0.001, 0.001, 0.002 and 0.001. LoD and LoQ were determined using equation 3.4

3.3.3 Interference of the excipients used:

Certain excipients may interfere with the estimation of drug(s). Hence, Interference of the excipients used in the formulation has been evaluated at highest concentration and the results are summarized in Table 3.10.

Table 3.10 Interference of excipients observed during estimation of donepezil by UV spectrophotometric method

Sr. No	Name of Excipient	Quantity Taken (% w/w)	Observation
1	Propylene glycol I.P.	60	No interference observed
2	Labrafil M 1944 CS®	10 – 20	No interference observed
3	Labrafac CC®	10 – 20	No interference observed
4	Cremophor RH 40®	20 – 50	No interference observed
5	Transcutol P®	10 – 30	No interference observed
6	Captex 355®	20 – 30	No interference observed
7	Capmul MCM®	10 – 30	No interference observed
8	Corn oil	10 – 20	No interference observed
9	Sunflower oil	10 – 20	No interference observed
10	Polysorbate 80 I.P. (Tween 80)	20 – 50	No interference observed

3.4 Estimation of Donepezil (Formulation/ Diffusion Medium)

Estimation of donepezil in formulation and biological fluid/tissues has been reported by many scientists (Matsui et al. 1999; Gotti et al. 2001; Yasui-Furukori et al. 2002; Pappa et al. 2002; Lu et al. 2004; Radwan et al. 2006; Pillai and Singhvi 2006; Abbas et al. 2006; Nakashima et al. 2006). Estimation of donepezil in formulation and diffusion medium has been carried out using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) as mentioned above in section 3.3 for estimation of drug substance.

3.4.1 Reagents:

Methanol analytical reagent grade was used to prepare the primary stock solution and subsequent dilutions for the estimation of donepezil.

3.4.2 Estimation of Donepezil (Formulation):

Donepezil formulation (solution, microemulsion, and mucoadhesive microemulsion -0.10 mL) was taken in a 10 mL volumetric flask. The formulation was diluted up to 10 mL using methanol (AR grade) and sonicated for 2 min at ambient temperature. The diluted solution (1 mL) was transferred in to 10 mL volumetric flask and volume was made up to 10 mL using methanol (AR grade) and analyzed using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) as mentioned above in section 3.3 for estimation of drug substance. The concentrations of the active ingredient (donepezil) were then back calculated (from absorbance) using the equation of standard calibration curve.

3.4.3 Estimation of Donepezil (Diffusion Medium):

Donepezil containing diffusion medium (0.50 mL) was taken in a 5 mL volumetric flask. Then it was diluted up to 5 mL using methanol (AR grade) and sonicated for 2 min at ambient temperature. The diluted solutions were analyzed using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) as mentioned above in section 3.3 for estimation of drug substance. The concentrations of donepezil were then back calculated (from absorbance) using the equation of standard calibration curve.

3.4.4 Estimation of Donepezil (Drug Retention at Stress and Accelerated Conditions):

Donepezil formulation (0.10 mL) was taken in a 10 mL volumetric flask. The formulation was diluted up to 10 mL using methanol (AR grade) and sonicated for 2 min at ambient temperature. The diluted solution (1 mL) was transferred in to 10 mL volumetric flask and volume was made up to 10 mL using methanol (AR grade). The estimation of donepezil was performed as previously mentioned under estimation of donepezil in formulation in this chapter (section 3.4.2).

3.5. Results and Discussion

3.5.1 Estimation of Tacrine Free Base (Drug Substance):

3.5.1.1 Determination of UV absorbance maxima and Preparation of calibration curve:

Tacrine free base (drug substance) was analyzed using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) using methanol as a vehicle. Based on the spectrophotometric scanning of an intermediate concentration (10 µg/mL) of tacrine solution in methanol, the maxima were obtained at three different wavelength viz. 243, 326 and 339 nm. The λ_{max} of 243 was not selected as it is close to UV region and it gives very high and fluctuating absorbance. Out of 326 and 339 nm, the λ_{max} of 326 nm was chosen for further analysis as it gives somewhat higher absorbance value as compared to λ_{max} 339 nm and is also reported in literature (Yang et al. 2001). The absorbance was found to be linear in the range of 2 – 20 µg/mL at λ_{max} of 326 nm (Figure 3.1) with r^2 value 0.9998 and equation of straight line $y = 0.0635x + 0.0022$.

3.5.1.2 Method Validation:

The method was validated for linearity, accuracy, precision, robustness and ruggedness. The validation parameters were found to meet the "readily pass criteria" specified in the USP and % RSD were found less than 2%.

3.5.1.2.1 Linearity of the Assay: The linearity of the assay was determined by plotting standard calibration curves for the concentration range 2 – 20 µg/mL at λ_{max} of 326 nm on three consecutive days (Table 3.2). The method for estimation of tacrine free base was found to be linear in the broad range of 2 – 20 µg/mL as suggested by the linear least-square regressions (> 0.9996) of the standard curves on all three days.

3.5.1.2.2 Accuracy of the Assay: The intra- and inter- day accuracies of the method are recorded in Table 3.3 and 3.4 respectively. As seen from Table 3.3 and 3.4, the % relative errors on intra- and inter-day, for three different concentration levels (LQC, MQC, and HQC), are very low (< 5 % absolute value), and intra- and inter- day accuracy is between

98.97% to 100.97% and 99.52% to 101.15%suggesting that the method was very accurate.

3.5.1.2.3 Precision of the Assay: The intra- and inter- day precisions of the method are recorded in Table 3.3 and 3.4 respectively. As seen from Table 3.3 and 3.4, the intra- and inter- day variability (expressed as % RSD) for LQC, MQC, and HQC are < 2 %. The low % RSD values for all the three concentration levels, intra- and inter-day, suggest the estimation method to be precise and reproducible.

3.5.1.2.4 Limit of Detection and Limit of Quantification: The LoD and LoQ for the assay of tacrine in this method are found to be 0.0244 µg/mL and 0.0813 µg/mL suggesting that the estimation method possesses sufficient sensitivity.

3.5.2 Estimation of Tacrine (Formulation and Diffusion Medium):

Tacrine formulations, tacrine in diffusion media (*in vitro* diffusion studies) and drug retention studies samples were analyzed using validated UV-Visible double beam spectrophotometry method. Microemulsions and samples of *in vitro* diffusion studies were analyzed by preparing dilution in methanol and measuring the absorbance at 326 nm. The ingredients used for microemulsion preparation or diffusion media were not found to interfere with the proposed method.

3.5.3 Estimation of Donepezil Free Base (Drug Substance):

3.5.3.1 Determination of UV absorbance maxima and Preparation of calibration curve:

Donepezil free base (drug substance) was analyzed using UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) using methanol as a vehicle. Based on the spectrophotometric scanning of an intermediate concentration (25 µg/mL) of donepezil solution in methanol, the maxima were obtained at four different wavelength viz. 207.5, 231, 268, and 313 nm. The λ_{max} of 207.5 and 231 were not selected as they are close to UV region and they give very high and fluctuating absorbance. Out of 268 and 313 nm, the λ_{max} of 313 nm was chosen for further analysis as it is reported in literature (Yasui-Furukori et al. 2002; Abbas et al. 2006). The absorbance was found to be linear in

the range of 5 – 50 µg/mL at λ_{max} of 313 nm (Figure 3.2) with r^2 value 0.9996 and equation of straight line y = 0.0261x + 0.0062.

3.5.3.2 Method Validation:

The method was validated for linearity, accuracy, precision, robustness and ruggedness. The validation parameters were found to meet the "readily pass criteria" specified in the USP and % RSD were found less than 2%.

3.5.3.2.1 Linearity of the Assay: The linearity of the assay was determined by plotting standard calibration curves for the concentration range 5 – 50 µg/mL at λ_{max} of 326 nm on three consecutive days (Table 3.7). The method for estimation of donepezil free base was found to be linear in the broad range of 5 – 50 µg/mL as suggested by the linear least-square regressions (≥ 0.9996) of the standard curves on all three days.

3.5.3.2.2 Accuracy of the Assay: The intra- and inter- day accuracies of the method are recorded in Table 3.8 and 3.9 respectively. As seen from Table 3.8 and 3.9, the % relative errors on intra- and inter-day, for three different concentration levels (LQC, MQC, and HQC), are very low (< 5 % absolute value), and intra- and inter- day accuracy is between 99.21% to 101.12% and 99.34% to 100.79% suggesting that the method was very accurate.

3.5.3.2.3 Precision of the Assay: The intra- and inter- day precisions of the method are recorded in Table 3.8 and 3.9 respectively. As seen from Table 3.8 and 3.9, the intra- and inter- day variability (expressed as % RSD) for LQC, MQC, and HQC are <2 %. The low % RSD values for all the three concentration levels, intra- and inter-day, suggest the estimation method to be precise and reproducible.

3.5.3.2.4 Limit of Detection and Limit of Quantification: The LoD and LoQ for the assay of tacrine in this method are found to be 0.0594 µg/mL and 0.1978 µg/mL suggesting that the estimation method possesses sufficient sensitivity.

3.5.4 Estimation of Donepezil (Formulation and Diffusion Medium):

Donepezil formulations, donepezil in diffusion media (*in vitro* diffusion studies) and drug retention studies samples were analyzed using validated UV-Visible double beam spectrophotometry method. Microemulsions and samples of *in vitro* diffusion studies were analyzed by preparing dilution in methanol and measuring the absorbance at 313 nm. The ingredients used for microemulsion preparation or diffusion media were not found to interfere with the proposed method.

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