Chapter 4
Development of Formulation for Tadalafil

4.1. Analytical Method Development for Tadalafil.

Tadalafil, an oral treatment for erectile dysfunction, is a selective inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type 5 (PDE5).Chemically Tadalafil is pyrazino[1′,2′:1,6]pyrido[3,4-b]indole-1,4-dione, 6-(1,3-benzodioxol-5- yl)-2,3,6,7,12,12a-hexahydro-2-methyl-, (6R,12aR)- and is not yet official in any Pharmacopoeia.[1-4]. In the market only tablet formulation of Tadalafil is available (10 mg/ 20 mg).So far only liquid phase extraction-liquid chromatography and solid-phase extraction–liquid chromatography–electron spray mass spectrometry methods have been reported for the estimation of Tadalafil[8-11].But these methods are comparatively more time consuming and expensive. So it was decided to develop simple, rapid and cost-effective analytical methods which can determine Tadalafil in bulk drug and its various dosage forms

4.1.1. Estimation of Tadalafil by spectrophotometry.

A simple, sensitive and accurate UV method for estimation of the actual amount of Tadalafil from its formulation (microemulsion and Inclusion complexes) was developed.

4.1.1.1. Methodology :

4.1.1.1.1. Reagents and Instrument :

Tadalafil working standard was obtained as a gift sample from Macleods Pharmaceuticals, Daman. **The reagents and instrument used are described in section 3.1.1.1.1. and 3.1.1.1.2.**

4.1.1.1.2. Preparation of working stock solution :

Tadalafil was weighed (approx. 100 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 using methanol to obtain standard stock solution (i.e. 1000 µg/mL). An aliquot (10 mL) of standard stock solution of Tadalafil was further diluted with 100 mL of methanol to get working stock solution (i.e. 100 µg/mL) of Tadalafil. The working stock solution was stored at 2°C to 8°C till assayed.

4.1.1.1.3. Preparation of Standard solution :

Suitable aliquots of the primary stock solutions of Tadalafil ranging from(0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, and 2.4) were transferred into a 10mL volumetric flask and volume were made up to 10 mL using methanol to obtained series of final concentrations of(2 - 24 µg/mL).

4.1.1.1.4. Determination of UV Absorbance Maxima of Tadalafil :

Tadalafil 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 and the absorbance maxima(λ_{max}) was found to be a 284.5 nm..

4.1.1.1.5. Calibration Curve :

Six different sets of working stock solutions (i.e.1000 µg/mL) of Tadalafil were prepared and suitable aliquots from each working stock solutions ranging from (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 µg/mL) were further diluted with 10 mL methanol to get standard solutions in the range of 5 – 50 µg/mL. The absorbance of samples was measured at λ_{max} 284.5 nm. Methanol was used as a blank. The solution was stored at 2°C to 8°C till assayed. The results are recorded in Table 4.1.1.1. Calibration curve is obtained by plotting mean absorbance vs. concentration (Figure 4.1.1.1.).

Table 4.1.1.1. Calibration curve of Tadalafil in methanol at 284.5 nm

Sr. No.	Concentration (µg/mL)	Absorbance ± SD (n=6)
1	0	0.000 ± 0.0005
2	2	0.067 ± 0.013
3	4	0.145 ± 0.012
4	6	0.214 ± 0.013
5	8	0.283 ± 0.014
6	10	0.352 ± 0.013
7	12	0.418 ± 0.016
8	14	0.5 ± 0.035
9	16	0.559 ± 0.022
10	18	0.632 ± 0.015
11	20	0.702 ± 0.021
12	22	0.778 ± 0.011
13	24	0.844 ± 0.023

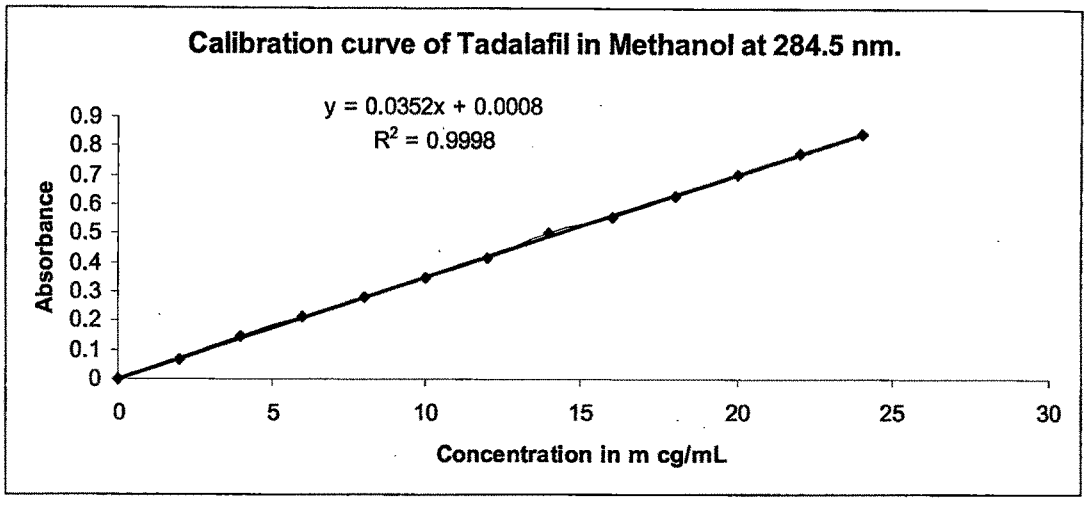


Figure 4.1.1.1. Calibration curve of Tadalafil in methanol at 284.5 nm

4.1.1.2. Method Validation :

4.1.1.2.1 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 – 24 µg/mL. Six different sets of primary stock solutions were prepared and final dilution was made using methanol. The absorbance of samples were measured on three consecutive days at λ_{max} 284.5 nm. 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 4.1.1.2.

Table 4.1.1.2. Calibration curves of Tadalafil in methanol at 284.5 nm on different days.

Day	Number of Runs (n)	Slope	Intercept	Linear Least Square Regression (r^2)
1	6	0.0352	0.0008	0.9998
2	6	0.0350	0.0029	0.9999
3	6	0.0336	0.0001	0.9998

4.1.1.2.2. 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 Tadalafil 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,

$$\%\ \text{Re}\ lative\ \ error\ = \frac{Observed\ \ \ value\ - True\ \ value}{True\ \ value} \times 100 \quad \textbf{(Equation 4.1)}$$

4.1.1.2.2.1. Intra-day Accuracy of the Assay :

Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2 (LQC), 12 (MQC) and 24 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} 285 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 4.1.1.3.

4.1.1.2.2.2. Inter-day Accuracy of the Assay :

Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2 (LQC), 12 (MQC) and 24 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} 284.5 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 4.1.1.4.

4.1.1.2.3. 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,

$$SD = \sqrt{\sum (X_i - X)^2 / (N-1)} \quad \textbf{(Equation 4.2)}$$

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:

$$\%RSD = \frac{SD}{Mean} \times 100 \quad \textbf{(Equation 4.3)}$$

The intra- and inter day precisions of the assay were calculated by replicate analysis of the solutions of known concentrations of Tadalafil 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 4.3.

4.1.1.2.3.1. Intra-day Precision of the Assay :

Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2 (LQC), 12 (MQC) and 24 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} 284.5 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 4.1.1.3.

4.1.1.2.3.2. Inter-day Precision of the Assay :

Primary stock solutions were appropriately diluted using methanol to obtain final concentrations of 2 (LQC), 12 (MQC) and 24 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} 285 nm 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 4.1.1.4.

Table 4.1.1.3. Intra day accuracy and precision for Tadalafil determination.

Run#	Tadalafil Concentration					
	Low QC, 2 µg/mL		Medium QC, 12 µg/mL		High QC, 24 µg/mL	
	Observed concentration	% Relative Error	Observed concentration	% Relative Error	Observed concentration	% Relative Error
Set I						
Run #1	1.98	-0.94	12.12	1.23	24.30	1.48
Run #2	2.04	2.20	12.25	2.49	23.89	-0.57
Run #3	1.97	-1.73	11.86	-1.45	23.54	-2.30
Run #4	2.03	1.42	12.11	1.07	23.60	-1.98
Run #5	2.04	2.20	12.17	1.70	23.78	-1.12
Run #6	1.97	-1.73	11.89	-1.13	23.67	-1.67
Mean	**2.00**		**12.066**		**23.79**	
SD	**0.038**		**0.146**		**0.301**	
Precision as % RSD	**1.91**		**1.58**		**1.32**	
Accuracy (%)	**100.24**		**100.55**		**99.125**	
Set II						
Run #1	2.04	2.20	12.25	2.49	24.39	1.95
Run #2	2.04	2.20	12.17	1.70	23.70	-1.51
Run #3	1.98	-0.94	12.22	2.17	23.51	-2.46
Run #4	1.97	-1.73	11.84	-1.61	23.81	-0.96
Run #5	2.03	1.42	12.19	1.86	24.30	1.48
Run #6	2.01	0.63	11.92	-0.82	23.48	-2.64
Mean	**2.01**		**12.098**		**23.86**	
SD	**0.033**		**0.167**		**0.384**	
Precision as % RSD	**1.64**		**1.71**		**1.99**	
Accuracy (%)	**100.63**		**100.81**		**99.41**	
Set III						
Run #1	2.04	2.20	12.08	0.76	24.22	1.09
Run #2	2.03	1.42	12.28	2.80	23.62	-1.91
Run #3	1.95	-2.52	11.87	-1.29	23.76	-1.20
Run #4	2.00	-0.16	12.23	2.33	23.49	-2.54
Run #5	2.01	0.63	11.81	-1.92	24.23	1.17
Run #6	2.06	2.99	12.08	0.76	23.71	-1.43
Mean	**2.02**		**12.058**		**23.83**	
SD	**0.039**		**0.209**		**0.343**	
Precision as % RSD	**1.94**		**1.88**		**1.58**	
Accuracy (%)	**100.76**		**100.48**		**99.29**	

Table 4.1.1.4. Inter day accuracy and precision for Tadalafil determination.

Run#	Tadalafil Concentration					
	Low QC, 2 µg/mL		Medium QC, 12 µg/mL		High QC, 24 µg/mL	
	Observed concentration	% Relative Error	Observed concentration	% Relative Error	Observed concentration	% Relative Error
Day 1						
Run #1	2.06	2.99	12.22	2.17	24.52	2.58
Run #2	2.03	1.42	12.15	1.54	23.57	-2.14
Run #3	2.00	-0.16	12.23	2.33	23.70	-1.51
Run #4	1.98	-0.94	11.79	-2.08	23.60	-1.98
Run #5	2.01	0.63	12.28	2.80	24.26	1.32
Run #6	2.03	1.42	11.95	-0.50	23.76	-1.20
Mean	**2.02**		**12.10**		**23.90**	
SD	**0.028**		**0.197**		**0.432**	
Precision as % RSD	**1.41**		**1.90**		**1.95**	
Accuracy (%)	**101.00**		**100.83**		**99.58**	
Day 2						
Run #1	1.97	-1.73	12.17	1.70	24.25	1.24
Run #2	2.04	2.20	12.22	2.17	23.54	-2.30
Run #3	1.95	-2.52	12.17	1.70	23.82	-0.88
Run #4	2.00	-0.16	11.86	-1.45	23.70	-1.51
Run #5	2.01	0.63	12.12	1.23	24.36	1.80
Run #6	2.03	1.42	11.89	-1.13	23.79	-1.04
Mean	**2.00**		**12.07**		**23.91**	
SD	**0.036**		**0.143**		**0.355**	
Precision as % RSD	**1.82**		**1.57**		**1.62**	
Accuracy (%)	**100.00**		**100.58**		**99.62**	
Day 3						
Run #1	2.00	-0.16	11.92	-0.82	24.47	2.35
Run #2	2.03	1.42	12.17	1.70	23.46	-2.69
Run #3	2.06	2.99	12.19	1.86	23.95	-0.25
Run #4	2.01	0.63	11.76	-2.39	23.60	-1.98
Run #5	1.98	-0.94	11.90	-0.98	24.23	1.17
Run #6	2.06	2.99	12.06	0.60	24.03	0.14
Mean	**2.02**		**12.00**		**23.95**	
SD	**0.033**		**0.185**		**0.421**	
Precision as % RSD	**1.61**		**1.68**		**1.89**	
Accuracy (%)	**101.00**		**100.00**		**99.79**	

4.1.1.2.4 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 using the proposed method. 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 0.001, 0.002, 0.001, 0.001, 0.002 and 0.001.

LoD and LoQ were determined using the following equation.

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

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

4.1.1.3. Results and Discussion :

The calibration curves of Tadalafil were constructed by plotting the absorbance of standard Tadalafil (*Y*) against concentration of Tadalafil (*X*) (Table 4.1.1.1.). The correlation coefficient and linear regression equation are shown in Fig. 4.1.1. Intraday and interday accuracy was carried out by determine the calculated values of % relative error and it was -2.64 – 2.99 and -2.52 – 2.99 respectively (Table 4.1.1.3 and 4.1.1.4). The developed method was validated for its intraday and interday precision in the ranges of 2 - 25 µg/ml. The intraday and interday (3 days, n = 3) precision were expressed as relative standard deviation in range of 1.32 – 1.99 % and 1.41 – 1.95 % respectively (Table 4.1.1.3. and 4.1.1.4).

Proposed method was rapid, economical, accurate and precise for the determination of Tadalafil. This method was later used for the estimation of Tadalafil in bulk drug and its pharmaceutical formulation.

4.1.1.4. Estimation of Tadalafil (Formulation/ Diffusion/Dissolution Medium)

Developed UV spectroscopic method was adopted for the estimation of Tadalafil in newly developed formulations (Microemulsions and Cyclodextrins Complexes). In which different calibration curve were prepared in Distilled water and acidic/basic buffers for estimation of Tadalafil from solution and its formulations. For Cyclodextrins complexes, the calibration curves were prepared for dissolution measurement study, inclusion efficiency measurement study and phase solubility measurement study and for microemulsion, the calibration curves were prepared for diffusion measurement study, excipients interference identification study and drug entrapment efficiency measurement study. This method was also applicable for estimation of Tadalafil for retention of drug from accelerated and stress study.

4.1.1.4.1. Preparation of working stock solution of Tadalafil :

The procedure was similar to that described in section 4.1.1.1.2.

4.1.1.4.2. Calibration curve of Tadalafil for inclusion efficiency measurement :

Aliquots of working stock solution of Tadalafil ranging from (0.50, 1.0, 1.5, 2.0 and 2.5 ml) were transferred into a series of 10 ml volumetric flasks and volume was made up to the mark with methanol to prepare a series of standard solutions (5-20 µg/mL) for calibration curve. The absorbance of the resulting solutions was measured at 284.5 nm against pure methanol as blank solution. The concentrations of Tadalafil were then back calculated (from absorbance) using the equation of standard calibration curve.

4.1.1.4.3. Calibration curve of Tadalafil for phase solubility measurement :

The procedure was similar to that described in section 4.1.1.4.2.

4.1.1.4.4. Calibration curve of Tadalafil for dissolution measurement :

Tadalafil containing dissolution medium (0.20 mL, 1.2 pH of HCl buffer) 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 as mentioned above in section 3.4 for estimation of drug substance. The concentrations of Tadalafil were then back calculated (from absorbance) using the equation of standard calibration curve.

4.1.1.4.5. Estimation of Tadalafil from its formulation :

Tadalafil formulation (solution, inclusion complex and 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 the mark by using methanol (AR grade) and analyzed it as mentioned above in section 4.1. for estimation of drug substance. The concentrations of the active ingredient (Tadalafil) were then back calculated (from absorbance) using the equation of standard calibration curve.

4.1.1.4.6 Calibration curve of Tadalafil for Diffusion measurement :

Tadalafil microemulsion containing diffusion medium (0.250 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 as mentioned above in section 4.1. for estimation of drug substance. The concentrations of Tadalafil were then back calculated (from absorbance) using the equation of standard calibration curve.

4.1.1.4.7 Estimation of Tadalafil (Drug Retention at Stress and Accelerated Conditions): The procedure was similar to that described in section 4.1.1.4.5.

4.1.1.4.8 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 by measuring their absorbance at the λ_{max} of the Tadalafil i.e. 284.5 nm and the results are summarized in Table 4.1.1.5.

Table 4.1.1.5. Interference of excipients observed during estimation of drug.

Sr. No	Name of Excipient	Quantity Taken (% w/w)	Observation
1	Labrafil M 2125 CS®	60	No interference observed
2	Labrafil M 1944 CS®	10 – 20	No interference observed
3	Labrafac PG®	10 – 20	No interference observed
4	Cremophor RH 40®	20 – 50	No interference observed
5	Cremophor EL®	20 – 50	No interference observed
6	Cotton seed oil	10 – 20	No interference observed
7	Peanut oil	10 – 20	No interference observed
8	Plurol	10 – 20	No interference observed
9	Transcutol P®	10 – 30	No interference observed
10	Capmul MCM C10®	10 – 20	No interference observed

11	Capmul MCM C8®	10 – 20	No interference observed
12	Tween 20	10 – 30	No interference observed
13	Tween 40	10 – 30	No interference observed
14	Tween 60	10 – 30	No interference observed
15	Tween 80	10 – 30	No interference observed
16	Captex 1000	10 – 20	No interference observed
17	Captex 200P	10 – 20	No interference observed
18	Captex 355EP/NF	10 – 20	No interference observed

4.1.2. Estimation of Tadalafil by HPLC method.

4.1.2.1 Introduction :

The HPLC and LC-MS-MS methods for pharmaceutical formulation and matrix (plasma/urine) have been reported in literature[5-11]. The availability of an HPLC method with high sensitivity and selectivity was desirable for the estimation of Tadalafil in pharmaceutical dosage forms.

4.1.2.2 Methodology :

4.1.2.2.1 Materials and reagents :

Tadalafil working standard was a gift sample from Macleods Pharmaceuticals, Daman. Methanol and acetonitrile (HPLC grade, Spectrochem Ltd, Bombay, India), and triple distilled water were used in the study. Commercially available Tadalafil tablets were procured from the local market.

4.1.2.2.2 Apparatus :

The apparatus are described in section 3.1.2.2.2.

4.1.2.2.3 Preparation of working stock solutions :

A primary stock solution of the Tadalafil (1000 µg/ml)was prepared by dissolving 25 mg of Tadalafil in a 25 ml of volumetric flask containing 10 ml of methanol, sonicated for about 15 min and diluted up to volume with methanol. Working stock solution of Tadalafil (100µg/ml) was prepared by taken a 5 mL aliquot of primary stock solution transferred it into a 50 ml volumetric flasks and volume was made up to the mark with the mobile phase. All stock solution were protected from light and kept at -20°C. They are stable for at least 6 months.

4.1.2.2.4 Chromatographic conditions :

The mobile phase was prepared by mixing acetonitrile and water containing 0.1 m.Mole of glacial acetic acid in the ratio of 40:60 v/v. The mobile phase was filtered through a 0.45 µm membrane filter, degassed by ultra sonication for 15 min and pumped from the solvent reservoir to the column at a flow rate of 1.00 ml/min. The run time was set at 15 min. The volume of injection loop was 20 µl. Prior to injection of the drug solutions; the column was equilibrated for at least 30 min with the mobile phase flowing through the systems. The eluents were monitored at 280 nm and the data were acquired, stored and analyzed with the software Spinchrom CFR version (Shimadzu).

4.1.2.2.5 Preparation of calibration curve :

Different aliquots of (0.05, 0.10, 0.50, 1.00, 2.00, 3.00, 4.00 and 5.00 ml) of working solution were taken in 10 ml volumetric flasks and diluted up to the mark with mobile phase to get concentrations of 0.5, 1.00, 5.00, 10.00, 20.00, 30.00, 40.00 and 50.00 µg/ml. Each of these drug solutions (20 µl) was injected three times into the column and the peak area and retention times were recorded.

4.1.2.2.6 Procedure for pharmaceutical formulations :

Twenty tablets were weighed to obtain the average tablet weight and powdered. A sample of the powdered tablets, equivalent to 25 mg of the Tadalafil was taken in a 25 ml volumetric flask containing 10 ml of methanol, sonicated it for 15 min and diluted to mark with methanol. The solution was filtered through a 0.45 µm membrane filter. An aliquot of solution (1.0 ml) was transferred to a 10 ml volumetric flask and diluted with mobile phase to get a concentration of 100µg/ml. From this, an aliquot (2.0 ml) was transferred to a 10 ml volumetric flask and diluted to the mark with mobile phase to obtain 20 µg/ml of test concentration. The resulting solution (20µl) was injected to HPLC system. All determinations were performed in triplicate.

4.1.2.3 Results and Discussion :

The HPLC method for the analysis of Tadalafil was developed by using the most commonly employed Phenomenex column with UV detection (280nm). The chromatographic procedure was optimized for various parameters and these parameters are shown in Table 4.1.2.1. The retention time (*t*R) of Tadalafil was found to be 11.7 -11.9 min. (Fig. 4.1.2.1). The calibration curve of Tadalafil was constructed by plotting the peak area of Tadalafil standard (*Y*) against concentration of Tadalafil (*X*) (Table 4.1.2.2.). It was found to be linear with a correlation coefficient of 0.9999, the representative linear regression equation being *Y* = 20.651X+3.1401 (Fig. 4.1.2.2).

The relative standard deviations based on the peak area for triplicate injections were found to be 0.22-1.34 % for calibration curve. The developed method was validated for its intraday and interday precision in the range of 0.5 -50.00 µg/ml. The intraday and interday (3 days, $n = 3$) precision were expressed as relative standard deviation in range of 0.18-1.89 % and 0.22-1.92 %, respectively (Table 4.1.2.3.).

The limit of quantification (LOQ) was calculated using the standard deviation of the intercepts and the mean slope of the calibration curves (LOQ = 3 x standard deviation of the intercepts/ mean slope) and it was 0.0931 µg/ml. The limit of detection (LOD) was calculated

using the standard deviation of the intercepts and the mean slope of the calibration curves (LOD = 10 x standard deviation of the intercepts/ mean slope) and it was 0.3105 µg/ml.

Robustness studies were performed for wavelength (+ 2 nm), flow rate (+ 0.01 unit) and analyst to analyst variations. The results of robustness studies are shown in Table 4.1.2.4. The HPLC method developed in the present study was used to quantify Tadalafil in tablet dosage forms. Tadalafil tablets (10 mg, and 20 mg) were analyzed. The obtained results are given in Table 4. The drug content was found to be 99.70–103.10 % of the labeled amount. No interfering peaks were found in the chromatogram, indicating that the tablet excipients did not interfere with the estimation of the drug by the proposed HPLC method. In the system suitability study ,six replicate injections of freshly prepared working stock solution of Tadalafil(30 µg/mL) were injected to the HPLC system, and the % relative standard deviation (%RSD) of peak areas, tailing factors, and theoretical plats were determine in Table 4.1.2.5.The results obtained from the system suitability study are in agreement with the USP requirements and the variation in the retention time among six replicate injections of Cilostazol working solutions was very low, rendering a R.S.D. of 1.1 %.

Also, when a known amount of the drug solution was added to a powdered sample of the tablet dosage form and subjected to an estimation of the drug by the proposed method, there was a high recovery of Tadalafil (99.66 −100.5%, Table 4.1.2.7.), indicating that the proposed procedure for the estimation of Tadalafil in the tablet dosage forms is accurate. The results of the study showed that developed RP-HPLC method was simple, rapid, precise and accurate and could be used for the determination of Tadalafil in its pharmaceutical dosage forms. Summary of method validation parameters is shown in Table 4.1.2.8.

Developed HPLC method was further adopted for the estimation of Tadalafil in newly developed formulations (Microemulsions and Cyclodextrins Complexes) and it could be used for estimation of Tadalafil from matrix (Plasma/Urine) required for pharmacokinetic study to estimate the actual amount of drug absorbed from G.I. Tract.

Table 4.1.2.1. Chromatographic parameters

HPLC parameters	
Column	Phenomenex Luna (Torrance U.S.A.), C_{18} ODS (250 mm × 4.6 ID,5µ) preceded with ODS guard column(10 mm × 5 mm ID)
Mobile Phase	Water containing 0.1 mM of glacial acetic acid (pH 2.5 – 2.7) : Acetonitrile (60 : 40)
Injection Volume	20 µl
Flow Rate	1.0 mL/min.
Analytical Wavelength (nm)	280
Retention Time (min.)	11.7 – 11.9
System	Shimadzu (Kyoto, Japan)
Detector	SPD-20A prominence UV/VIS
Pump	LC-20AT prominence solvent delivery module
Injector	A manual rheodyne injector with 20 µl fixed loop
Software	Spyncrom chromatographic station CFR version-2.4.0.193 (Spinchrom Pvt. Ltd., Chennai, India).
Temperature	Ambient

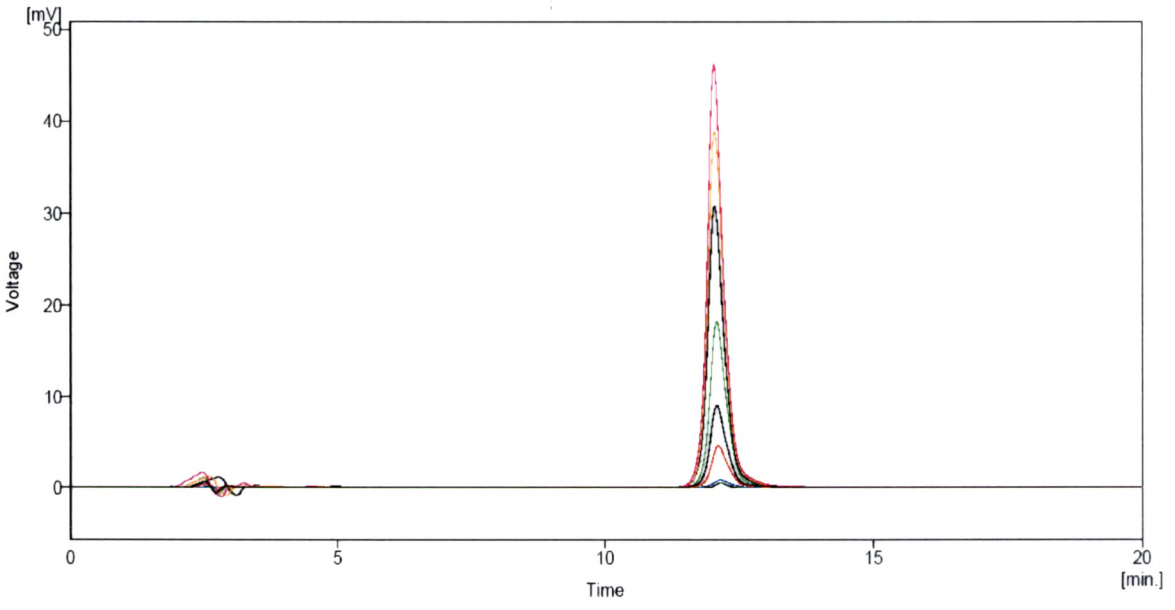


Fig. 4.1.2.1 The overlain chromatograms of standard Tadalafil from 0.5 to 50.0µg/ml concentration in mobile phase

Table 4.1.2.2. Calibration data of Tadalafil by the proposed HPLC method

Concentration of Tadalafil (µg/ml)	Mean peak area in mV (n=3)	% RSD
0.5	8.154	1.344
1	18.7513	1.0797
5	100.4187	0.1644
10	199.5943	0.5176
20	407.2243	0.452
30	620.5523	0.397
40	825.382	0.621
50	1026.67	0.227

Regression equation: $Y = 20.651X - 3.1401$ ($r = 0.9999$)

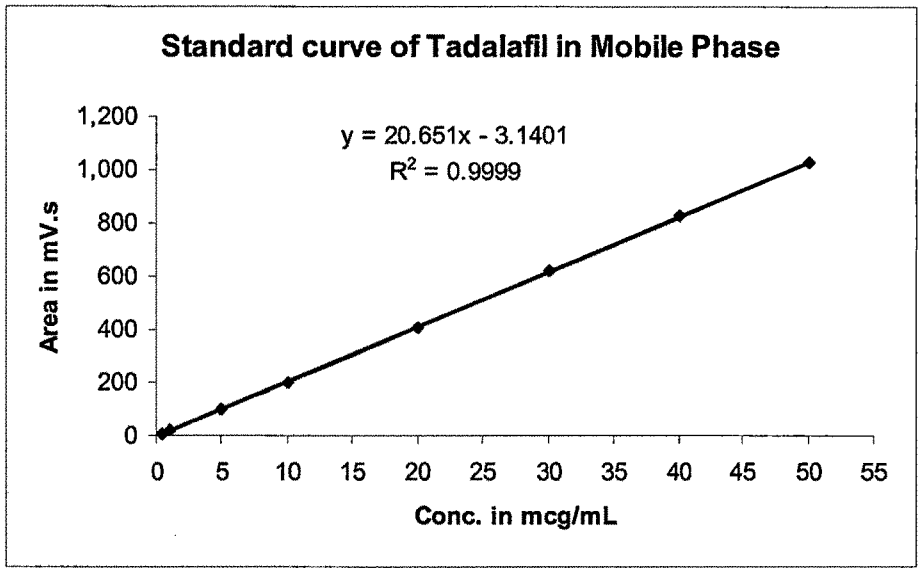


Fig. 4.1.2.2Calibration curve for Tadalafil in mobile phase

Table 4.1.2.3. Interday and intraday precision of proposed HPLC method

Concentration of Tadalafil (µg/ml)	Amount of Tadalafil found (µg/ml)			
	Intraday precision		Interday precision	
	Mean ($n = 3$)	%RSD	Mean ($n = 3$)	%RSD
0.5	8.031	1.300819	8.24	1.543
1	18.669	2.7486	18.603	0.228
5	100.257	1.83829	100.412	0.927

10	200.758	2.0820	199.24	1.923
20	409.153	1.055442	405.478	1.552
30	622.986	0.217113	618.053	0.324
40	829.008	0.180186	821.756	1.672
50	1029.035	1.897	1024.375	1.258

Table 4.1.2.4. Robustness studies of Tadalafil by proposed HPLC method

Parameters	Retention time variation (min)			Peak area variation		
	Mean area	S.D.	% RSD	Mean area	S.D.	% RSD
Wavelength (+ 2 nm)	11.91	0.03	0.251	407.042	17.26	4.24
Flow rate (+ 0.01 unit)	11.63	0.05	0.429	411.36	18.27	4.441
Analyst to Analyst	11.86	0.05	0.421	406.990	17.63	4.331

Table 4.1.2.5. System suitability parameter.

Parameter	
RT(min ± SD)	12.0466 ± 0.128
Tailing factor ± SD	1.382 ± 0.0355
Theoretical plats ± SD	7476 ± 27.098
%RSD	1.1

Table 4.1.2.6. Analysis of marketed formulations by proposed HPLC method

Dosage Forms Code	Labeled amount (mg/tab)	Amount found* (mg/tab)	% Assay*
Tablet-1	10	10.31 ± 0.53	103.1 ± 1.23
Tablet-2	10	9.970 ± 0.91	99.70 ±1.06
Tablet-3	20	20.15 ± 0.66	100.75 ± 1.23

Tablet-1 were the 36-Hours(10mg.) tablets from Cadila pharmaceuticals Ltd., Ahmedabad,
Tablet-2 were the Tadora-10(10mg.) tablets from German Remedies, Mumbai and
Tablet-3 were the Tadora-20(20mg.) tablets from German Remedies, Mumbai
*Mean ± S.D. of three determinations

Table 4.1.2.7. Recovery studies of Tadalafil tablets by proposed HPLC method

Concentration of Tadalafil (µg/ml)			Amount found# (µg/ml)	% Recovery#
Initial	Added	Total		
10	5	15	14.95 ± 0.86	99.66
10	10	20	20.10 ± 1.23	100.5
10	20	30	29.95 ± 0.55	99.83

#Mean ± S.D. of three determinations

Table 4.1.2.8. Summary of method validation parameters

Method validation parameters	Results
Linearity and Range	
• Linearity (µg/ml)	0.5 mcg/mL-50.00mcg/mL
• Linear equation (Y= MX +C)	$Y = 20.651X-3.1401$
• Regression co-efficient (r)	0.9999
Precision (% RSD)	
• Interday precision	0.80 – 1.80
• Intraday precision	0.22 – 1.92
Robustness for retention time (% RSD)	
• Wavelength (+2 nm)	0.251
• Flow rate (+ 0.01 unit)	0.429
• Analyst to Analyst	0.421
Robustness for peak area ratio (% RSD)	
• Wavelength (+2 nm)	4.24
• Flow rate (+ 0.1 unit)	4.44
• Analyst to Analyst	4.33
Limit of Quantification (µg/ml)	0.093
Percentage Recovery	99.66 – 100.50
Percentage Assay	99.70 – 103.1

4.1.3. Estimation of Tadalafil in Human Plasma by HPLC method.

4.1.3.1 Methodology :

4.1.3.1.1 Reagents :

The reagents are described in section 4.2.2.1.1.

4.1.3.1.2 Apparatus :

The apparatus are described in section 4.1.2.2.2.

4.1.3.1.3. Preparation of working stock solutions :

A working stock solution of the Tadalafil (1000 µg/ml) was prepared by dissolving 25 mg of Tadalafil in a 25 ml of volumetric flask containing 10 ml of mobile phase (water containing 0.1 m. mole of glacial acetic acid and acetonitrile in the ratio of 60:40 v/v), sonicated for about 15 min and diluted up to volume with mobile phase. A working stock solution was protected from light and kept at -20°C. They are stable for at least 6 month.

4.1.3.1.4. Plasma Calibration Standards :

Plasma calibration standards of 0.25, 0.5,1, 2, 5, 10, and 20 µg/ml of Tadalafil were obtained by diluting the suitable aliquots of working stock solution ranging from (0.025, 0.05, 0.1, 0.2 0.5, 1.0, and 2.0 mL) with up to the 100 mL of drug-free human plasma. Stability of the drug in plasma at -20 °C over 6 months was documented.

4.1.3.1.5. Sample extraction Procedure :

0.1 mL of Internal standard solution and 1.0 mL of calibration standard were mixed with 0.5 mL of drug-free human plasma in 5 mL-polypropylene centrifuge tube with flat caps and mixed for 3 minutes on vortex. 50 microliters of 1N sodium hydroxide solution was immediately added with a micro pipette while gently vortexing the tubes. To each tube 3 mL of Methyl tertiary Butyl ether was added, and the tube was vortexed for 30 sec. at high speed. The sample was finally shaken on a rotating shaker (50 rotations/minute) for 3 minutes. After centrifugation for 20 minutes at 2000 rpm at 4 °C, the clear Methyl Tert. Butyl ether layer was transferred with a calibration pipette to a disposable glass tube and evaporated under a gentle stream of nitrogen. The dried residue was taken up with 25µL of mobile phase, and 20 µg of this mixture were injected in the HPLC system.

4.1.3.1.6. System suitability study.

In this study, 1.0 mL of calibration standard Tadalafil (10µg/mL) was mixed with 0.5 mL of drug-free human plasma in 5 mL-polypropylene centrifuge tube with flat caps and mixed for 3 minutes on vortex. 50 microliters of 1N sodium hydroxide solution was immediately added with a micro pipette while gently vortexing the tubes. To each tube 3 mL

of methyl tertiary butyl ether was added, and the tube was vortexed for 30 sec. at high speed. The sample was finally shaken on a rotating shaker (50 rotations /minute) for 3 minutes. The sample was centrifuged for 20 minutes at 2000 rpm at 4°C the clear methyl tert. butyl ether layer was transferred with a calibrated pipette to a disposable glass tube and evaporated under a gentle stream of nitrogen. The dried residue was taken up with 25 µL of mobile phase, and 20 µL of this mixture were injected in the HPLC system. The study was repeated for six times and the % relative standard deviation (%RSD) of peak areas, resolution factors, tailing factors, and theoretical plats were determined and shown in Table .4.1.3.4.

4.1.3.1.7. Chromatographic conditions :

The chromatographic condition is similar to that described in section 4.1.2.2.4. and Table 4.1.2.1.

4.1.3.2. Result and Discussion :

4.1.3.2.1 Assay Validation :

For assay validation, Tadalafil was mixed with drug-free human plasma over the concentration ranges 0.25 – 20 µg/ml. Concentration height then 20 µg/ml were not tested for linearity. The retention time (*t*R) of Tadalafil was found to be 11.80 min. (Fig. 4.1.3.1). The calibration curve of Tadalafil in human plasma was constructed by plotting the peak area of Tadalafil standard (*Y*) against concentration of Tadalafil (*X*) (Table 4.1.3.1). It was found to be linear with a correlation coefficient of 0.9989, the representative linear regression equation being $Y = 16.741+6.9269$ (Fig. 4.1.3.2).

The relative standard deviations based on the peak area for triplicate injections were found to be 0.07 – 1.98 % for calibration curve. The developed method was validated for its intraday and interday precision in the range of 0.25-20.00 µg/ml. The intraday and interday (3 days, $n = 3$) precision were expressed as relative standard deviation in range of 1.24 – 4.55 % and 0.89-4.89 %, respectively (Table 4.1.3.2).

The limit of quantification (LOQ) was calculated using the standard deviation of the intercepts and the mean slope of the calibration curves (LOQ = 3 x standard deviation of the intercepts/ mean slope) and it was 0.007293 µg/ml. The limit of detection (LOD) was calculated using the standard deviation of the intercepts and the mean slope of the calibration curves (LOD = 10 x standard deviation of the intercepts/ mean slope) and it was 0.024330 µg/ml.

4.1.3.2.2 Extraction Yield :

Several extraction solvents were attended: diethylether, methanol, chloroform, n-hexane, di chloromethane and methyl tertiary butyl ether. Best result was obtained with methyl tertiary butyl ether. Extraction recoveries determined by comparing the peak height heights obtained by direct injection of standard Tadalafil solution with those obtained after methyl tertiary butyl ether extraction of plasma samples were not less then 90.00% over the 0.25 – 20 µg/ml concentration range. (Table 4.1.3.1.)

4.1.3.2.3 Stability :

The amount of Tadalafil recovered over a 6-month period in plasma samples stored at -20°C did not show significant differences from the initial concentrations. (Table 4.1.3.3)

4.1.3.2.4. System suitability study :

The results obtained from the system suitability study (Table 4.1.3.4.) are in agreement with the USP requirements and the variation in the retention time among six replicate injection of Cilostazol working solutions was very low, rendering a R.S.D. of 0.9 %.

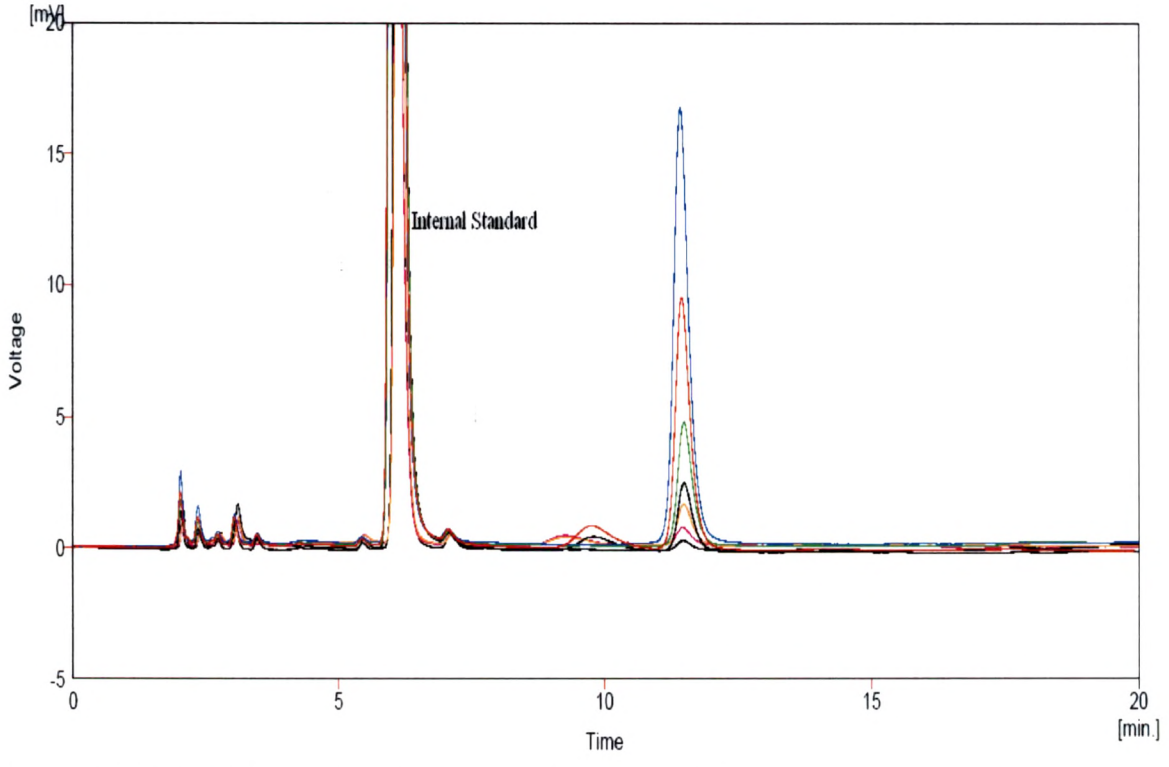


Fig. 4.1.3.1 The overlain chromatograms of standard Tadalafil from 0.25 to 20.0µg/ml concentration in mobile phase

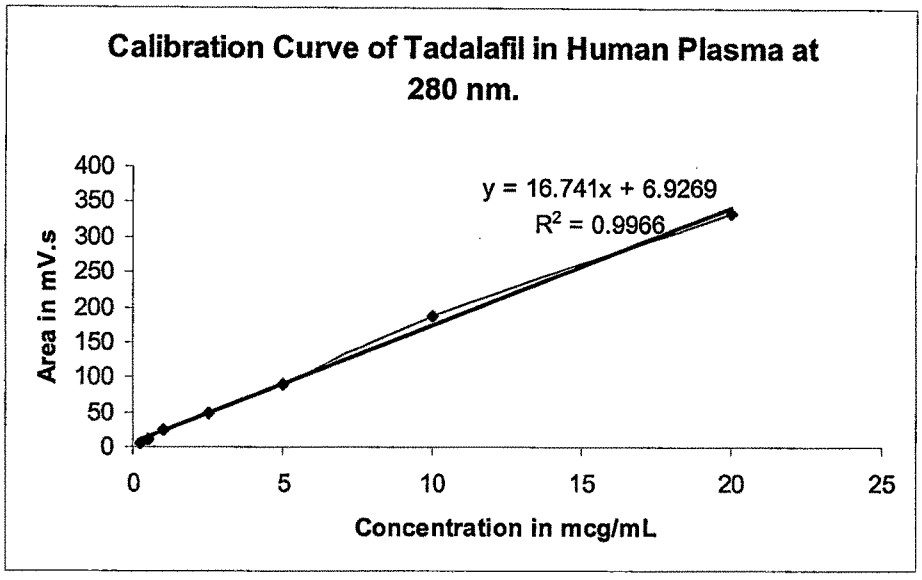


Fig. 4.1.3.2 Calibration data of Tadalafil in human plasma at 284.5 nm.

Table 4.1.3.1 Tadalafil recovery after extraction: Drug-free human plasma was spiked with Tadalafil at different concentrations and extraction coefficient calculated.

Concentration of Tadalafil (µg/ml)	Average Extraction Coefficient (%) (n=3 for each level)	Mean peak area in mV (n=3)	Standard Deviation
0.250	96.325	5.3815	0.07
0.500	97.02715	10.901	0.79
1	94.235.	25.5985	0.60
2	97.64885	50.005	0.91
5	90.40099	90.7795	1.89
10	94.28751	188.1925	1.96
20	82.19377	334.731	0.73

Table 4.1.3.2 Interday and intraday variability of the assay for Tadalafil in plasma (3 series)

Concentration of Tadalafil (µg/ml)	Amount of Tadalafil found (µg/ml)			
	Intraday precision		Interday precision	
	Mean Area ($n = 3$)	SD	Mean Area ($n = 3$)	SD
0.250	5.332	3.26	5.431	0.89
0.500	11.461	2.59	10.341	2.63
1	22.528	1.86	21.669	1.51
2	50.655	4.55	49.355	3.89
5	92.116	2.21	89.443	4.89
10	189.597	1.24	186.788	3.55
20	339.233	1.36	388.193	2.99

Table 4.1.3.3 Drug-free human plasma spiked with 3 different concentration of Tadalafil and Stored at -20°C Over 6- Month Period.

Concentration Added (µg/ml)	Concentration Obtained in µg/ml along with Mean Area(n = 3)			
	Day 1	Day 30	Day 90	Day 180
0.250	0.246(5.29)	0.251(5.40)	0.241(5.18)	0.239(5.14)
2	2.06(51.25)	1.98(49.54)	2.13(53.30)	2.09(52.30)
20	19.57(327.53)	20.43(341.92)	18.96(317.32)	20.55(343.93)

Table 4.1.3.4. System suitability parameter.

Parameter	
RT(min ± SD)	11.781 ± 0.0101
Resolution factor ± SD	11.090 ±0.090
Tailing factor ± SD	1.5353 ± 0.007
Theoretical plats ± SD	8360.33 ± 113.22
%RSD	0.86

4.1.4. Estimation of Tadalafil from its formulations and in human plasma by Spectrofluorophotometric method[12-13].

4.1.4.1. Methodology :

4.1.4.1.1. Material and Reagent :

An authentic working standard of Tadalafil was procured as a gift sample from Macleods Pharmaceuticals, Daman. The reagents used were of Analytical-Reagent grade. Methanol and sulphuric acid were purchased from Allied chemicals Corporation, Vadodara. 0.1 M methanolic H_2SO_4 (0.5 ml conc. H_2SO_4 in 100 ml methanol) was prepared as per IP-1996[14]. Film coated tablets of TFL having brand names "36 Hour" of Zydus Cadila and "Tadora-10" of German Remedies containing 10 mg of Tadalafil were procured from local drug store.

4.1.4.1.2. Instrument :

A Shimadzu Spectrofluorophotometer (Model RF-540 with DR-3 data recorder), equipped with a 1 cm fluorescence free quartz cell having four transparent side was used for all spectral and fluorescence measurements was used for through out the study.

4.1.4.1.3. Preparation of working stock solution :

Tadalafil (50 mg) was accurately weighed and transferred to 50 mL volumetric flask. About 30 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 50 mL using methanol to obtained standard stock solution (i.e. 1000 µg/mL). An aliquot of 0.1 mL of standard stock solution was further diluted with 100 mL of 0.1 M methanolic H_2SO_4 to get working stock solution containing 1µg/mL of the drug.

4.1.4.1.4. Preparation of Standard solution :

Suitable aliquots of the working stock solution ranging from 0.1, 0.2, 0.3, 0.4 and 0.5 mL were taken into 10 mL volumetric flask and volumes were made up with 0.1 M methanolic H_2SO_4 to prepare a series of standard solutions (10-50 ng/mL) for calibration curve. All the solutions and working standard solution of Tadalafil were protected from light by wrapping in aluminum foil.

4.1.4.1.5. Determination of analytical wavelength for Tadalafil :

Standard solution (1µg/mL) of Tadalafil was scanned in the range of 200-350 nm for determination of excitation wavelength and it was found to be 315 nm. Same solution was scanned for determination of emission wavelength in the range of 315-400 nm taking 315 nm as excitation wavelength and it was found to be 332 nm. The relative fluorescence intensity

of all the standard solutions was measured in the range of 315-400 nm and calibration curve was prepared by plotting concentration against fluorescence intensity.

4.1.4.1.6. Procedure for pharmaceutical formulations :

Twenty tablets were weighed, powdered and tablet powder equivalent to 50 mg of Tadalafil was taken and stirred with 20 mL of methanol using magnetic stirrer for 30 min at room temperature. Then it was filtered through whatman filter paper No 42. into 50 mL volumetric flask. Filter paper was rinsed thrice with 2 mL of methanol and the solution was diluted to 50 mL with methanol to give a stock solution of 1 mg/mL. Taken 0.1 mL of this solution was further diluted with 100 mL of 0.1 M methanolic H_2SO_4 to get stock solution containing 1µg/mL of the drug. From above solution suitable three aliquots (0.2 mL, 0.3 mL and 0.4 mL) were taken and diluted to 10 mL with 0.1M methanolic H_2SO_4 to prepare the sample solutions 20 ng/mL (low), 30 ng/mL(middle) and 40 ng/mL (high) in the range of calibration curve.

4.1.4.1.7. Preparation of Tadalafil working stock solution in spiked human plasma :-

Standard stock solution (5 mg/ml) of Tadalafil was prepared by dissolved 50 mg Tadalafil into 10 ml of methanol, from which 0.2 ml of this stock solution was taken and diluted it up to 100 ml with human plasma to get working stock solution containing 10 µg/ml of Tadalafil.

4.1.4.1.8. Plasma Calibration Standards :

Suitable aliquots of the working stock solution ranging from 0.1, 0.2, 0.3, 0.4 and 0.5 mL were taken into disposable polypropylene micro centrifuge tube. Shake well for 5 min, then add 1.0 mL of 4% trichloro acetic acid for precipitation of plasma. Shake the mixture on a vortex mixer for 1 min, and centrifuge it for 6 min at 5000 rpm in a micro centrifuge. Precipitated plasma was settled down into micro centrifuge tube. Then taken 550,600,650,700 and 750 µL of supernatant containing (0.5 -2.5 µg of Tadalafil) from each micro centrifuge tube and diluted it up to 50 ml with 0.1 M methanolic H_2SO_4 to get a series of standard solutions (10-50 ng/ml) for calibration curve.

4.1.4.2. Results and Discussion :

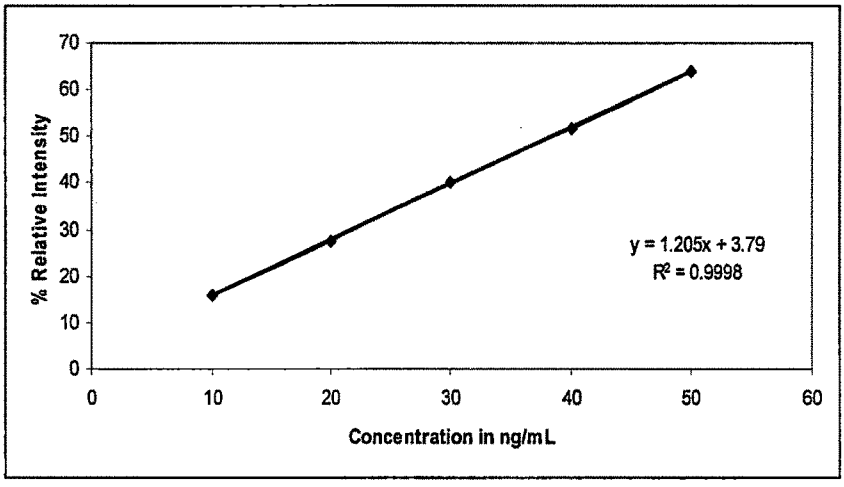
The calibration curves for Tadalafil showed linearity in the concentration range of 10-50 ng/mL at 332 nm as shown in Figure. 4.1.4.1. and Figure 4.1.4.2. The other parameters like excitation wavelength, emission wavelength, regression equation, coefficient of determination (r^2), correlation coefficients (r), limit of detection (LOD), limit of quantification (LOQ) etc. are enumerated in Table 4.1.4.1. Calibration curves were repeated

five times and RSD of each concentration level was found to be less than 1%, which indicates that methods can be used for analysis of bulk drug samples and applicable also for matrix(plasma/serum). The correlation coefficient values were highly significant for both the method. Recovery study for commercial samples was carried out by adding a known amount of standard drug to different concentration of sample solutions at three different levels. The total amount of drug was then determined by these method and the amount of added drug found by difference. Results of recovery studies for commercial samples are given in Table 4.1.4.3.

Recovery study for Tadalafil in plasma was carried out by protein precipitation method. The aliquots of plasma working stock solution of Tadalafil were mixed with 1 mL of 0.4 % of trichloro acetic acid solution. Shake the mixture on a vortex mixer for 1 min, and centrifuge it for 6 min at 5000 rpm in a micro centrifuge. Collect the supernatant diluted it with suitable solvent and determine the total amount of drug. Result of recovery studies for Tadalafil in human plasma are given in Table 4.1.4.4.

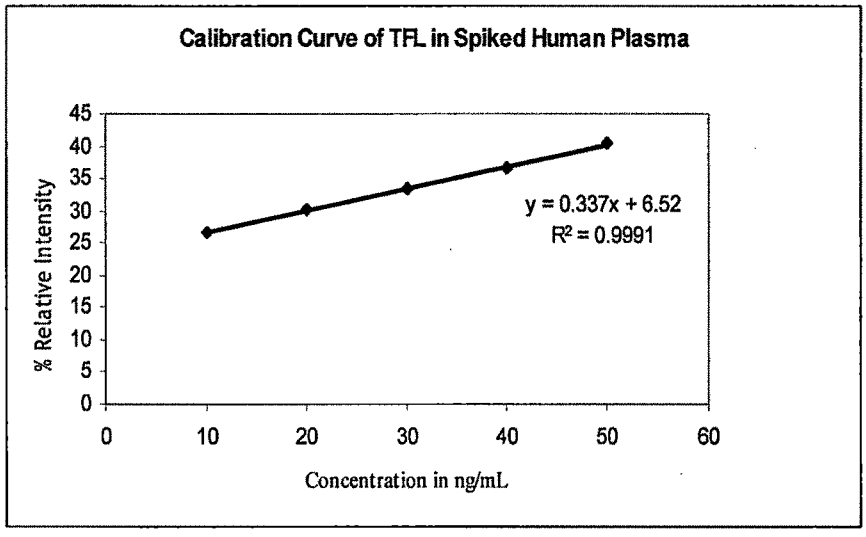
The methods was validated for analytical parameters such as accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ). Inter-day and Intra-day RSD were also found out to ascertain precision and accuracy of the developed method. In this method accuracy was grater than 98 % and RSD did not exceed 2% in any of the case. The low values of standard deviation and coefficient of variation establish the precision of the proposed methods. Standard, stocks and working standards of Tadalafil for both methods (pure drug and plasma method) did not show significant change in relative fluorescence intensity on storage and hence were stable for up to 6 Hr. when wrapped in aluminum foil.

From the discussion above, it is clear that the developed method is accurate, precise, repeatable, reproducible, linear, quick, inexpensive and simple. Though, the results obtained from this method suggest that developed spectrofluorophotometric method was highly sensitive for estimation of bulk drug as well as plasma analysis but method method was not used for later study.



a) 10 ng/mL, (b) 20 ng/mL, (c) 30 ng/mL, (d) 40 ng/mL, (e) 50 ng/mL.

Figure 4.1.4.1 Calibration curve of Tadalafil in methanol



a) 10 ng/mL, (b) 20 ng/mL, (c) 30 ng/mL, (d) 40 ng/mL, (e) 50 ng/mL.

Figure 4.1.4.2. Calibration curve of Tadalafil in Spiked Human plasma.

Table 4.1.4.1. : OPTICAL CHARACTERISTICS AND ANALYTICAL DATA

Parameter	Result for Standard Drug	Result for Standard Drug Spiked in Human Plasma
Excitation wavelength, λ_{Ex} (nm)	315	315
Emission wavelength, λ_{Ex} (nm)	332	332
Linearity range (ng/ml)	10 – 50	10 – 50
Regression equation (Y^a)	Y = 1.205X + 3.79	Y = 0.337X +6.52
Slope (b)	1.205	0.337
Intercept (a)	3.79	6.52
Coefficient of determination (r^2)	0.9998	0.9991
Correlation coefficient (r)	0.9998	0.9995
Limit of detection, LOD (ng/ml)	0.208	0.235
Limit of quantification, LOQ (ng/ml)	0.694	0.701
Inter-day % RSD	<1.38%	< 1.12%
Intra-day % RSD	<1.47%	<1.56%
Accuracy	>98.13%	-

[a]Y=a+bX, Where X is the concentration (ng/mL). LOD=3σ/S, LOQ=10σ/S, Where σ is standard deviation of blank, and S is a slope of concentration.

Table 4.1.4.2.: Result of Commercial Samples Analysis.

Marketed Formulation of TFL	Drug/Label Claim (mg/Tablet)	*Amount found (mg)	% R.S.D.
36 Hours (TFL-1)	Tadalafil/ 10	9.81	0.89
Tadora-10 (TFL-2)	Tadalafil/ 10	9.86	0.95

Asterisk (*) denotes mean of five determinations, % R.S.D. = Relative Standard Deviation (n = 5).

Table 4.1.4.3 : Recovery Study of Commercial Samples.

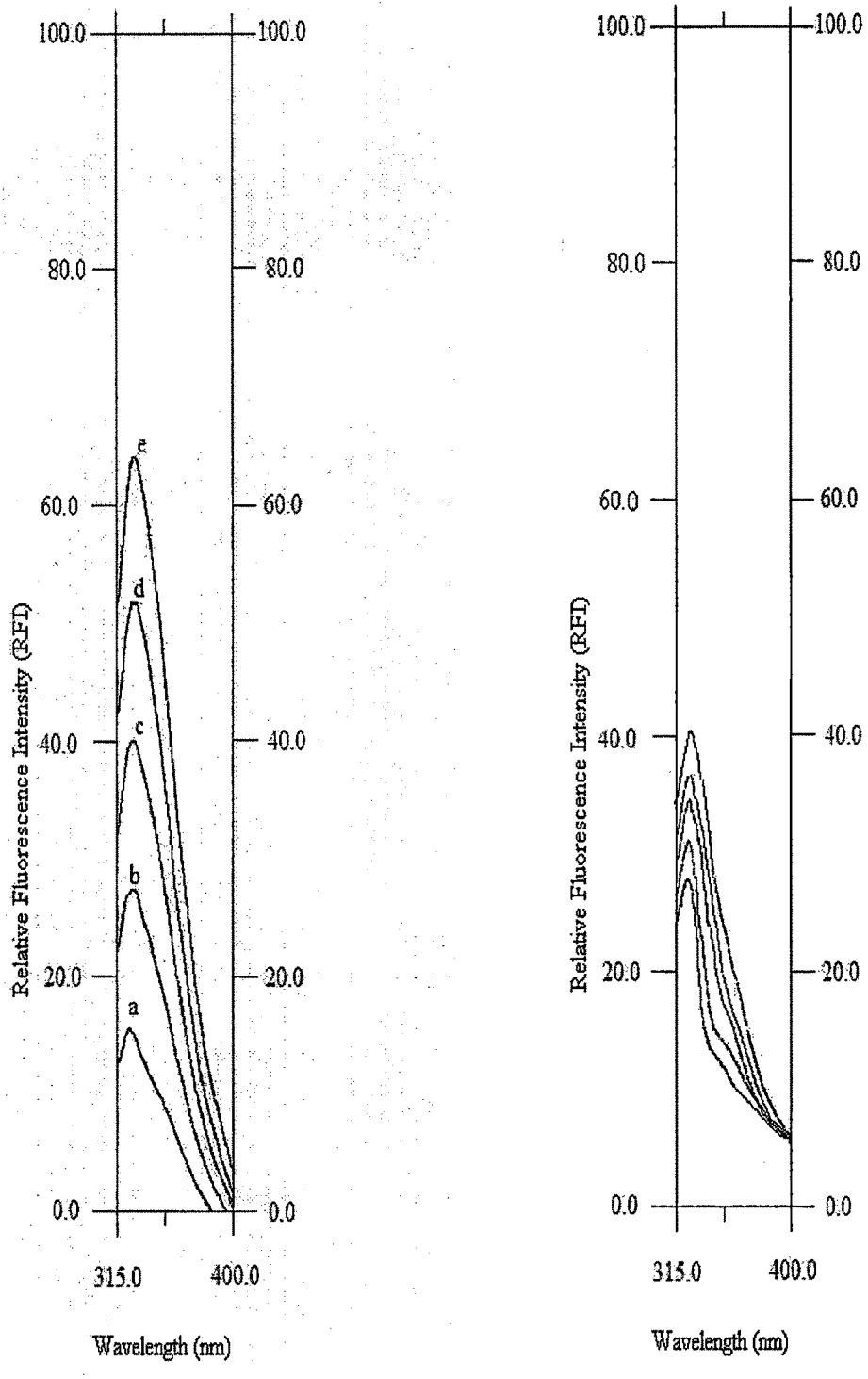
Formulation	Standard drug added in final solution (ng/mL)	Drug found* ±SD (ng/mL)	*Recovery (%)
TFL-1	8	8.016 ± 0.86	100.2
	10	9.78 ± 0.96	97.81
	12	11.74 ± 0.52	97.85
TFL-2	8	7.979 ± 0.69	99.6
	10	9.854 ± 0.42	98.54
	12	11.86 ± 0.16	98.84

*Average of three determinations at three levels.

Table 4.1.4.4. Tadalafil Plasma Recovery by Protein Precipitation Method.

Concentration of Tadalafil (ng/ml)	% Recovery (n=3 for each level)	Standard Deviation
10	76.5	3.57
20	79.02	4.35
30	77.9.	4.29
40	81.1	2.91
50	83.3	3.89

Figure 4.1.4.3: EMISSION FLUORESCENCE SPECTRA OF TADALAFIL AT 332 nm.



4.1.5. References

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4.2.1. Preparation of Oral Microemulsion of Tadalafil (TME)

4.2.1.1. Solubility of Tadalafil in various oils/surfactants/co-surfactants :

The procedure used for measuring the solubility of Tadalafil in various oils/surfactants/co-surfactants was similar to that described in section 3.2.1.1.

Table 4.2.1.1.Solubility profile of Tadalafil in different oils/surfactants/co-surfactants:

Sr. No.	Name of oil/surfactant/co-surfactant	Solubility of Tadalafil(mg/mL) in oil /surfactant/co-surfactant
1	Peanut oil	2.73
2	Cotton seed oil	0.54
3	Captex 355 EP/NF	0.5
4	Captex 200 P	0.63
5	Labrafac PG	0.7
6	Labrafil M 2125	1.4
7	Cremophor EL	2.43
8	Cremophor RH 40	1.26
9	Labrafil M 1944	2.21
10	Plurol	0.9
11	Transcutol P	21.80 $\sqrt{}$ **(Co - S)**
12	Capmul MCM (C8)	10.20 $\sqrt{}$ **(O)**
13	Capmul MCM (C10)	11.05 $\sqrt{}$ **(O)**
14	Captex 1000	1.86
15	Tween 80	16.32 $\sqrt{}$ **(S)**
16	Tween 20	17.97 $\sqrt{}$ **(S)**

The solubility of Tadalafil was found to be better in Transcutol P, Capmul MCM (C8), Capmul MCM (C10), Tween 80 and Tween 20, these were further used for the preparation of ME.

4.2.1.2. Preparation of Tadalafil Microemulsions :

TME (system 1, TME 1) were prepared by titration method using Capmul MCM C8® as an oil phase (O), Tween - 20® as a surfactant (S), Transcutol P® as a co-surfactant (CoS) and distilled water as an aqueous phase (AQ). Tadalafil (15 mg/mL) was dissolved in oil phase containing surfactant and co-surfactant at room temperature with continuous stirring. To the resultant mixture distilled water was added gradually with continuous stirring.

Similarly, another set of TME (system 2, TME 2) was prepared using Capmul MCM C10® as oil phase, Tween - 80® as surfactant, Transcutol P® as co-surfactant and distilled water as an aqueous phase. TME system 3 (TME 3) was prepared by replacing oil phase of TME 1 with Capmul MCM C10®. The process was identical as TME 1. Tadalafil (15 mg/mL) was dissolved in oil phase containing surfactant and co-surfactant at room temperature with continuous stirring. To the resultant mixture distilled water was added gradually with continuous stirring. The excipient profile for TME system 1, 2and 3 is shown in Table 4.2.1.2.

Table 4.2.1.2. Excipient profile for three different systems of Tadalafil microemulsions:

Ingredients	System 1	System 2	System 3
Tadalafil	√	√	√
Capmul MCM C10® (O)	☒	√	√
Capmul MCM C8® (O)	√	☒	☒
Tween 20® (S)	√	☒	√
Tween 80® (S)	☒	√	☒
Transcutol P® (Co-S)	√	√	√
Water (AQ)	√	√	√

S: CoS – 1:1, 2:1 and 3:1 for System 1, 2, 3 and System 4.

√ Ingredients used ☒ Ingredients not used

For optimization of ME composition, distilled water was added with stirring to the mixture of oil and surfactant / co-surfactant (at different mass ratios viz. 1:1, 2:1 and 3:1) containing Tadalafil. Visually clear and transparent MEs were considered as acceptable. The concentrations of selected batches are recorded in Table 4.2.1.3.

Table 4.2.1.3. Composition of TME 1, TME 2 and TME 3 along with different S:CoS ratios.

Foemulation	S:CoS ratio	O (%)	S (%)	CoS (%)	AQ (%)	Cilostazol (mg/mL)
TME 1	1:1	12	16.66	16.66	55.55	15
	2:1	12	22.22	11.11	55.55	15
	3:1	**12**	**25**	**8.33**	**55.55**	**15**
TME 2	1:1	16.66	16.68	16.66	50	15
	2:1	16.66	22.24	11.11	50	15
	3:1	**16.66**	**25.02**	**8.33**	**50**	**15**
TME 3	1:1	15.09	18.86	18.86	47.16	15
	2:1	15.09	25.15	12.57	47.16	15
	3:1	**15.09**	**28.30**	**9.43**	**47.16**	**15**

The concentrations of various phases which yielded clear MEs were plotted as two dimensional pseudo ternary phase diagram as shown in Figure 4.2.1.1. (TME 1), Figure 4.2.1.2. (TME 2) and 4.2.1.3. (TME 3) respectively, to obtain ME region. Phase study indicated that, the ME region for TME 1, TME 2 and TME 3 (3:1 S:CoS ratio) were found to be highest among all formulations and hence selected for further characterization study.

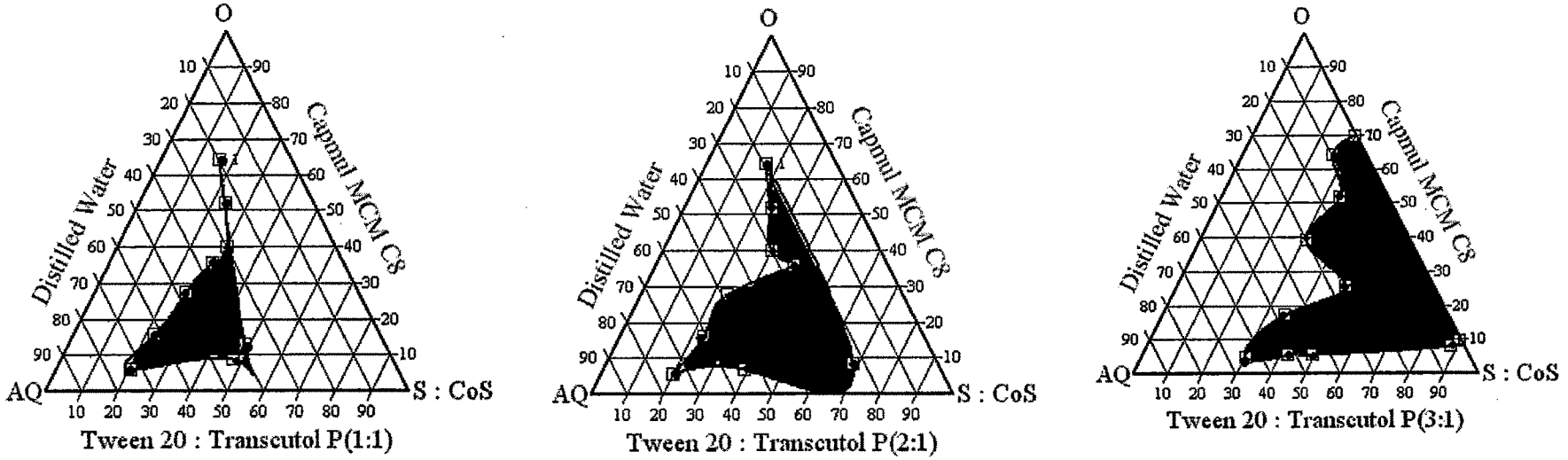


Figure 4.2.1.1. Pseudo-ternary phase diagrams for TME 1 (System 1) showing ME regions (Shaded) at S: CoS ratio 1:1, 2:1 and 3:1.

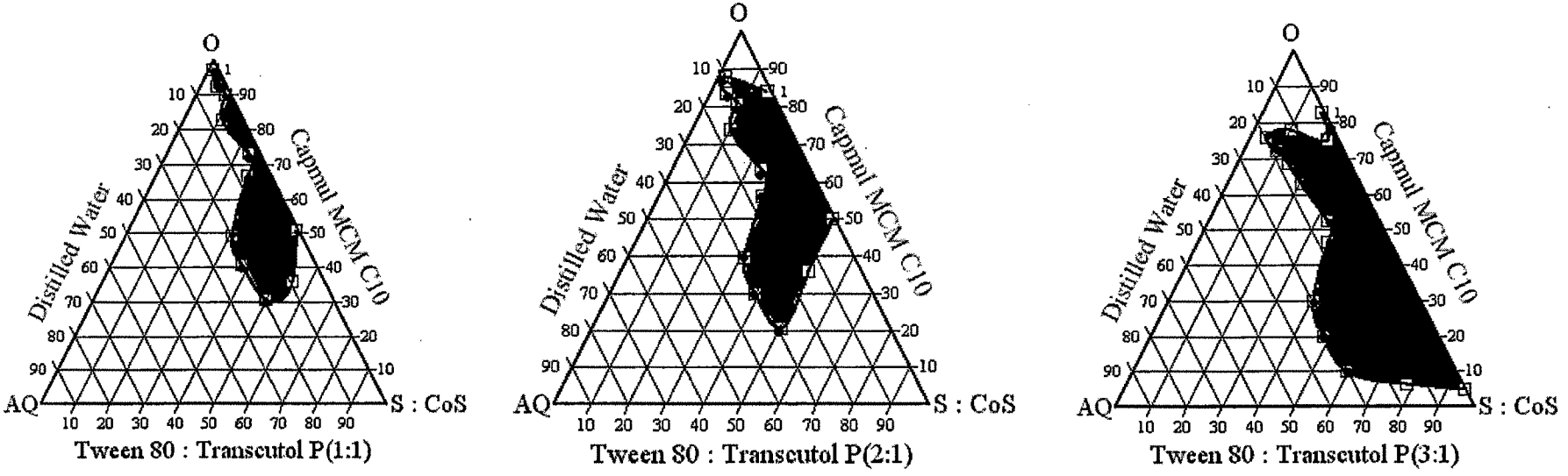


Figure 4.2.1.2. Pseudo-ternary phase diagrams for TME 2 (System 2) showing ME regions (Shaded) at S: CoS ratio 1:1, 2:1 and 3:1.

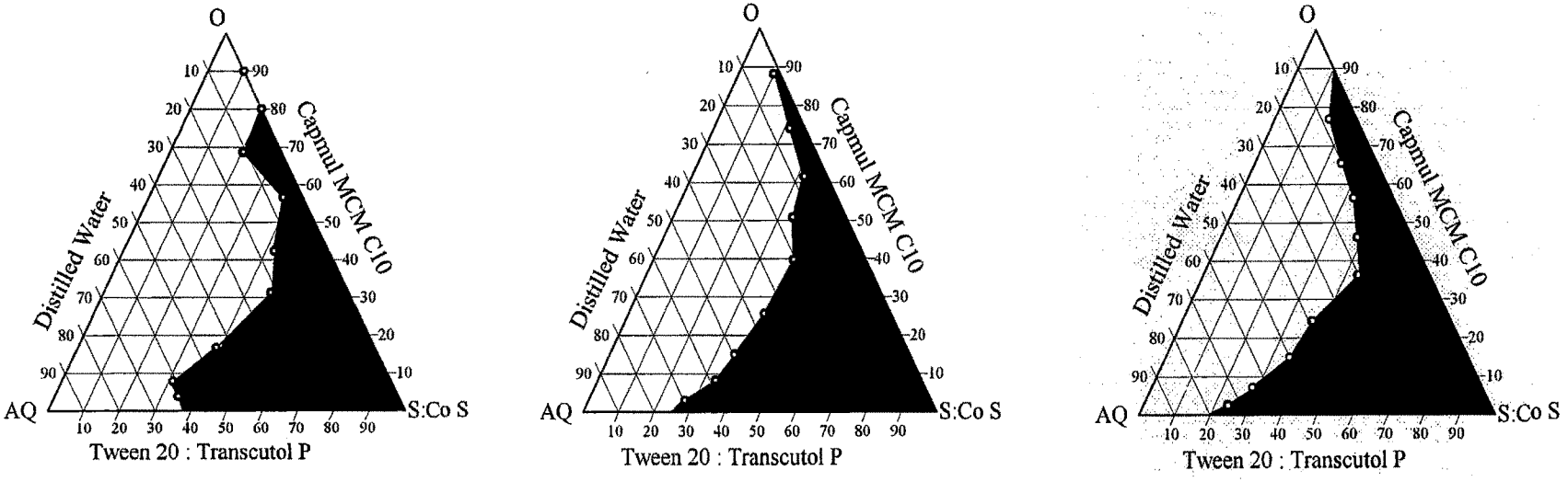


Figure 4.2.1.3. Pseudo-ternary phase diagrams for TME 3 (System 3) showing ME regions (Shaded) at S: CoS ratio 1:1, 2:1 and 3:1.

4.2.2.Characterization

Prepared Oral microemulsions of Tadalafil were further characterized for pH, globule size, zeta potential, viscosity, electroconductivity and % Transmittance.

4.2.2.1. Appearance :

Appearances of TME 1, TME 2 and TME 3 were tasted against white and black background and turbidity were checked. The test was carried out as described in the Indian Pharmacopoeia (1996) and United States Pharmacopoeia (2003).

4.2.2.2. Stability as per stomach condition and pH Determination :

The pH of TME 1, TME 2 and TME 3 were measured by diluting the 5 mL of respective test sample of ME with 10 parts, 100 parts and 1000 parts of distilled water. The resultant solution/dispersion was stirred for 5 min and the stability was checked. The pH were recorded for stable solutions/dispersions by using calibrated digital pH meter at 25° C ± 1°C[1]. The pH was recorded in triplicate when the pH gets stabilized. pH meter was calibrated daily using standard buffer solutions (pH 4.2, pH 7.00 and pH 9.2) prior to recording the observations.

4.2.2.3. Globule size Determination :

The globule size[2,3]of TME 1,TME 2 and TME 3 were determined using photon correlation spectroscopy (PCS) method with in-built Zetasizer (Model: Nano ZS, Malvern Instruments, UK) at 633 nm. The equipment was filled with 18 mm width, helium-neon gas laser source having intensity of 4 mW. The mean PCS diameter is the so-called intensity-weighted "z-average" (mean particle size). Average of three measurements of each sample was used for derivation of mean particle size. Latex dispersion having mean particle size 60 nm ± 5 nm was used as a standard. The standard was evaluated after every 60 min during measurement of test samples in order to validate the equipment.

4.2.2.4. Zeta Potential Determination :

The Nano ZS Zetasizer (Malvern Instrument, UK) was used to measure the zeta potential by electrophoresis and electrical conductivity of the formed ME was also performed using in built conductivity option (Zeta potential) of the Zetasizer[2]. The electrophoretic mobility (μm/s) was converted to zeta potential by in-built software using Helmholtz-Smoluchowski equation. Measurements were performed using small volume disposable zeta cell. Average of three measurements of each sample was used to derive average zeta potential. Latex dispersion having zeta potential -50 mV ± 2.5 mV was used as a standard.

The standard was evaluated after every 60 min during measurement of test samples in order to validate the results of test formulation.

4.2.2.5. Viscosity Measurement :

The rheological property of the TME 1, TME 2 and TME 3 were evaluated[1] by a Brookfield LVDV 111 + CP viscometer (Stoughton, MA) at 30 °C using a CPE 42 spindle at 5 rpm. Experiment was performed in triplicate for each sample, and results were presented as average ± standard deviation.

4.2.2.6. Electroconductivity Measurement :

The electroconductivity of the resultant system was measured by an electroconductometer (CM 180 conductivity meter, Elico, Mumbai, India). For the conductivity measurements, the tested microemulsions were prepared with a 0.01N aqueous solution of sodium chloride instead of distilled water.

4.2.2.7. % Transmittance Measurement :

The percent transmittance of TME 1, TME 2 and TME 3 were measured at 650 nm using UV spectrophotometer (UV 1601, Shimadzu, Japan) keeping distilled water as a blank.

4.2.2.8 Active Ingredient Analysis :

TME 1, TME 2 and TME 3 were analyzed for presence of Tadalafil ingredient using the developed spectrophotometric method of analysis for analyzing formulations described under section 4.1.1.4.5.

Table 4.2.2.1. Compositions and characterization of Tadalafil microemulsion system 1 (TME 1) as per stomach condition.

System	O (%)	S (%)	CoS (%)	AQ (%)	Globule size (nm) ± SD		Zeta potential (mV) ± SD		Transmittance (%) ± SD	
					Initial	After 3 Hr.	Initial	After 3 Hr.	Initial	After 3 Hr.
TME 1 (S:CoS ratio 3:1)	**12**	**25**	**8.33**	**55.55**	22.1 ± 0.27	28.2 ± 0.35	-2.61 ± 0.51	-2.10 ± 0.42	99.55 ± 0.55	99.34 ± 0.59
	TME 1 DILUTED BY 10 TIMES WITH AQUEOUS PHASE									
	4.83	10.88	3.62	80.64	31.5 ± 0.37	38.25 ± 0.53	-3.82 ± 0.77	-3.44 ± 0.31	99.26 ± 0.32	99.22 ± 0.85
	TME 1 DILUTED BY 100 TIMES WITH AQUEOUS PHASE									
	0.58	1.31	0.43	97.62	47.11 ± 0.88	55.46 ± 0.13	-5.01 ± 0.26	-4.91 ± 0.14	99.12 ± 0.35	99.03 ± 0.63
	TME 1 DILUTED BY 1000 TIMES WITH AQUEOUS PHASE									
	0.059	0.134	0.044	99.76	52.82± 0.56	63.33 ± 0.72	-6.36 ± 0.97	-5.21 ± 0.11	98.76 ± 0.84	98.66 ± 0.96

The results are mean values ± SD derived from three different experimental batches. O is denoted for Oil Phase (Capmul MCM C8®), S for surfactant (Tween - 20®), Co-S for co-surfactant (Transcutol P®) and AQ is denoted for aqueous phase (Distilled Water). The TME formulations contain Tadalafil – 15 mg/mL.

Table 4.2.2.2. Compositions and characterization of Tadalafil microemulsion system 2 (TME 2) as per stomach condition.

System	O (%)	S (%)	CoS (%)	AQ (%)	Globule size (nm) ± SD		Zeta potential (mV) ± SD		Transmittance (%) ± SD	
					Initial	After 3 Hr.	Initial	After 3 Hr.	Initial	After 3 Hr.
TME 2 (S:CoS ratio 3:1)	**16.66**	**25.02**	**8.33**	**50**	28.5 ± 0.67	32.35 ± 0.63	0.140 ± 0.22	0.348 ± 0.61	99.96 ± 0.78	99.74 ± 0.79
	TME 2 DILUTED BY 10 TIMES WITH AQUEOUS PHASE									
	7.6	11.53	3.84	76.92	33.9 ± 0.33	35.55 ± 0.98	-1.08 ± 0.74	0.22 ± 0.91	99.61 ± 0.58	99.44 ± 0.79
	TME 2 DILUTED BY 100 TIMES WITH AQUEOUS PHASE									
	0.97	1.45	0.48	97.08	41.5 ± 0.12	42.29 ± 0.66	-2.26 ± 0.44	-1.96 ± 0.99	99.51 ± 0.57	99.22 ± 0.35
	TME 2 DILUTED BY 1000 TIMES WITH AQUEOUS PHASE									
	0.099	0.149	0.049	99.70	48.5 ± 0.35	51 ± 0.56	-4.88 ± 0.42	-3.56 ± 0.24	99.21 ± 0.75	99.16 ± 0.29

The results are mean values ± SD derived from three different experimental batches. O is denoted for Oil Phase (Capmul MCM C10®), S for surfactant (Tween - 80®), Co-S for co-surfactant (Transcutol P®) and AQ is denoted for aqueous phase (Distilled Water). The TME formulations contain Tadalafil – 15 mg/mL.

Table 4.2.2.3. Compositions and characterization of Tadalafil microemulsion system 3 (TME 3) as per stomach condition.

System	O (%)	S (%)	CoS (%)	AQ (%)	Globule size (nm) ± SD		Zeta potential (mV) ± SD		Transmittance (%) ± SD	
					Initial	After 3 Hr.	Initial	After 3 Hr.	Initial	After 3 Hr.
TME 3 (S:CoS ratio 3:1)	**15.09**	**28.30**	**9.43**	**47.16**	11.5 ± 0.67	12.1 ± 0.63	-5.88 ± 0.62	-4.56 ± 0.41	99.86 ± 0.48	99.34 ± 0.69
	TME 3 DILUTED BY 10 TIMES WITH AQUEOUS PHASE									
	6.25	11.71	3.90	78.125	12.1 ± 0.67	13.8 ± 0.63	-6.5 ± 0.45	-5.67 ± 0.55	99.56 ± 0.68	99.48 ± 0.59
	TME 3 DILUTED BY 100 TIMES WITH AQUEOUS PHASE									
	0.778	1.45	0.48	97.27	14.8 ± 0.67	15.3 ± 0.63	-7.80 ± 0.78	-7.16 ± 0.32	99.32 ± 0.88	99.11 ± 0.56
	TME 3 DILUTED BY 1000 TIMES WITH AQUEOUS PHASE									
	0.079	0.149	0.049	99.72	21.4 ± 0.67	24.2 ± 0.63	-8.10 ± 0.21	-7.32 ± 0.44	99.08 ± 0.23	98.91 ± 0.33

The results are mean values ± SD derived from three different experimental batches. O is denoted for Oil Phase (Capmul MCM C10®), S for surfactant (Tween - 20®), Co-S for co-surfactant (Transcutol P®) and AQ is denoted for aqueous phase (Distilled Water). The TME formulations contain Tadalafil – 15 mg/mL.

Table 4.2.2.4. Characterization of Tadalafil microemulsions (TME 1, TME 2 and TME 2).

System	O (%)	S (%)	CoS (%)	AQ (%)	pH ± SD	Conductivity (μ semence) ± SD	Viscosity (Cps)± SD
TME 1	12	25	8.33	55.55	4.63 ± 0.35	136.3 ± 0.72	29.2 ± 1.2
TME 2	16.66	25.02	8.33	50	4.58 ± 0.64	119.3 ± 0.56	31.33 ± 0.9
TME 3	**15.09**	**28.30**	**9.43**	**47.16**	**4.52 ± 0.82**	**101.4 ± 0.66**	**28.5 ± 1.4**

4.2.3. Physical Stability

TMEs were evaluated for their physical and chemical stability[4].The prepared ME were subjected to accelerated centrifugation for the assessment of physical phase separation, if any between the oil and aqueous phase. Some of the batches meeting the criteria mentioned below were selected for further studies[5].

Criteria for selection of batches

1. Microemulsions having mean globule size below 100 nm; and
2. Zeta potential at least -5 mV

Microemulsions having least globule size are expected to have larger surface area and therefore, may get absorbed or may transverse rapidly across the intestinal mucosa. Moreover, literature citation revealed that ME which are negatively charged and having zeta potential close to -5 mV or less exhibits moderate to excellent physical stability[1,6]. Therefore, both the selection criteria were used as a filter prior to assessment of accelerated physical stability. These experimental batches are marked in bold face fonts (Table 4.2.1.3.).

Method

The procedure used for measurement of physical stability was similar to that described in section 3.2.3. and the results of globule size following accelerated centrifugation for selected batches of TME are recorded in Table 4.2.3.1.

Table 4.2.3.1. Accelerated physical stability of Tadalafil microemulsions.

System	Ratio of S:CoS	O (%)	S (%)	CoS (%)	AQ (%)	Globule size (nm)		
						Top layer	Middle layer	Bottom layer
TME 1	**3:1**	12	25	8.33	55.55	22.39 ± 0.65	20.12 ± 0.37	19.35 ± 0.67
TME 2	**3:1**	16.66	25.02	8.33	50	28.76 ± 0.52	26.34 ± 0.35	23.67 ± 0.92
TME 3	**3:1**	**15.09**	**28.30**	**9.43**	**47.16**	**11.74 ± 1.45**	**10.26 ± 1.24**	**9.50 ± 1.68**

4.2.4. Chemical Stability (Drug Retention Studies)

Tadalafil microemulsions were subjected to accelerated temperature and stress conditions (http://www.nihs.go.jp/dig/ich/quality/q1e/Q1E). The ME were analyzed for physical and chemical stability. Approximately 10 mL of the formulation was filled in USP type III glass vials and sealed using VP6 crimp on spray pump fitted with 10 µm actuator. Physical stability was assessed using accelerated centrifugation technique as described previously in this chapter (section 4.2.3.)[2].

The stress stability was conducted at 60° C ± 2° C in an incubator. The accelerated stability was performed at 30° C ± 2° C / 65% ± 5% relative humidity (RH) and 40° C ± 2° C / 75% ± 5% RH. The duration of stability was 6 months and samples were withdrawn at predetermined time intervals after 1 month, 2 months, 3 months and 6 months (http://www.nihs.go.jp/dig/ich/quality/q1e/Q1E). The parameters such as physical separation at accelerated gravitational force, active ingredient content, globule size determination, zeta potential measurement, appearance, cracking or physical separation, solidification/ gel formation etc. were assessed. These parameters were evaluated as per the methods described in the section 4.2.2 and 4.2.3. The results for Tadalafil microemulsions drug retention studies are recorded in Table 4.2.4.1. and 4.2.4.2.

Table 4.2.4.1. Accelerated chemical stability of Tadalafil microemulsions at 40°C/75% RH and 30°C/65% RH

System	Ratio of S:CoS	O (%)	S (%)	CoS (%)	AQ (%)	Period (month)	40°C/75% RH				30°C/65% RH			
							Globule size (nm) ± SD	Zeta potential (mV) ± SD	Trans-mittance (%) ± SD	Drug content (%) ± SD	Globule size (nm) ± SD	Zeta potential (mV) ± SD	Trans-mittance (%) ± SD	Drug content (%) ± SD
TME 1	3:1	12.00	25.00	8.33	55.55	0	23.55 ± 0.57	-2.50 ± 0.51	99.56 ± 0.22	99.67 ± 0.35	23.31 ± 0.88	-2.65 ± 0.21	99.88 ± 0.22	99.91 ± 0.55
						1	24.56 ± 0.68	-2.36 ± 0.69	99.63 ± 0.75	99.54 ± 0.44	25.55 ± 0.20	-2.82 ± 0.87	99.33 ± 0.48	99.31 ± 0.69
						2	23.88 ± 0.53	-2.51 ± 0.55	99.51 ± 0.48	99.17 ± 0.50	24.15 ± 0.22	-3.41 ± 0.77	99.32 ± 0.84	99.02 ± 0.33
						3	24.27 ± 0.56	-2.85 ± 0.91	99.15 ± 0.31	99.01 ± 0.34	24.85 ± 0.56	-2.92 ± 0.22	99.88 ± 0.58	98.85 ± 0.72
						6	25.36 ± 0.72	-3.15 ± 0.33	99.04 ± 0.23	98.66 ± 0.82	25.18 ± 0.62	-2.66 ± 0.72	99.18 ± 0.39	98.62± 0.25
TME 2	3:1	18.66	25.02	8.33	50.00	0	28.01 ± 0.45	0.126 ± 0.27	99.22 ± 0.25	99.49 ± 0.33	29.56 ± 0.27	0.26 ± 0.17	99.42 ± 0.66	99.59 ± 0.74
						1	29.46 ± 0.33	-0.112 ± 0.18	99.06 ± 0.64	99.09 ± 0.66	28.88 ± 0.72	0.180 ± 0.67	99.36 ± 0.44	99.15 ± 0.48
						2	29.84 ± 0.56	0.197 ± 0.58	98.90 ± 0.54	98.87 ± 0.54	28.68 ± 0.54	0.219 ± 0.68	99.24 ± 0.53	98.79 ± 0.84
						3	28.55 ± 0.83	-0.33 ± 0.66	99.05 ± 0.83	98.58 ± 0.97	30.11 ± 0.67	-0.17 ± 0.66	99.57 ± 0.51	98.36 ± 0.49
						6	29.92 ± 0.53	0.13 ± 0.94	98.99 ± 0.88	98.26 ± 0.58	29.06 ± 0.74	-0.253 ± 0.63	99.19 ± 0.39	98.06 ± 0.67

Table 4.2.4.2. Accelerated chemical stability of Tadalafil microemulsions at 40°C/75% RH and 30°C/65% RH

System	Ratio of S:CoS	O (%)	S (%)	CoS (%)	AQ (%)	Period (month)	40°C/75% RH				30°C/65% RH			
							Globule size (nm) ± SD	Zeta potential (mV) ± SD	Trans-mittance (%) ± SD	Drug content (%) ± SD	Globule size (nm) ± SD	Zeta potential (mV) ± SD	Trans-mittance (%) ± SD	Drug content (%) ± SD
TME 3	3:1	15.09	28.30	9.43	47.16	0	11.73 ± 0.56	-5.65 ± 0.21	99.88 ± 0.82	99.59 ± 0.53	13.79 ± 0.90	-6.05 ± 0.21	99.91 ± 0.22	99.65 ± 0.895
						1	12.06 ± 0.74	-6.87 ± 0.71	99.53 ± 0.59	99.28 ± 0.84	12.85 ± 0.58	-5.22 ± 0.87	99.56 ± 0.46	99.45 ± 0.73
						2	12.42 ± 0.62	-5.91 ± 0.55	99.37 ± 0.79	99.07 ± 0.50	14.35 ± 0.52	-6.84 ± 0.67	99.32 ± 0.74	99.12 ± 0.33
						3	14.87 ± 0.88	-6.85 ± 0.156	99.08 ± 0.71	98.92 ± 0.61	14.85 ± 0.41	-6.72 ± 0.32	99.69 ± 0.89	98.85 ± 0.80
						6	15.28 ± 0.38	-5.98 ± 0.84	99.04 ± 0.38	98.56 ± 0.86	14.18 ± 0.42	-5.66 ± 0.82	99.26 ± 0.53	98.32± 0.55

4.2.5. Drug Diffusion study

In Vitro drug diffusion study was carried by using two different methods.

1. Dialysis bag study.
2. Intestinal permeability study.

In this investigation, all the test formulations were assessed for *in vitro* diffusion across the dialysis technique and *in vitro* permeation across Male Sprague Dawley rat's duodenum in triplicate and the physicochemical parameters were calculated as mentioned below[7].

(A) Percent Drug Diffused

The percent drug diffused across the sheep nasal mucosa at predetermined sampling time interval was determined using formula mentioned below.

$$\%\,Drug\ Diffused = \frac{c_r v_r}{c_d v_d} \times 100$$

Where, Cr = Concentration of the drug in receptor compartment

Vr = Volume of the receptor compartment

Cd = Initial concentration of the drug in donor compartment

Vd = Initial volume in the donor compartment

(B) Kinetics of Release

In order to investigate the mechanism of drug release from the formulation, the release rates were integrated into each of the following equation and the regression coefficient was investigated from each of the regressed graph.

Zero-order equation:

$$Q = K_0 t$$

Where, Q = Amount of drug released at time t

t = Time in hours

K_0 = Zero-order release rate constant

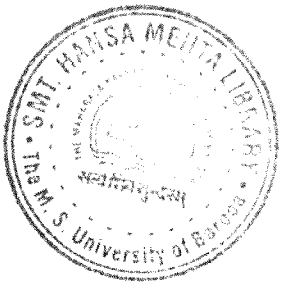
First-order equation:

$$Q = Q_0 e^{-K_1 t}$$

Where, Q = Amount of drug released at time t

t = Time in hours

K_1 = First-order release rate constant



Higuchi's equation:

$$Q = K_H \times \sqrt{t}$$

Where, Q = Amount of drug released at time t

t = Time in hours

K_H = Higuchi's diffusion rate constant

The order of drug release was determined by performing the regression over the mean values of percent drug diffusion vs. time (for Zero-order), log percent drug diffusion vs. time (for First-order) and percent drug diffusion vs. square root of time (for Higuchi).

4.2.5.1. Experimental design.

4.2.5.1.1 Dialysis Bag Technique[8,9].

In vitro diffusion study was performed for all prepared ME of Tadalafil by using the dialysis bag technique. Experiments were carried out by using a cellulose dialysis bag (7 cm in length), having a circular tubing shape with both open ends as shown in Figure 3.2.5.1.(a). Dialysis bag was soaked for over night into a phosphate buffer pH 6.8 for saturation purpose and then it was used for further experimental study. The dialysis medium was 30 mL of Phosphate buffer pH 6.8.One end of the prepared dialysis bag was tied with thread, and then 1 mL of concentrated ME (2 mg of drug)was placed into it along with 0.5 mL of diffusion medium. The other end of the dialysis bag was also secured with thread and was allow to rotate freely in 30 mL of dialysis medium and stirred continuously for 100 rpm with magnetic bead on magnet plate at 37 ^{o}C(Figure 3.2.5.1.(b)). Aliquots of 0.250 mL were withdrawn at different time interval and volume of aliquots replaced with fresh dialysis medium each time. The samples were analyzed quantitatively for Tadalafil dialyzed across the membrane at corresponding time by using UV-Visible spectrophotometer (Shimadzu UV 1601, Japan) as mentioned in Chapter 4.1, Section 4.1.1.4.6.The experiments were run in triplicate and the mean cumulative % drug diffused along with SD of Pure Tadalafil, Marketed formulation (Tadora-20) and prepared MEs are shown in Table 4.2.5.2 and Table 4.2.5.3.There are represented graphically in Figure 4.2.5.2.(a). The release kinetics of diffusion was studied by calculating the regression coefficient for zero order, first order, and Higuchi's equations. The regression coefficients for the different formulations of Tadalafil are recorded in Table 4.2.5.4.

4.2.5.1.2 Intestinal Permeability Study

The experimental procedure described by P. smith[10] and P. K. Ghish[11] was modified for permeation study. Male Sprague Dawley rats (250 – 300 gm) were killed by over dose with pentobarbitone administrated by intravenous injection. Our basic aim was to check the intestinal permeability of the drug, orally administered as ME base. To check the intraduodenal permeability, the duodenal part of the small intestine was isolated and used for *in vitro* intestinal study. Separated duodenal part was washed with cold ringer solution to remove mucous and lumen contents and one end of the duodenum was tied with thread. Prepared concentrated ME of Tadalafil was diluted outside with 1 mL of phosphate buffer pH 6.8 for 5 minute by vortex mixture. A suspension of marketed formulation (tablet, Tadora-20) was formed by using 1 mL of phosphate buffer pH 6.8. The resultant solution (2mg/mL) was injected into the lumen of the duodenum using a syringe and another side of the lumen was tightly closed with the thread. The tissue was placed in a chamber of organ bath with continuous aeration and a constant temperature of 37 $^{\circ}$ C. The receiver compartment was filled with 30 mL of Phosphate buffer pH 6.8. Aliquots of 0.250 mL were withdrawn at different time intervals and volume of aliquots replaced with fresh dialysis medium each time. The samples were analyzed quantitatively for Tadalafil and Tadalafil dialyzed across the membrane at corresponding time by using UV-Visible spectrometric method as mentioned in Chapter 4.1, Section 4.1.1.4.6. The experiments were run in triplicate and the mean cumulative % drug diffused along with SD of Tadalafil, Marketed formulation (Tadora-20) and prepared MEs are shown in Table 4.2.5.2. and Table 4.2.5.3.and graphically it is represented in Figure 4.2.5.2(b)The release kinetics of diffusion was studied by calculating the regression coefficient for zero order, first order, and Higuchi's equations. The regression coefficients for the different formulations of Tadalafil and Tadalafil are recorded in Table 4.2.5.5.[12,13].

Table 4.2.5.1. Promising compositions of Tadalafil microemulsions for *in vitro* diffusion study :

System	Ratios of S : CoS	O %	S %	CoS %	AQ %
TME 1	3 : 1	12	25	8.33	55.55
TME 2	3 : 1	16.66	25.02	8.33	50
TME 3	3 : 1	15.09	28.30	9.43	47.16

Table 4.2.5.2. Cumulative % drug diffused for Pure Tadalafil and its marketed formulation(Tadora-20) at different time intervals.

Time (h)	Cumulative % drug diffused[#] from System (Formulation)			
	Pure Tadalafil		Tadora – 20 (Tablet)	
	Dialysis bag Technique	Intestinal Permeability Study	Dialysis bag Technique	Intestinal Permeability Study
0.15	0.98 ± 0.10	1.35 ± 0.21	3.51 ± 0.73	4.47 ± 0.44
0.30	1.88 ± 0.32	1.51 ±0.62	4.85 ± 0.84	4.7 ± 0.91
0.45	3.11 ± 0.59	1.564 ± 0.33	5.11 ± 0.59	5.32 ± 0.43
1	4.47 ± 0.50	1.715 ± 0.78	6.26 ± 0.26	6.65 ± 0.56
2	5.72 ± 0.88	2.225 ± 0.81	7.79 ± 0.87	7.554 ± 0.55
3	6.99 ± 1.01	2.31 ± 0.77	8.86 ± 0.93	8.625 ± 0.63
4	7.54 ± 1.23	3.495 ± 0.91	12.03 ± 0.37	11.375 ± 0.82
5	8.62 ± 0.99	5.05 ± 0.82	15.21 ± 1.01	13.69 ± 1.11
6	9.855 ± 1.55	7.83 ± 0.74	18.66 ± 0.96	17.88 ± 0.99
7	11.375 ± 1.86	9.7 ± 0.51	21.43 ± 0.88	19.61 ± 0.73
8	13.695 ± 1.46	12.21 ± 0.88	23.76 ± 0.43	21.38 ± 1.36

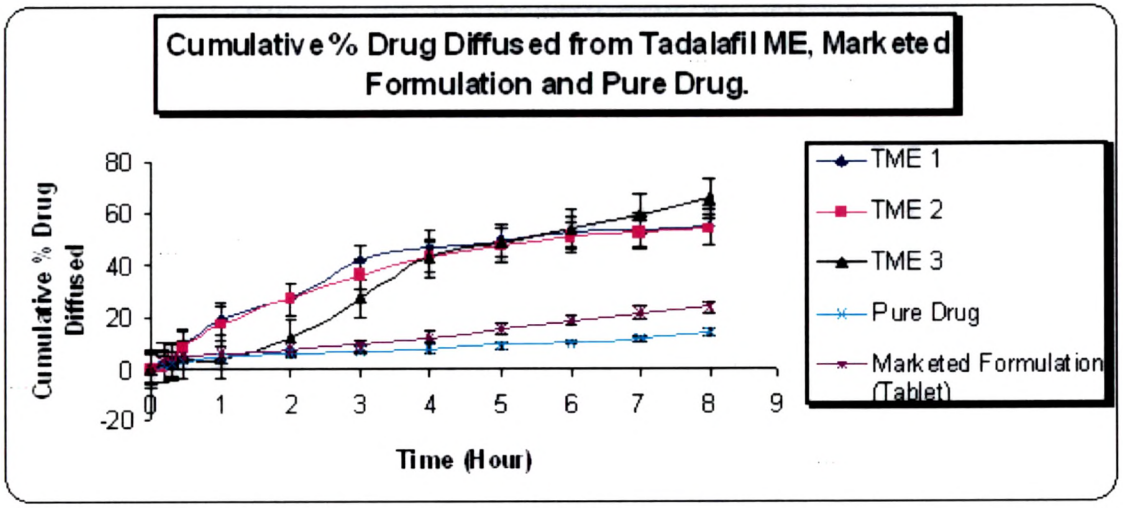
[#] All values are represented as mean ± SD (±n=3).

Table 4.2.5.3. Cumulative % drug diffused for different Tadalafil microemulsions at different time intervals.

Time (h)	Cumulative % drug diffused[#] from Systems (Formulation)					
	TME 1		TME 2		TME 3	
	Dialysis bag Technique	Intestinal Permeability Study	Dialysis bag Technique	Intestinal Permeability Study	Dialysis bag Technique	Intestinal Permeability Study
0.15	0.68 ± 0.17	1.23 ± 0.17	0.58 ± 0.40	0.52 ± 0.31	**2.29 ± 0.58**	**2.9 ± 0.25**
0.30	2.61 ± 0.47	2.81 ± 0.44	2.79 ± 0.68	2.12 ± 0.57	**2.89 ± 0.92**	**3.61 ± 0.75**
0.45	8.49 ± 0.84	8.46 ± 0.85	8.42 ± 1.08	5.87 ± 0.98	**3.61 ± 0.36**	**6.65 ± 0.46**
1	19.33 ± 0.18	18.85 ± 0.78	17.35 ± 0.57	16.23 ± 1.33	**3.75 ± 0.66**	**14.27 ± 0.63**
2	27.34 ± 1.51	25.64 ± 1.41	27.33 ± 1.12	20.21 ± 1.51	**11.87 ± 0.72**	**27.36 ± 1.12**
3	41.67 ± 1.87	36.28 ± 1.27	36.68 ± 1.58	28.30 ± 1.47	**27.36 ± 1.31**	**43.07 ± 1.22**
4	46.79 ± 1.28	41.59 ± 1.58	43.30 ± 1.28	37.23 ± 1.68	**43.07 ± 1.22**	**48.84 ± 1.46**
5	49.82 ± 1.34	46.92 ± 1.14	47.60 ± 1.28	41.56 ± 1.36	**48.84 ± 1.45**	**54.28 ± 1.55**
6	52.36 ± 1.19	50.31 ± 1.69	50.79 ± 1.23	44.38 ± 1.55	**54.28 ± 1.78**	**58.6 ± 1.11**
7	53.56± 1.51	53.29 ± 1.33	52.34 ± 1.27	47.04 ± 1.83	**59.60 ± 1.52**	**61.95 ± 1.57**
8	54.89 ± 1.23	56.55 ± 1.75	53.60 ± 1.55	49.76 ± 1.69	**65.95 ± 1.63**	**63.38 ± 1.39**

[#] All values are represented as mean ± SD (n=3).

Figure – 4.2.5.2 Cumulative % drug diffused from Tadalafil ME, Marketed Formulation (Tadora-20) and Pure Drug at different time intervals. Error bars represent SD (n=3). (a) Cumulative % Drug Diffused by Dialysis Bag Study.



(b) Cumulative % Drug Diffused by Intestinal Permeability Study.

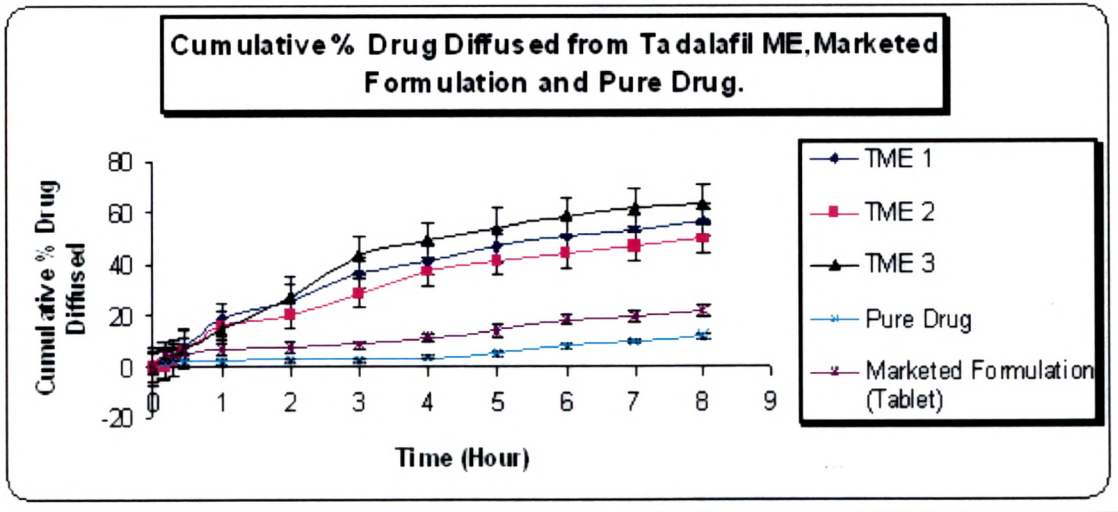


Table 4.2.5.4. Regression coefficients of different Tadalafil microemulsions (TME 1, TME 2, TME 3), marketed formulation (Tadora-20), Pure drug derived using regressed graphs by Dialysis Bag Method.

System (Formulation)	Zero-order equation	First-order equation	Higuchi's equation
	r^2	r^2	r^2
TME 1	0.8825	0.6267	**0.9663**
TME 2	0.907	0.6292	**0.9783**
TME 3	0.9288	0.8628	**0.9765**
TADORA-20	0.9311	0.7937	**0.9743**
Pure Drug	0.9551	0.7711	**0.9737**

Table 4.2.5.5. Regression coefficients of different Tadalafil microemulsions (TME 1, TME 2, TME 3), marketed formulation (Tadora-20), Pure drug derived using regressed graphs by Intestinal Permeability Study.

System (Formulation)	Zero-order equation	First-order equation	Higuchi's equation
	r^2	r^2	r^2
TME 1	0.9297	0.6648	**0.9858**
TME 2	0.9417	0.6645	**0.982**
TME 3	0.9326	0.7227	**0.9766**
TADORA-20	0.9298	0.7395	**0.9616**
Pure Drug	0.9082	0.7397	**0.9792**

4.2.6. Results and Discussion

Tadalafil microemulsions (TME 1, TME 2 and TME 3) were successfully prepared using titration technique and the ME regions are plotted using pseudo-ternary phase diagrams[14]. As per the solubility data shown in Table 4.2.1.1, the solubility of drug in cotton seed oil, peanut oil and Labrafac PG was less than 3 mg/mL but showed maximum solubility in Capmul MCM C8® and Capmul MCM C10 (> 10 mg/mL), hence it was selected to formulate MEs. As oral formulations should be dose dependent and compatible with stomach condition (acidic pH as well as excess amount of aqueous phase (> 1000 ml)), the ME base was selected on the merits of stability of micells when concentrated ME was contacted with excess of aqueous phase as well as solubilization capacity of drug. The selection of surfactant and co-surfactant mixture was on the basis of HLB values.

To screen out a drug vehicle suitable for oral delivery of Tadalafil, three different TME systems (TME 1, TME 2, and TME 3) were prepared wherein system 1 comprises of Capmul MCM C 8® as oil phase, Tween 20 as surfactant, Transcutol P® as co-surfactant and distilled water as an aqueous phase. System 2 and system 3 were prepared using Capmul MCM C 10 ® an oil phase respectively, Tween 80 and Tween 20® as surfactant, Transcutol P® as a co-surfactant and distilled water as an aqueous phase. All the MEs (TME 1, TME 2, and TME 3) were formulated at different S:CoS ratios: 1:1, 2:1 and 3:1 and phase studies were done to investigate the effect of S:CoS ratio on the existence ranges of stable o/w ME region. The transparent ME area is presented in the phase diagrams as shaded region. No distinct conversion from w/o to o/w ME was seen; therefore, this single isotropic region is considered as a bicontinuous ME. The rest of the region on the phase diagram represented the viscous gel area or turbid and conventional emulsions based on visual identification. The pseudo-ternary phase diagrams of TME 1(three formulations) TME 2 (three formulations) and TME 3(three formulations) are displayed in Figures. 4.2.1.1., 4.2.1.2. and 4.2.1.3. respectively.

It can be concluded from the phase diagrams, 3:1 S:CoS ratios of TME 1, TME 2 and TME 3 showed maximum microemulsion region and their compositions are shown in Table 4.2.6.1. The data obtained from the phase diagram study suggested that, increased ME region was towards the oil–water axis, there by indicating at optimum surfactant concentration, the amount of water and oil that could be solubilized into the ME is increased[15,16].

Table 4.2.6.1: Excipients and its composition for the preparation of TME 1,TME 2 and TME 3.

System	Oil (%)	Surfactant (%)	Co-surfactant (%)	Aqueous phase (%)	Amount of Tadalafil (mg/mL)
TME 1 (S: CoS ratio 3:1)	Capmul MCM C8 (12 %)	Tween 20 (25.00 %)	Transcutol P (8.33 %)	Water (55.55 %)	15
TME 2 (S: CoS ratio 3:1)	Capmul MCM C10 (16.66 %)	Tween 80 (25.02 %)	Transcutol P (8.33 %)	Water (50.00%)	15
TME 3 (S: CoS ratio 3:1)	Capmul MCM C10 (15.09 %)	Tween 20 (28.30 %)	Transcutol P (9.43 %)	Water (47.16 %)	15

The optimized formulations of TME 1,TME 2 and TME 3 were further selected for characterization studies like, globule size, zeta potential and % Transmittance and the results along with ± SD are mentioned in Table 4.2.6.2.

Table 4.2.6.2. Characterization of Tadalafil microemulsion systems (TME 1, TME 2 and TME 3).

Formulations	S:CoS ratio	Globule size (nm) ± SD		Zeta potential (mV) ± SD		% Transmittance ± SD	
		Initial	After 3 Hr.	Initial	After 3 Hr.	Initial	After 3 Hr.
TME 1	3:1	22.1 ± 0.27	28.2 ± 0.35	-2.61 ± 0.51	-2.10 ± 0.42	99.55 ± 0.55	99.34 ± 0.59
TME 2	3:1	28.5 ± 0.67	32.35 ± 0.63	0.140 ± 0.22	0.348 ± 0.61	99.96 ± 0.78	99.74 ± 0.79
TME 3	3:1	11.5 ± 0.67	12.1 ± 0.63	-5.88 ± 0.62	-4.56 ± 0.41	99.86 ± 0.48	99.34 ± 0.69

From the globule size analysis of different Tadalafil formulations with varying concentrations of S:CoS mixture, it was observed that increase in the S:CoS mixture concentrations results in the decrease in the globule size. This may be due to increase in the

concentration of co-surfactant added into it. Co-surfactant can reduce the interfacial tension and provide a large interface between oil and water because of the small droplet size; they can only be thermodynamically stable if the interfacial tension is so low. The globule size and zeta potential were fairly reproducible within ± 5 nm / ± 1 mV range respectively for initially and after 3 Hr. It was also found that increase in the total concentration of S:CoS (up to medium range), leads to increase in absolute zeta potential. This may be due to increase in the concentration of co-surfactant resulting into formation of bicontinuous or o/w system due to higher HLB value hence, the negative charge of the system also increases[17].

The pH of the prepared ME systems were similar to the pH of water which proved the ME systems were stable at stomach condition. Viscosity of TME 1, TME 2 and TME 3 were found to be 29.2, 31.33 and 28.5 cps which were suitable for the preparation of oral liquid formulation. Electroconductivity of the prepared MEs (TME 1, TME 2 and TME 3) were found to be 136.3, 119.3 and 101.4μsemence. From the above results of viscosity and electroconductivity it can be concluded that the ME systems were purely o/w type.

Concentrated TME 1, TME 2 and TME 3 were further diluted with 10 times, 100 times and 1000 times of aqueous phase and the results are recorded in Table 4.2.2.1., 4.2.2.2. and 4.2.2.3. After each dilution, the globule size was increased TME 1 (63.33 ± 0.72 nm), TME 2 (51.00 ± 0.56 nm) and TME 3 (24.2 ± 0.63 nm) because of decrease in the interface between oil and water phase by adding excess of water. Zeta potential was reduced up to -8.1 mV because with increase in anionic phase, the negative charge was increased into the MEs. Diluted MEs showed decrease in the % Transmittance (between 98.66 to 99.08 %) with increase in the globule size and they have been further evaluated for physical and chemical stability.

TME 1, TME 2 and TME 3 were subjected to accelerated centrifugation for assessment of physical stability study and the results are shown in Table 4.2.3.1..The data revealed that there was no appreciable change before and after centrifugation for 15 min at accelerated conditions. Moreover, the layers from top, middle and bottom following centrifugation were sampled and analyzed to determine homogeneity. The globule size of the TME 1, TME 2 and TME 3 in top, middle and bottom layer were found to be within ± 5 nm from the initial values. The data clearly suggested that TME 1, TME 2 and TME 3 were physically stable under the testing conditions. The MEs were selected on the basis of globule size. All the batches of MEs were having globule size less than 30 nm and zeta potential close to -4 mV or less.

Drug retention study was performed on physically stable TME 1, TME 2 and TME 3 by subjecting them at 30°C / 65% RH and 40°C / 75% RH. The samples were withdrawn at the period of 1, 2, 4 and 6 months respectively and were subjected to globule size, size distribution, zeta potential, percent transmittance and drug content. The data was recorded in Table 4.2.4.1. and Table 4.2.4.2.. As seen from the table, globule size for all TME 1, TME 2 and TME 3 were within the range of ± 5 nm from the initial values and no abnormal changes in the globule size were noticed at both the accelerated testing conditions. The zeta potential values were also found to be consistent and within the range ± 5 mV from the initial values. The data clearly indicated that the formulations were physically stable at 30°C / 65% RH and 40°C / 75% RH without noticeable change in the zeta potential values. Percent transmittances for all the experimental batches were found to be greater than 99% which indicated the clarity of the tested ME and indirectly gives an indication that no separation was observed in the Tadalafil microemulsions. Drug content for different TME formulations were found to be more than 95 %. It was concluded form the above data that the formulations were found to meet the general monograph of Pharmacopoeia and criteria stipulated therein for the liquid preparations. Physically and chemically stable TME 1, TME 2 and TME 3 were further taken up for the *in vitro* diffusion studies to evaluate the potential.

Diffusion kinetics of Tadalafil formulations (Table 4.2.5.1) was studied and cumulative drug diffused up to 8 h across the diffusion bag and intestinal mucosa is recorded in Table 4.2.5.2 and 4.2.5.3..These are graphically shown in Figure 4.2.5.2. It is evident from the data, that TME 3 showed better drug diffusion across the dialysis bag (65.95%) and intestinal mucosa (63.38%). The mechanism of drug diffusion was also predicted by inputting the regressed data into the excel spread sheet and the results are recorded in Table 4.2.5.2 and Table 4.2.5.3. It was found that all the tested formulations of Tadalafil follow Higuchi's kinetics. The results (Table 4.2.5.2 and Table 4.2.5.3.) showed that TME 3 has 2.77-fold higher diffusion compared to marketed formulation (Tadora - 20) and 4.81-fold higher diffusion compared to Tadalafil solution (Figure 4.2.5.2.) by dialysis bag study, 3.00 fold higher diffusion compared to marketed formulation (Tadora - 20) and 5.19-fold higher diffusion compared to Tadalafil solution (Figure 4.2.5.2.) by intestinal permeability study. This may be attributed to the fact that microemulsion enhances transport of drug across mucosa.

In conclusion, *in vitro* diffusion study across the dialysis bag and intestinal mucosa may be a reasonable tool for comparative evaluation of different formulations. The non linearity of percent drug diffused vs. time graphs suggested that the diffusion pattern does not

follow zero order kinetics[19]. However, the correlation coefficients indicated that Higuchi's model was found to be the best-fit curve for all the tested formulations. This may be attributed to the fact that the systems tested has reservoir compartment, dialysis bag and intestinal mucosa as a barrier or controlling membrane hence, the drug diffusion will more mimic and closer to reservoir system rather than zero-order or first-order (concentration gradient) diffusion[20].

It was observed from the results of characterization and evaluation for TME 1, TME 2 and TME 3, TME 3 showed less globule size and low zeta potential as compared to TME 1 and TME 2. Its viscosity, pH, % Transmittance and Conductivity data were found to be suitable for oral delivery of Tadalafil. In vitro diffusion study supported the fact that TME 3(3:1 S: CoS ratio) is more convincing formulation for oral drug delivery then all tested formulations and hence, it was selected for in vivo pharmacokinetic study.

4.2.7. References

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4.3.1. Preparation of Inclusion Complexes :

4.3.1.1. Material and Reagents :

Tadalafil was generously provided by Macloid Pharmaceutical Laboratory, Daman as a gift sample. **The other materials and reagents are described in section 3.3.1.1.**

4.3.1.2. Preparation of Tadalafil Inclusion Complexes :

Tadalafil-CDs inclusion complex were prepared in 1:1, 1:2 and 1:3 molar ratios by using two different method (1) Kneading Method and (2) Co-precipitation method and compared with physical mixtures of Tadalafil-CDs[5].

(1) Physical Mixture :

The method used to prepare physical mixtures was similar to that described in section 3.3.1.2.

(2) Kneading Method :

The method used to prepare inclusion complexes was similar to that described in section 3.3.1.2.

(3) Co – precipitation method :

The method was used to prepare inclusion complexes was similar to that described in section 3.3.1.2.

4.3.2. Characterization of prepared Tadalafil-Cylodextrins Inclusion Complexes

Prepared CDs inclusion complexes of Tadalafil were further characterized by Phase solubility Study, Inclusion efficiency study, FT-IR Spectroscopy study, Differential scanning calorimetry study and X-ray powder diffraction study.

4.3.2.1. Phase solubility study :

The phase solubility of Tadalafil was conducted according to Higuchi and Connors[1].An excess amount of Tadalafil (50 mg) was added to 5 mL of water or aqueous solutions of CDs and its derivatives (10 – 50 mM/L) individually in 10 mL stoppered glass tubes, and the tubes were shaken for 24 h at 50 cycle/min in a water bath at 37 ± 0.5° C. At equilibrium after 2 days, aliquots were withdrawn, filtered (0.45- µm cellulose nitrate filtres) and suitably diluted. Concentration of Tadalafil and Tadalafil were determined spectrophotometrically. The phase solubility study was further carried out in HCl buffer pH1.2 and Phosphate buffer pH 6.8.

A plot of total molor concentration of the drug against the total molar concentration of CDs gave phase-solubility diagrams from where the apparent solubility constant, K_C were calculated for all the pH values using their regression lines to the following equation.

$$\text{Stability constant } (K_C) = \frac{Slope}{S_o(1 - Slope)}$$

Where S_0 is the intrinsic solubility of the drug studied under the conditions[2,3,4].

4.3.2.2. Inclusion efficiency study :

All inclusion complexes of Tadalafil and their physical mixtures (25 mg) were taken in 25 ml volumetric flasks. 10 mL of methanol was added to it, mixed thoroughly and sonicated it for 30 min at ambient temperature. The volume was made up to mark with methanol. The solution was suitably diluted with methanol to get the concentration of 10µg of drug per ml of solution and spectrophotometrically assayed for drug content at 284.5 nm. Inclusion efficiency was calculated using the formula :-

Inclusion efficiency = (estimated % drug content /theoretical % drug content) x 100

4.3.2.3. FTIR spectroscopy study :

The procedure was similar to that described in section 3.3.2.3.

4.3.2.4. Differential scanning calorimetry :

The procedure was similar to that described in section 3.3.2.4.

4.3.2.5. X-ray powder diffraction study :

The procedure was similar to that described in section 3.3.2.5.

4.3.2.1. Phase solubility diagrams of Tadalafil (Each point represent the mean of 5 experiments).

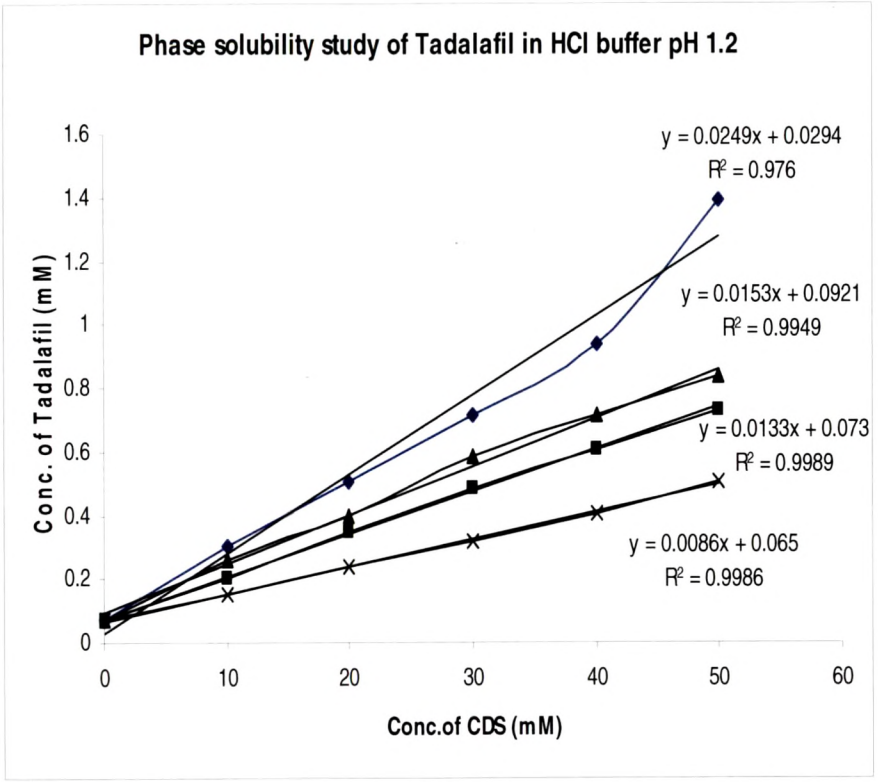

Figure 4.3.2.1.1. Phase solubility study of Tadalafil in 1.2 pH HCl buffer at 284.5 nm. Key (♦) β-CD; (▲) HP-β-CD;(■) γ-CD; (×)DM-β-CD.

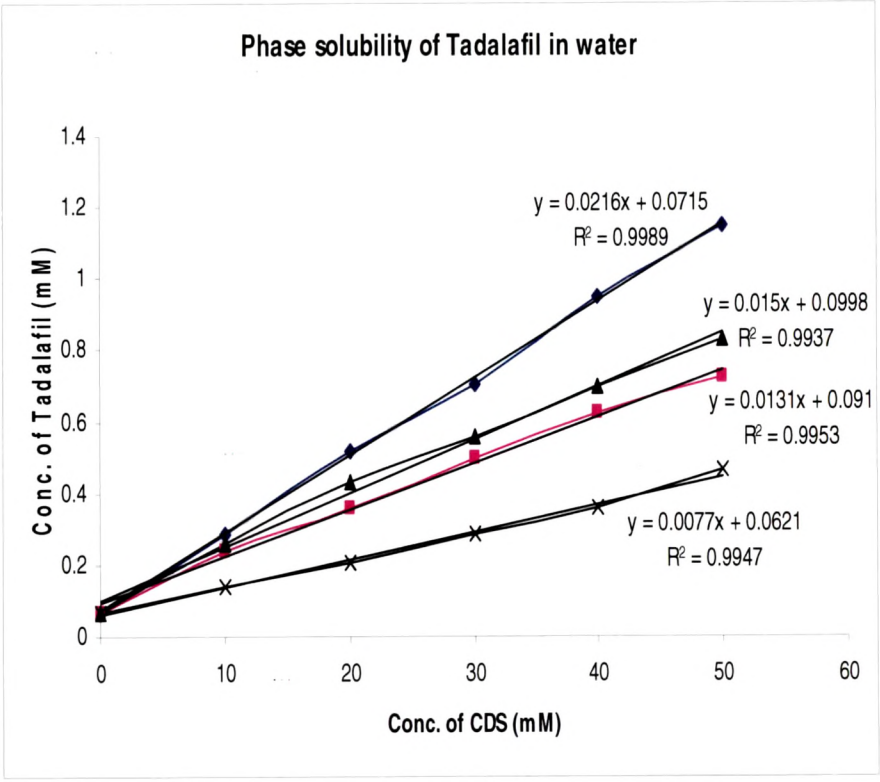

Figure 4.3.2.1.2. Phase solubility study of Tadalafil in Water at 284.5 nm. Key (♦) β-CD;(▲) HP-β-CD;(■) γ-CD; (×)DM-β-CD.

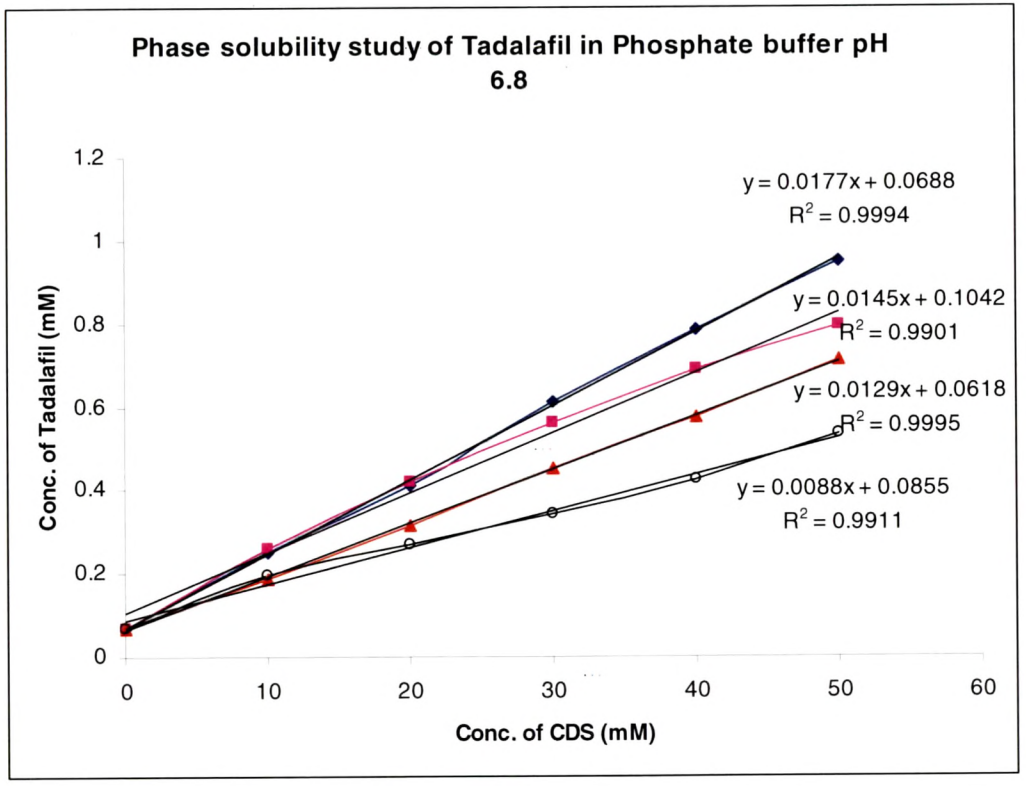

Figure 4.3.2.1.3. Phase solubility study of Tadalafil in Phosphate buffer pH 6.8 at 284.5 nm. Key (♦) β-CD ;(▲) HP-β-CD ;(■) γ-CD; (O)DM-β-CD.

Table 4.3.2.1.1. Apparent Inclusion complex Stability constant (Kc) of Tadalafil with different CDs at 37 ° C and types of phase solubility curves for Tadalafil with various CDs at different pH solutions.

CDs	Tadalafil			
	Solutions	K 1:1 [M^{-1}] ± SD	R^2	Type of Curve
B-CD	HCl buffer pH 1.2	**351.88 ± 21**	0.976	A_P
	Water	306.28 ± 17	0.9989	A_L
	Phosphate buffer pH 6.8	251.89 ± 11	0.9994	A_L
γ-CD	HCl buffer pH 1.2	**190.19** ± 13	0.9989	A_L
	Water	187.36 ± 15	0.9953	A_L
	Phosphate buffer pH 6.8	184.54 ± 7	0.9995	A_L
HP-β-CD	HCl buffer pH 1.2	**218.34** ± 10	0.9949	A_L
	Water	214.13 ± 14	0.9937	A_L
	Phosphate buffer pH 6.8	207.09 ± 12	0.9901	A_L
DM-β-CD	HCl buffer pH 1.2	**126.41** ± 15	0.9911	A_L
	Water	123.56 ± 19	0.9986	A_L
	Phosphate buffer pH 6.8	110.73 ± 16	0.9947	A_L

4.3.2.2. Inclusion efficiency study.

Table 4.3.2.2.1. Inclusion efficiency data for Tadalafil-β CD Inclusion complex.

Tadalafil-β-CD (w/w)	Inclusion efficiency ± SD#	%RSD#
1:1	69.45 ± 1.17	1.17.
1:2	74.70 ± 0.62	0.63
1:3	**89.10 ± 1.64**	**1.65**
1:1*	78.53 ± 0.46	0.47
1:2*	91.48 ± 0.70	0.71
1:3*	98.98 ± 0.35	0.36

mean of three determinations *physical mixture

Table 4.3.2.2.2. Inclusion efficiency data for Tadalafil – γ-CD Inclusion complex.

Tadalafil-γ-CD (w/w)	Inclusion efficiency ± SD#	%RSD#
1:1	60.20 ± 0.11	1.39
1:2	71.16 ± 1.63	0.33
1:3	**83.89 ± 0.33**	**0.67**
1:1*	76.46 ± 1.19	0.46
1:2*	84.89 ± 1.45	0.81
1:3*	96.22 ± 0.82	1.01

mean of three determinations *physical mixture

Table 4.3.2.2.3. Inclusion efficiency data for Tadalafil–HP–β-CD Inclusion complex.

Tadalafil-HP-β-CD (w/w)	Inclusion efficiency ± SD#	%RSD#
1:1	68.0 ± 1.69	1.72
1:2	79.2 ± 1.91	1.97
1:3	**88.1 ± 1.48**	**1.51**
1:1*	77.7 ± 1.95	2.00
1:2*	85.0 ± 1.93	1.83
1:3*	97.4 ± 1.78	1.81

mean of three determinations *physical mixture

Table 4.3.2.2.4. Inclusion efficiency data for Tadalafil–DM-β-CD Inclusion complex.

Tadalafil-DM-β-CD (w/w)	Inclusion efficiency ± SD#	%RSD#
1:1	55.87 ± 1.49	
1:2	69.10 ± 0.89	1.73
1:3	**78.82 ± 0.79**	**0.35**
1:1*	81.20 ± 0.66	1.25
1:2*	90.69 ± 0.92	1.52
1:3*	98.19 ± 1.11	0.66

mean of three determinations *physical mixture

4.3.2.3. IR Spectrum of CDs, Physical mixtures and Inclusion complexes of Tadalafil.

Figure 4.3.2.3.1. IR Spectrum of Pure β-CD.

IR spectrum of pure β-CD was shown in 3.3.2.3.1.

Figure 4.3.2.3.2. IR Spectrum of Pure γ-CD.

IR spectrum of pure γ-CD was shown in 3.3.2.3.2.

Figure 4.3.2.3.3. IR Spectrum of Pure HP- β-CD.

IR spectrum of pure HP-β-CD was shown in 3.3.2.3.3.

Figure 4.3.2.3.4. IR Spectrum of Pure DM-β-CD.

IR spectrum of pure DM-β-CD was shown in 3.3.2.3.4.

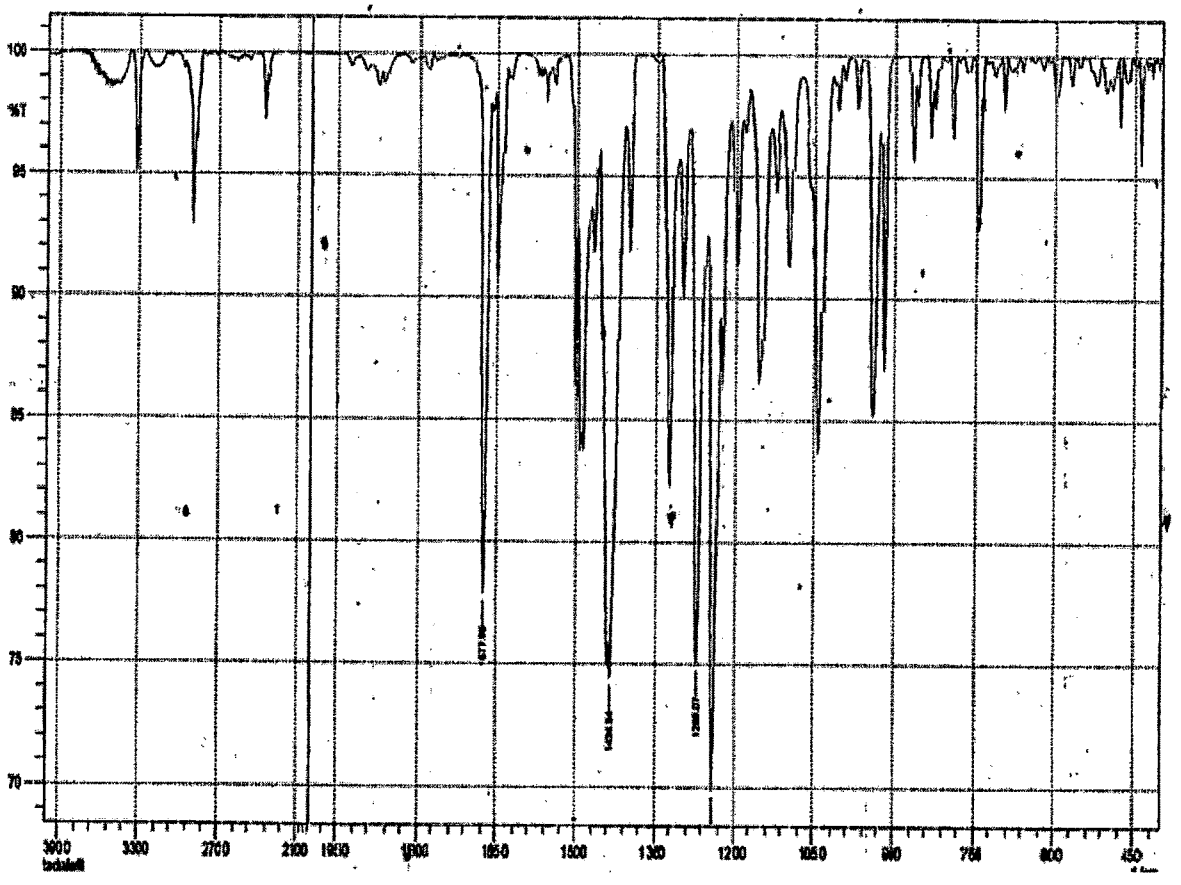

Figure 4.3.2.3.5. IR Spectrum of Pure Tadalafil.

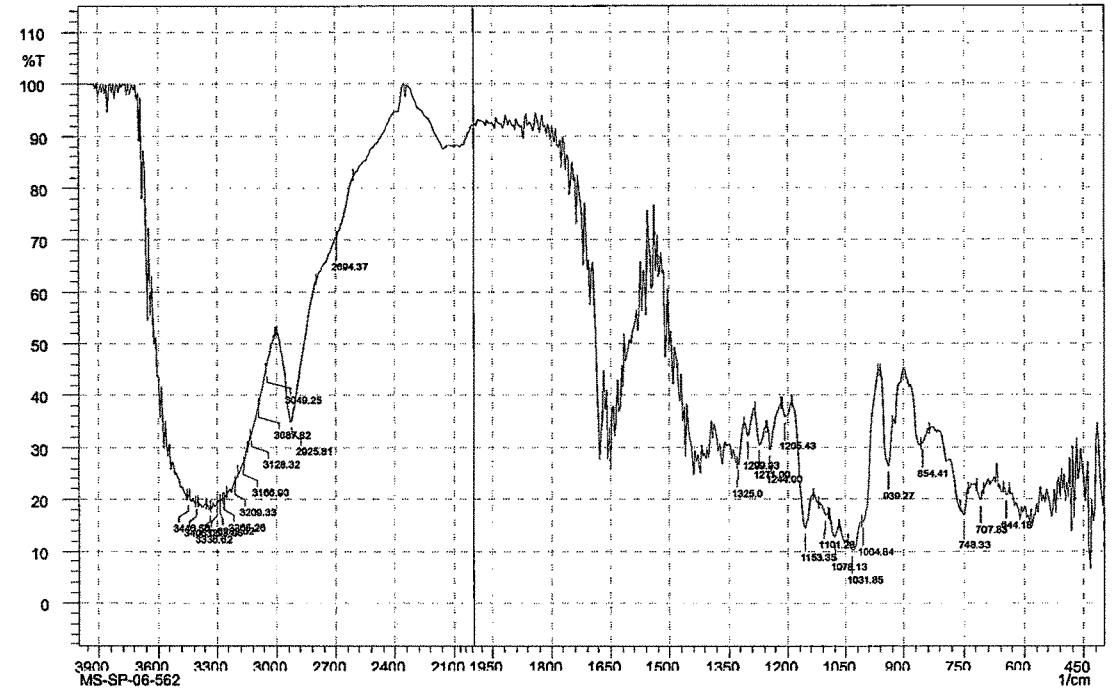

Figure 4.3.2.3.6. IR Spectrum of β-CD-Tadalafil physical mixture(3:1).

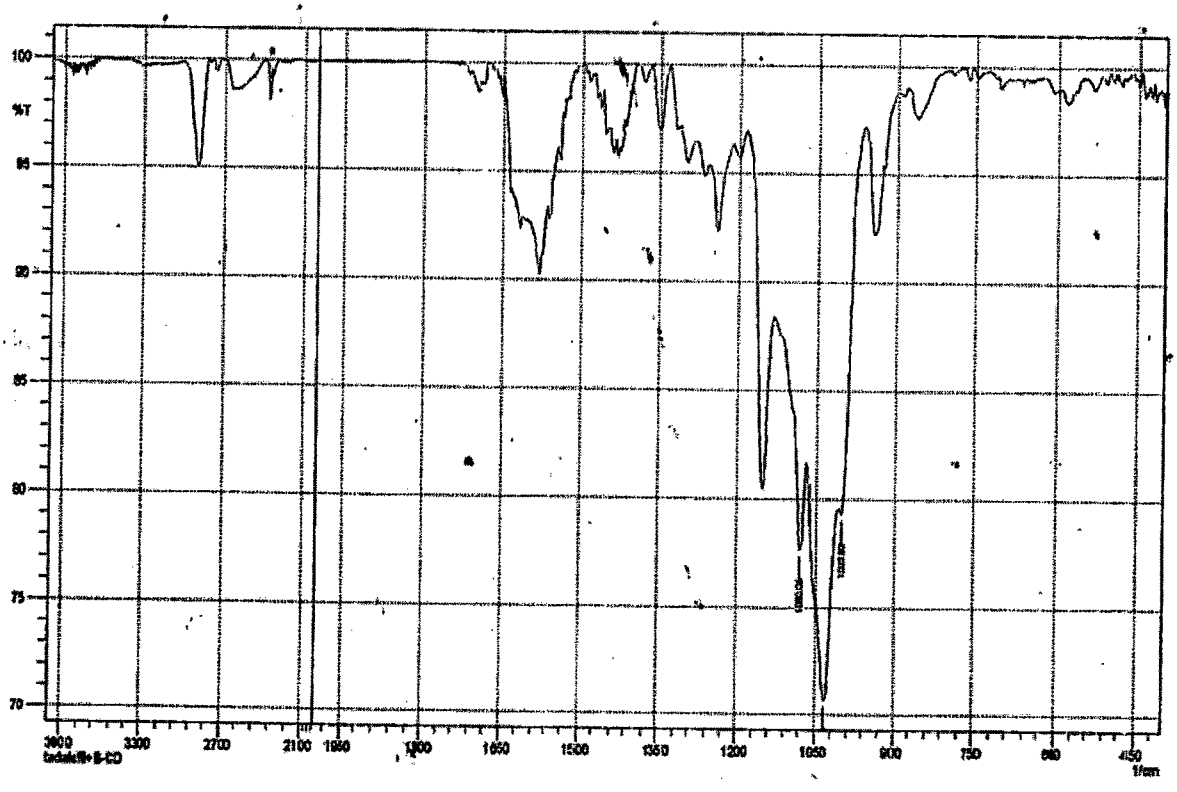

Figure 4.3.2.3.7. IR Spectrum of β-CD-Tadalafil inclusion complex(3:1).

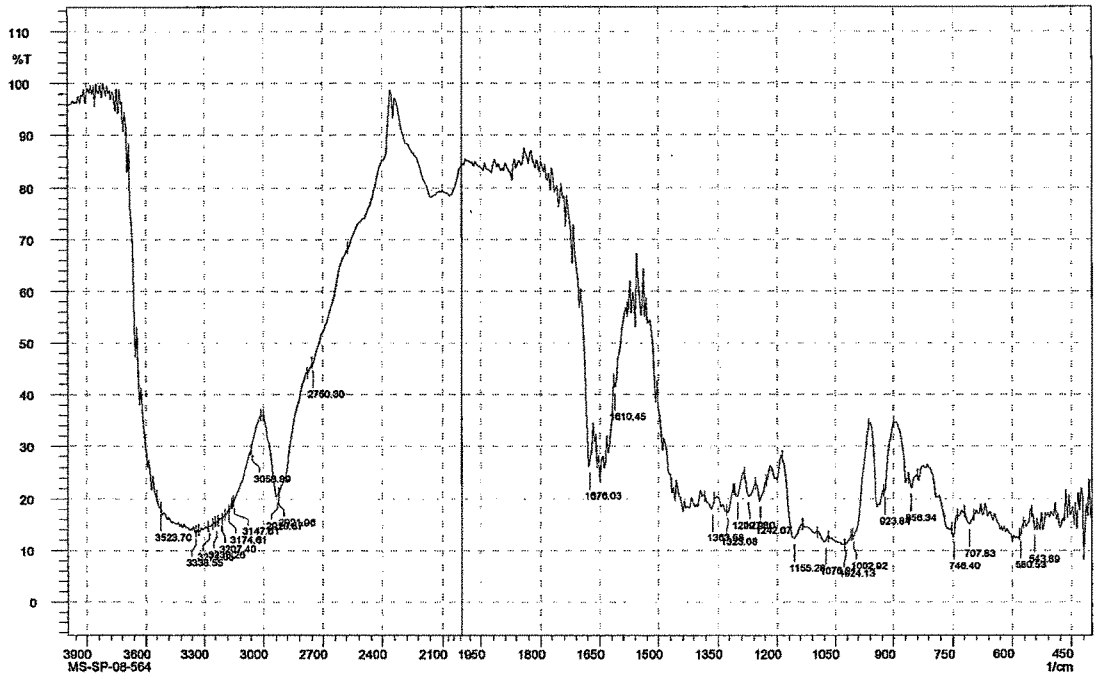

Figure 4.3.2.3.8. IR Spectrum of γ-CD-Tadalafil physical mixture(3:1).

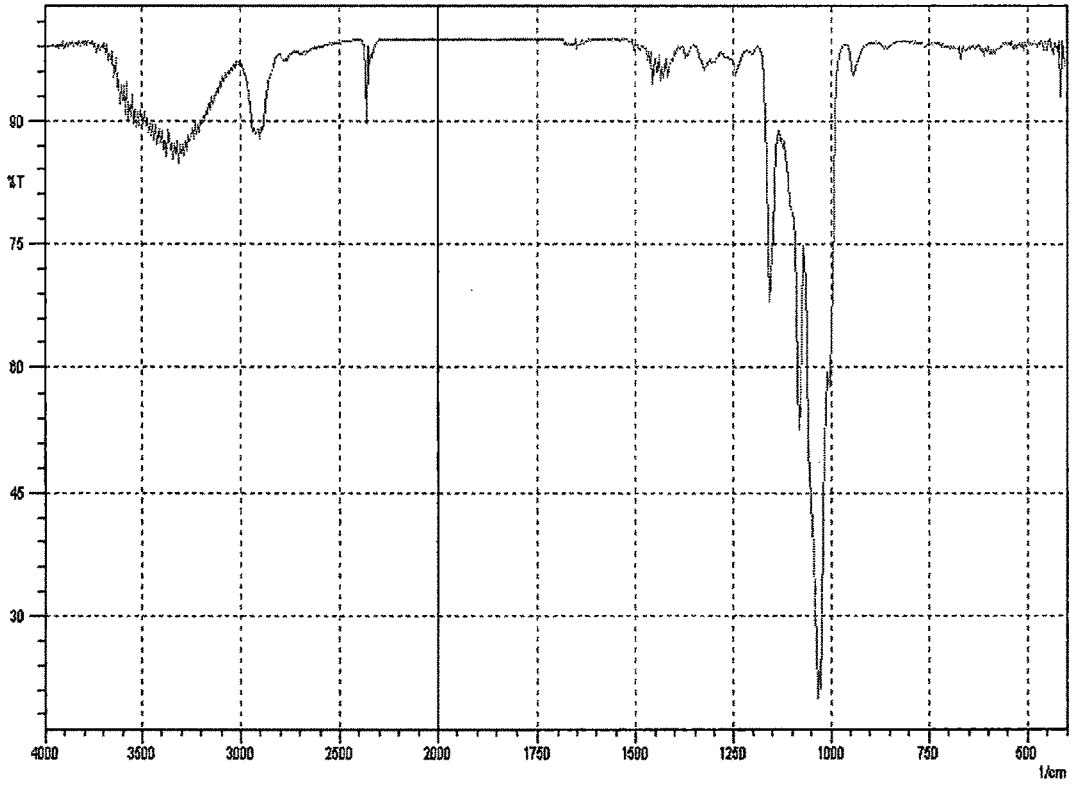

Figure 4.3.2.3.9. IR Spectrum of γ-CD-Tadalafil inclusion complex(3:1).

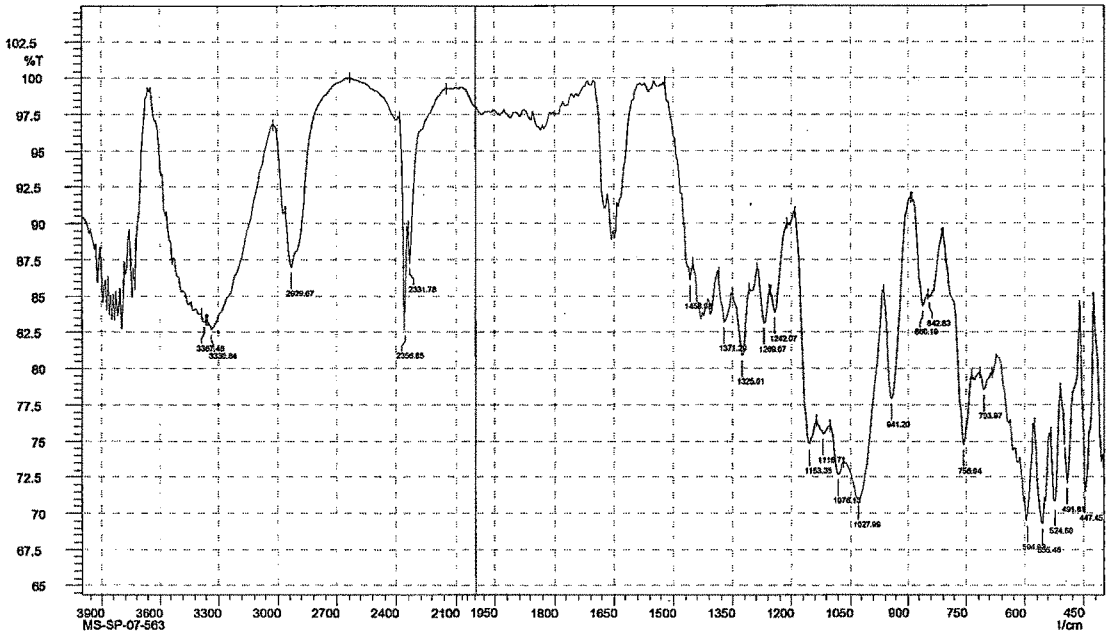

Figure 4.3.2.3.10. IR Spectrum of HP-β-CD-Tadalafil physical mixture(3:1).

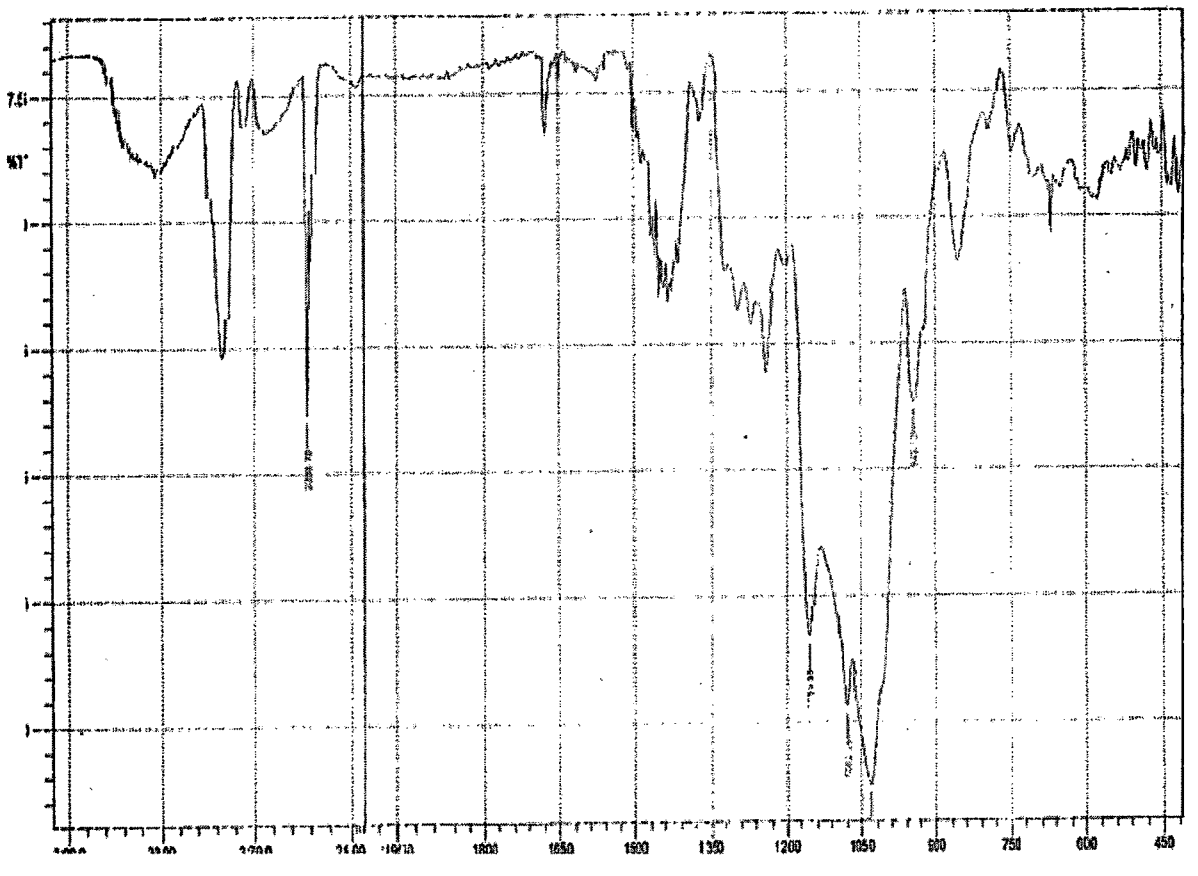

Figure 4.3.2.3.11. IR Spectrum of HP-β-CD-Tadalafil inclusion complex(3:1).

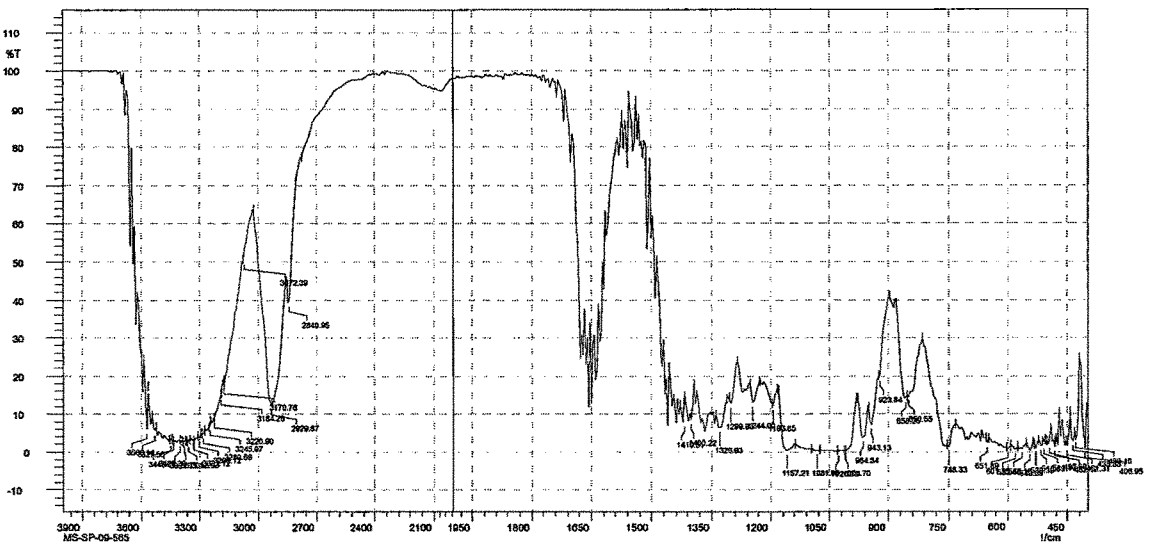

Figure 4.3.2.3.12. IR Spectrum of DM-β-CD-Tadalafil physical mixture(3:1).

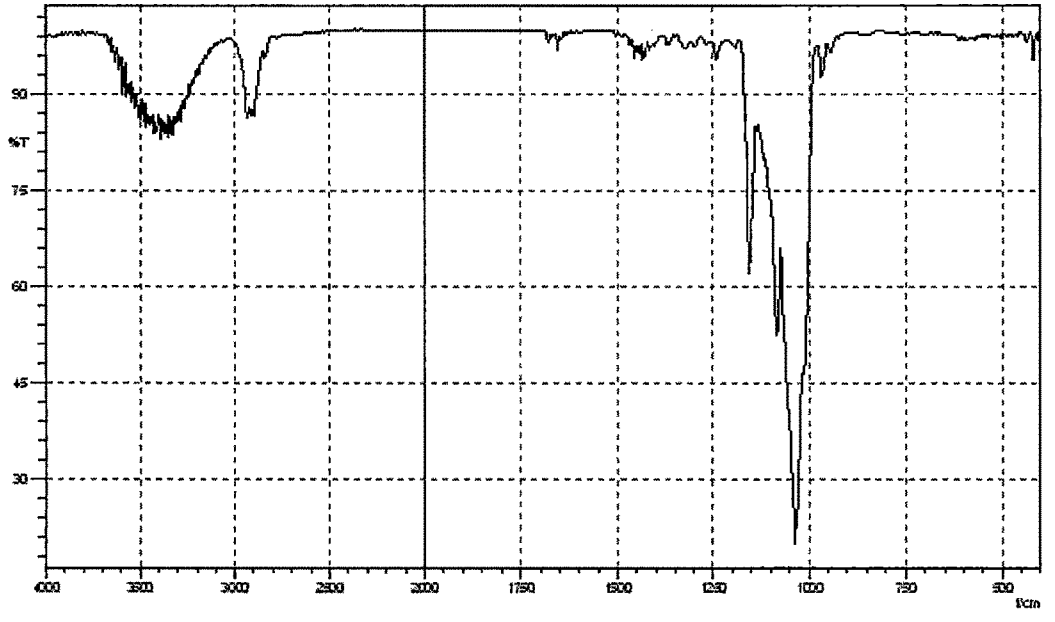

Figure 4.3.2.3.13. IR Spectrum of DM-β-CD-Tadalafil inclusion complex(3:1).

4.3.2.4. DSC thermograms of Tadalafil, CDs, it's Physical mixtures and it's Inclusion complexes.

Figure 4.3.2.4.1. DSC thermogram of pure β-CD.

DSC thermogram of β-CD was shown in 3.3.2.4.1.

Figure 4.3.2.4.2. DSC thermogram of pure γ-CD.

DSC thermogram of γ-CD was shown in 3.3.2.4.2.

Figure 4.3.2.4.3. DSC thermogram of pure HP-β-CD.

DSC thermogram of HP-β-CD was shown in 3.3.2.4.3.

Figure 4.3.2.4.4. DSC thermogram of pure DM-β-CD.

DSC thermogram of DM-β-CD was shown in 3.3.2.4.4.

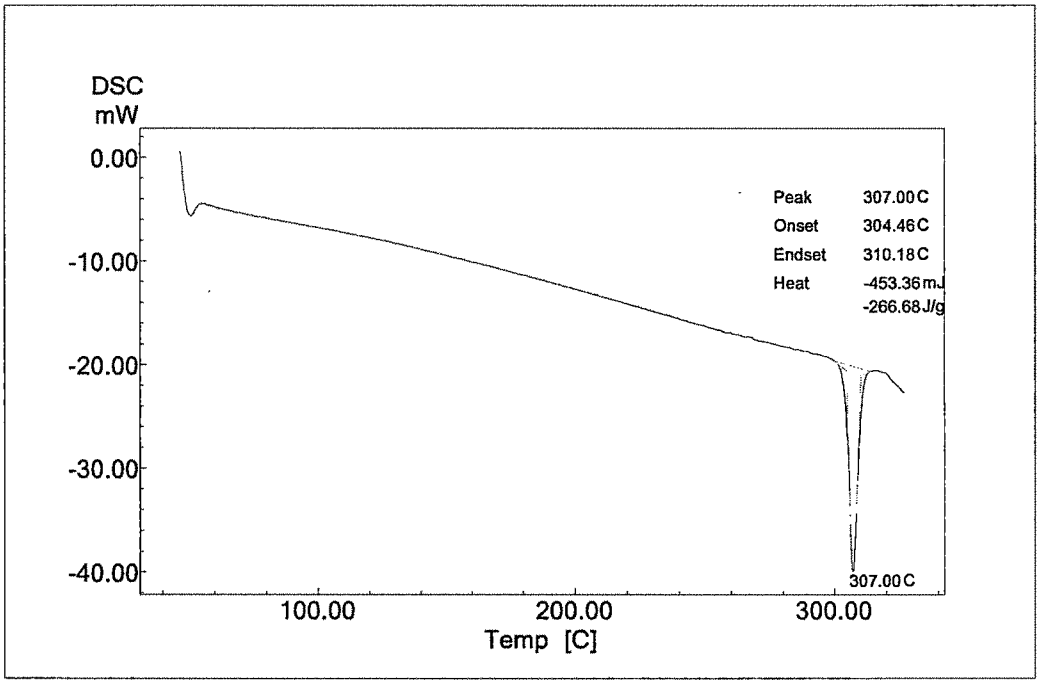

Figure 4.3.2.4.5. DSC thermogram of pure Tadalafil.

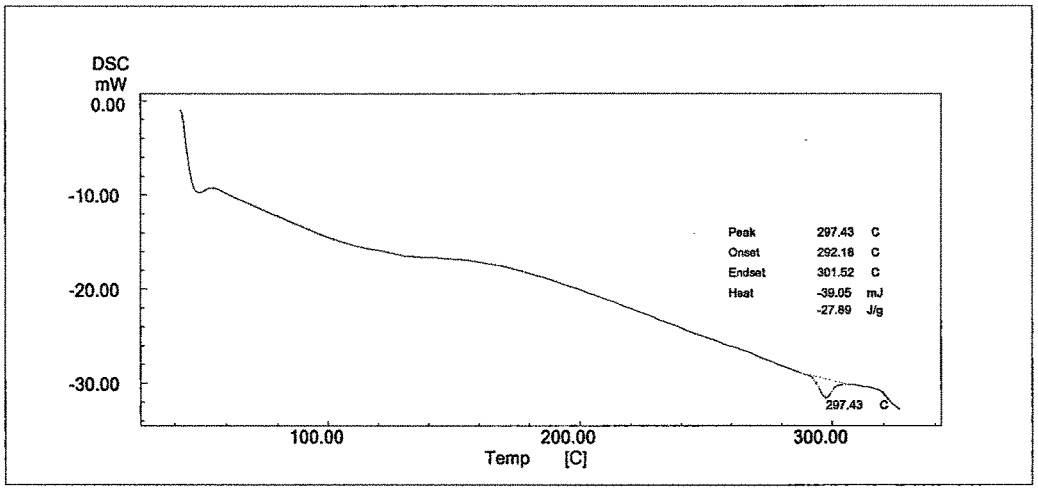

Figure 4.3.2.4.6. DSC thermogram of β-CD-Tadalafil Physical mixture (3:1).

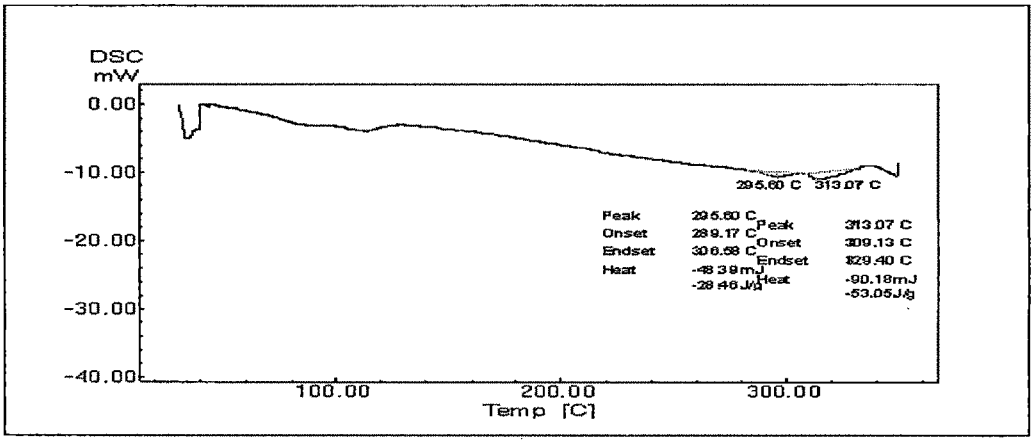

Figure 4.3.2.4.7. DSC thermogram of β-CD-Tadalafil Inclusion complex by co-precipitation method (3:1)

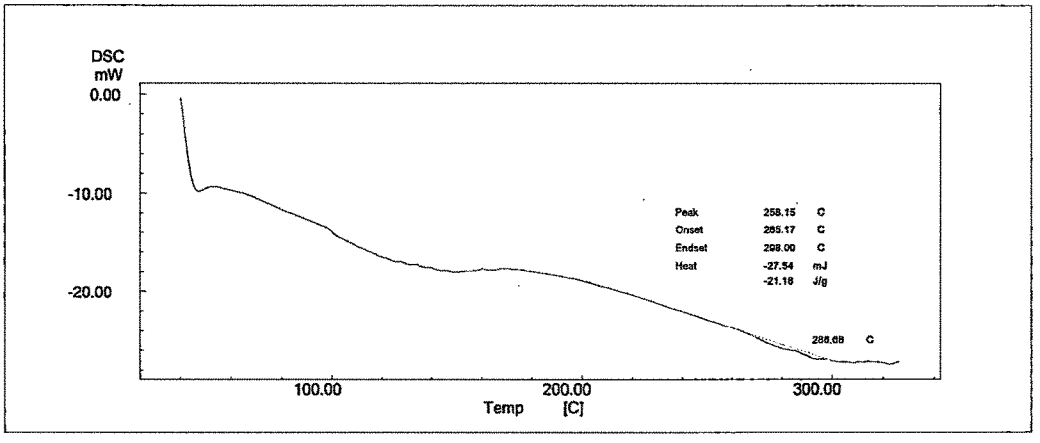

Figure 4.3.2.4.8. DSC thermogram of β-CD-Tadalafil Inclusion complex by kneading method (3:1)

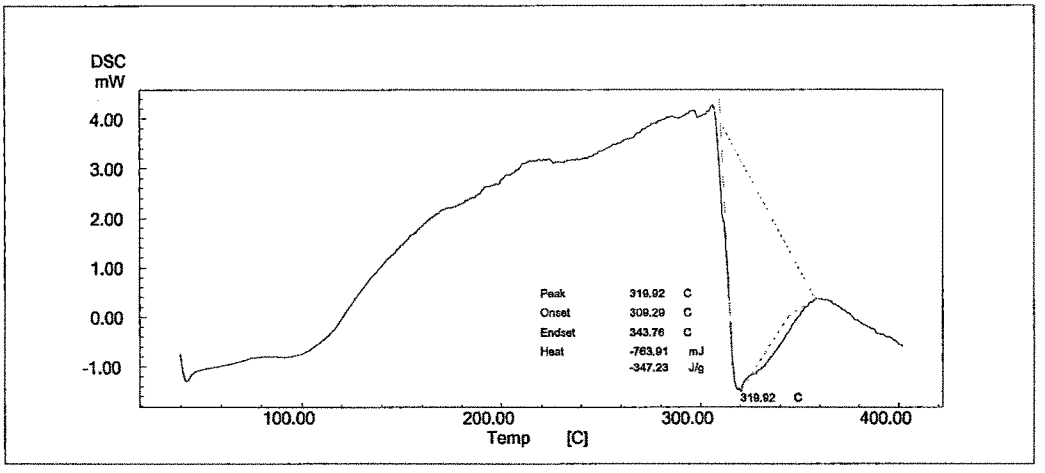

Figure 4.3.2.4.9. DSC thermogram of γ-CD-Tadalafil Physical mixture (3:1).

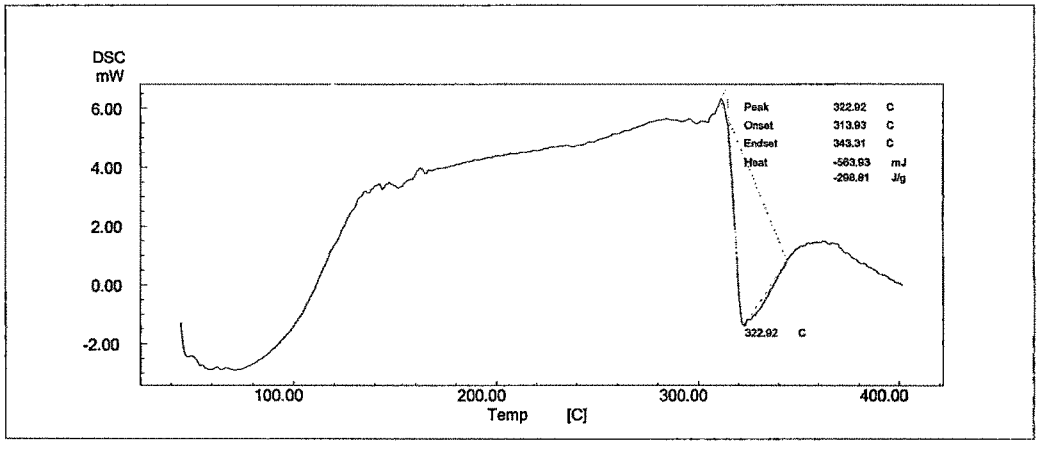

Figure 4.3.2.4.10. DSC thermogram of γ-CD-Tadalafil Inclusion complex by co-precipitation method (3:1)

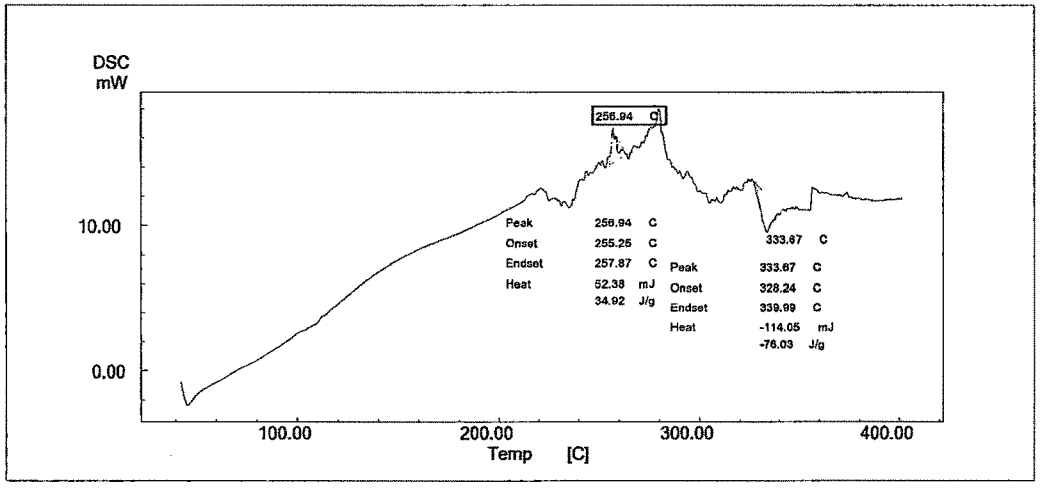

Figure 4.3.2.4.11. DSC thermogram of γ-CD-Tadalafil Inclusion complex by kneading method (3:1)

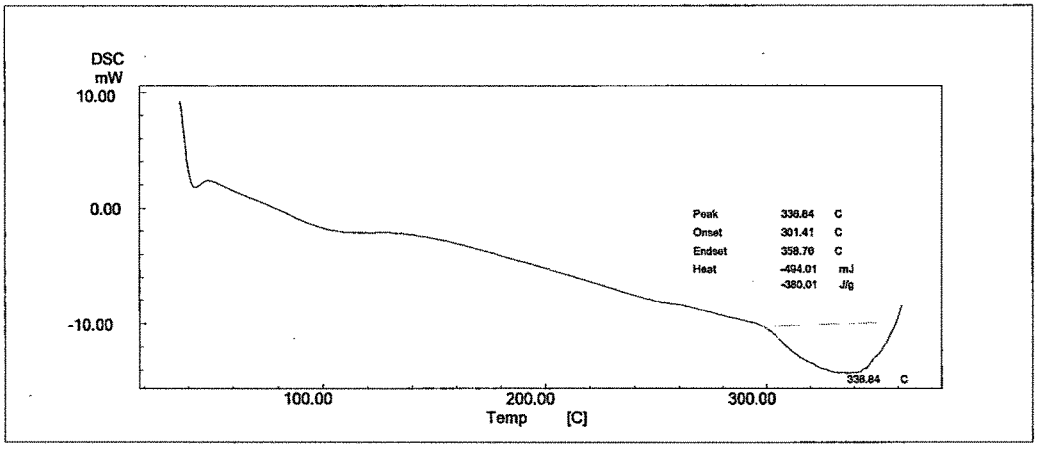

Figure 4.3.2.4.12. DSC thermogram of HP-β-CD-Tadalafil Physical mixture (3:1).

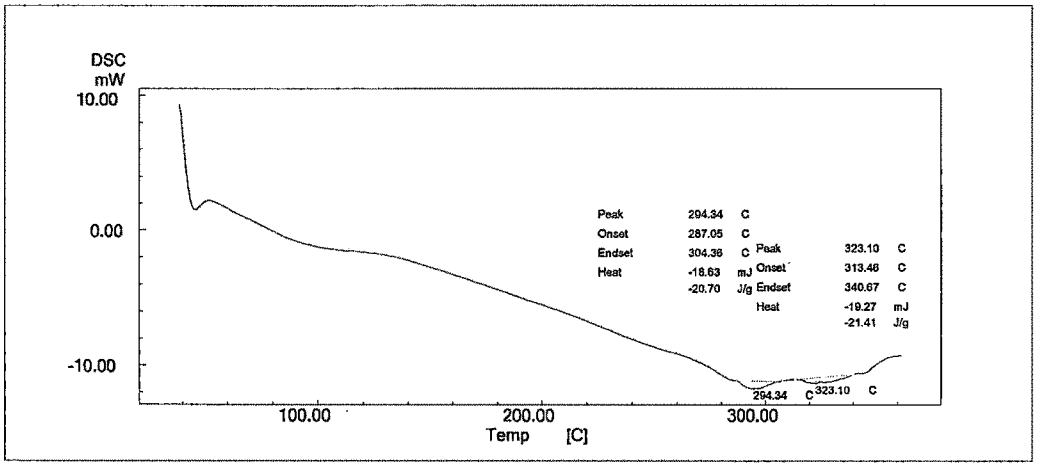

Figure 4.3.2.4.13. DSC thermogram of HP-β-CD-Tadalafil Inclusion complex by co-precipitation method (3:1)

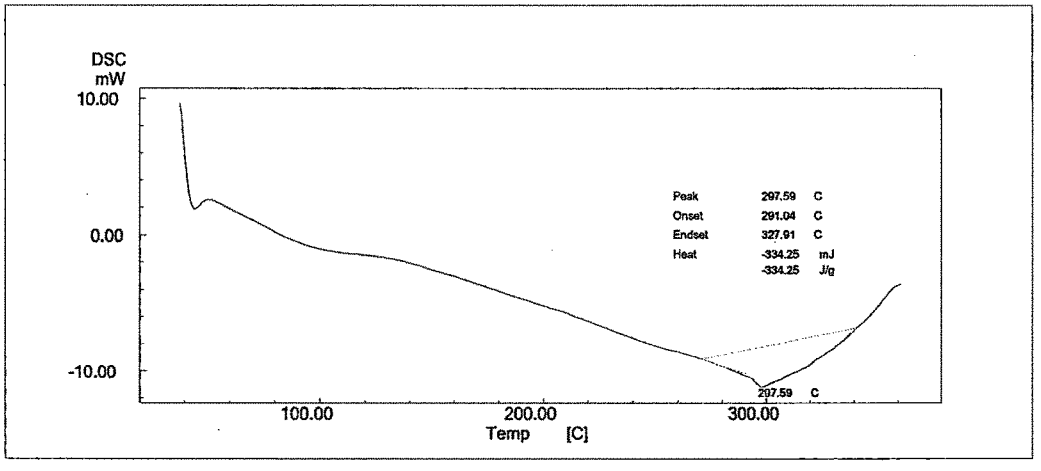

Figure 4.3.2.4.14. DSC thermogram of HP-β-CD-Tadalafil Inclusion complex by kneading method (3:1)

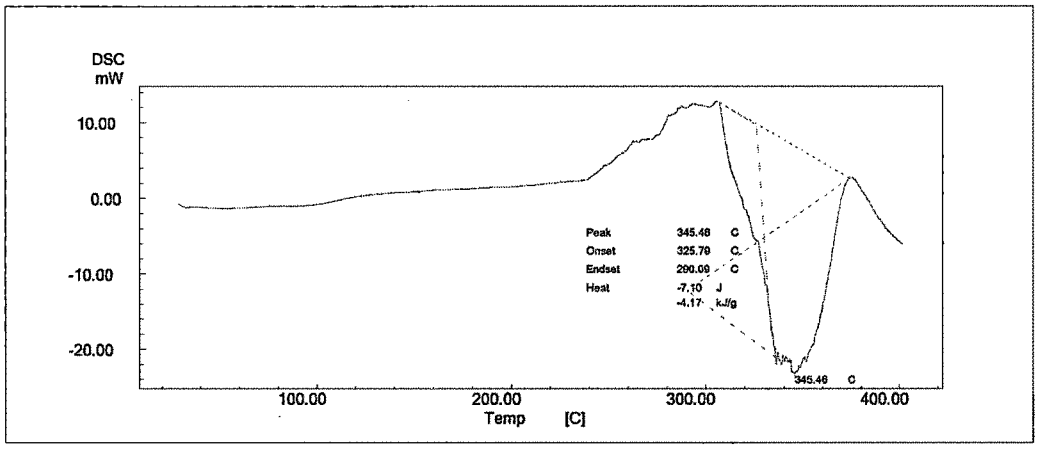

Figure 4.3.2.4.15. DSC thermogram of DM-β-CD-Tadalafil Physical mixture (3:1).

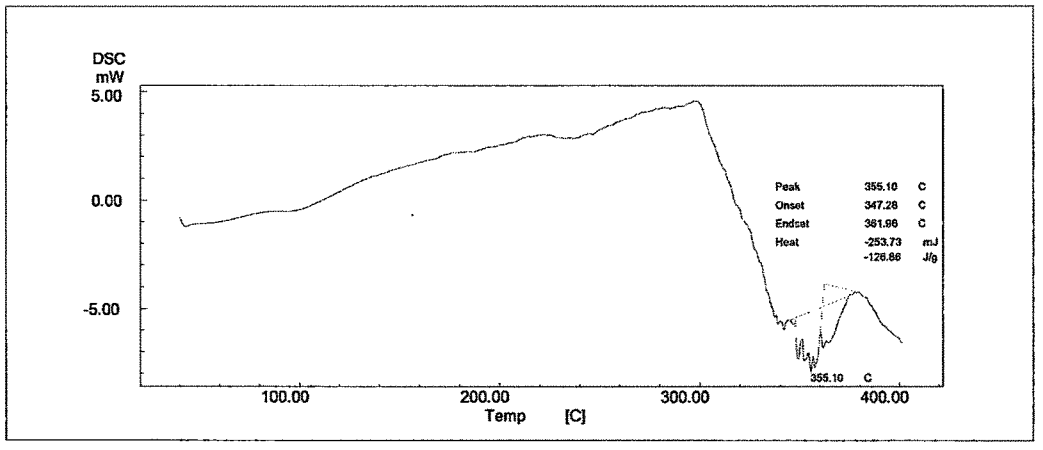

Figure 4.3.2.4.16. DSC thermogram of DM-β-CD-Tadalafil Inclusion complex by co-precipitation method (3:1)

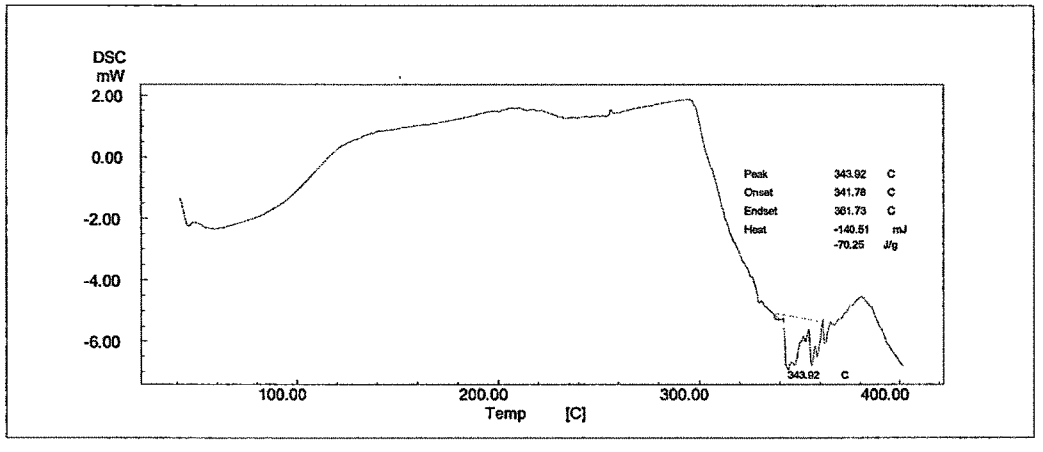

Figure 4.3.2.4.17. DSC thermogram of DM-β-CD-Tadalafil Inclusion complex by kneading method (3:1)

4.3.2.5. X-Ray diffraction pattern of Tadalafil, CDs, its Physical mixtures and its Inclusion complexes.

Figure 4.3.2.5.1. X-Ray diffraction pattern of Pure β-CD.

X-Ray diffraction pattern of Pure β-CD was shown in 3.3.2.5.1.

Figure 4.3.2.5.2. X-Ray diffraction pattern of Pure γ-CD.

X-Ray diffraction pattern of Pure γ-CD was shown in 3.3.2.5.2.

Figure 4.3.2.5.3. X-Ray diffraction pattern of Pure HP-β--CD.

X-Ray diffraction pattern of Pure HP-β-CD was shown in 3.3.2.5.3.

Figure 4.3.2.5.4. X-Ray diffraction pattern of DM-β--CD.

X-Ray diffraction pattern of Pure DM-β-CD was shown in 3.3.2.5.4.

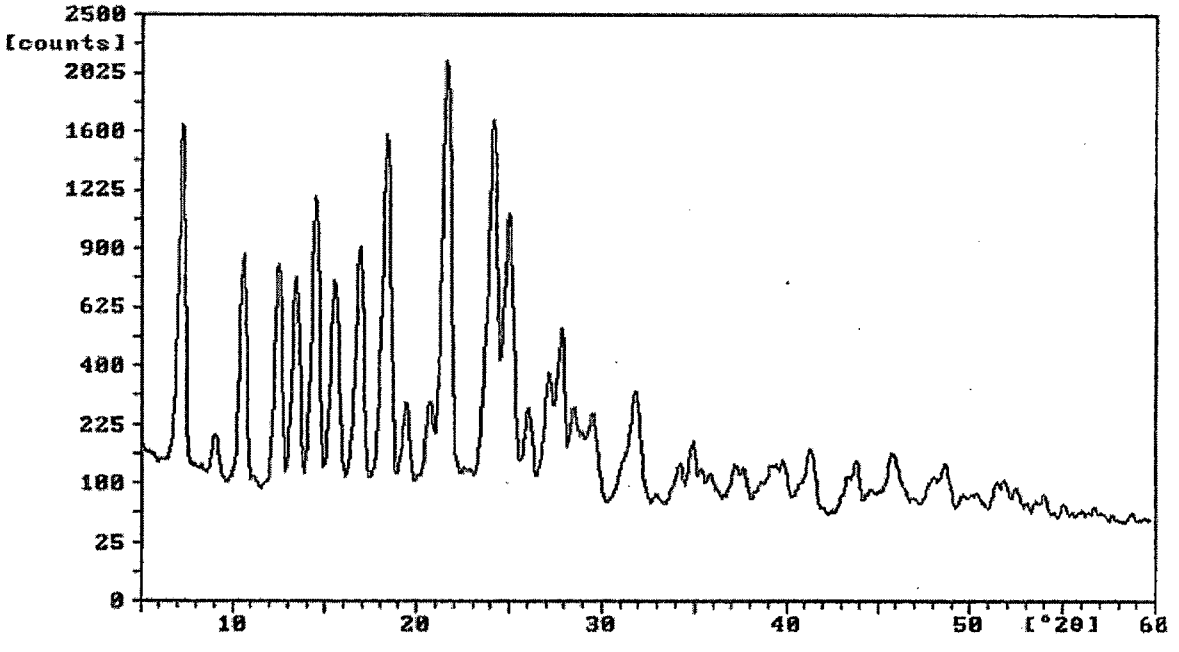

Figure 4.3.2.5.5. X-Ray diffraction pattern of Tadalafil.

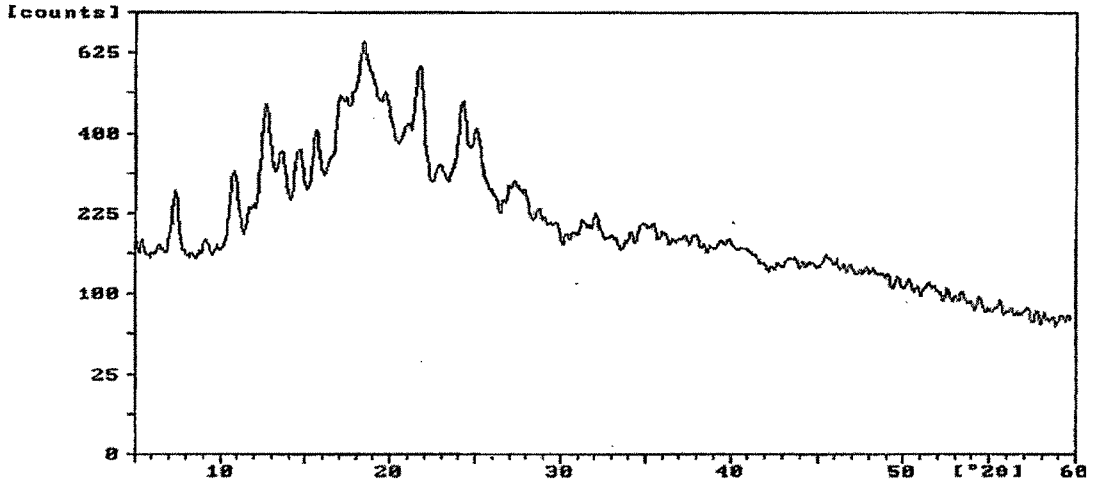

Figure 4.3.2.5.6. X-Ray diffraction pattern of β-CD - Tadalafil physical mixture (3:1).

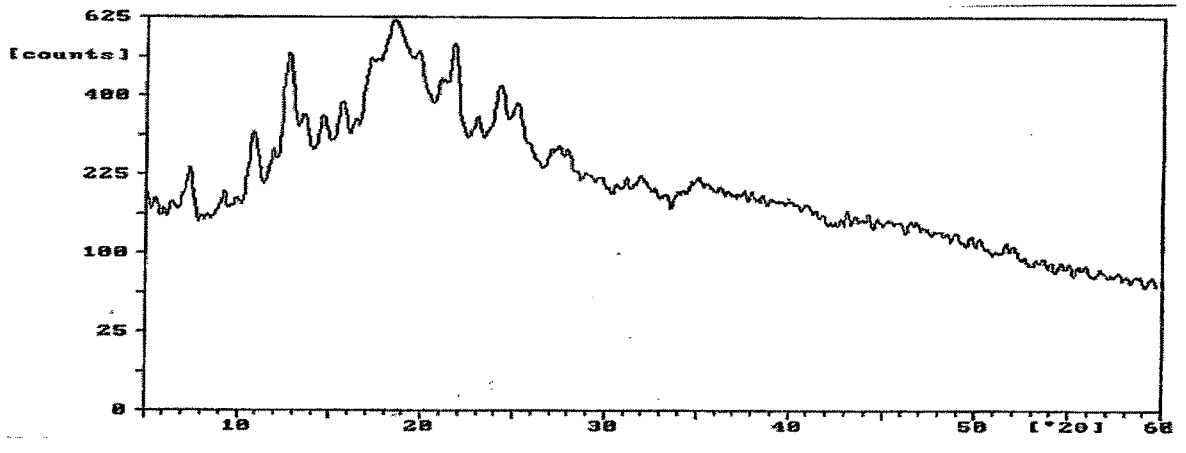

Figure 4.3.2.5.7. X-Ray diffraction pattern of β-CD - Tadalafil Inclusion complex (2:1).

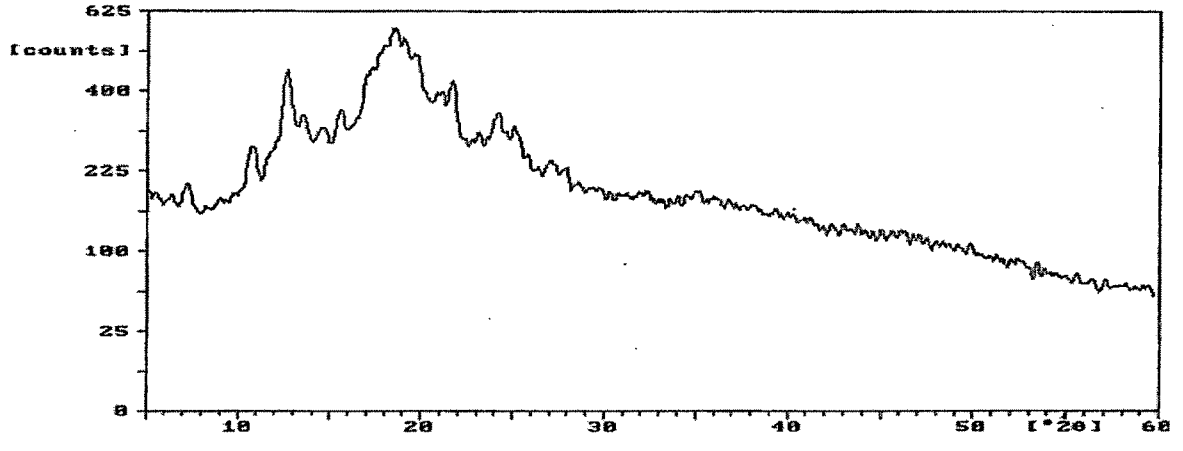

Figure 4.3.2.5.8. X-Ray diffraction pattern of β-CD - Tadalafil Inclusion complex(3:1).

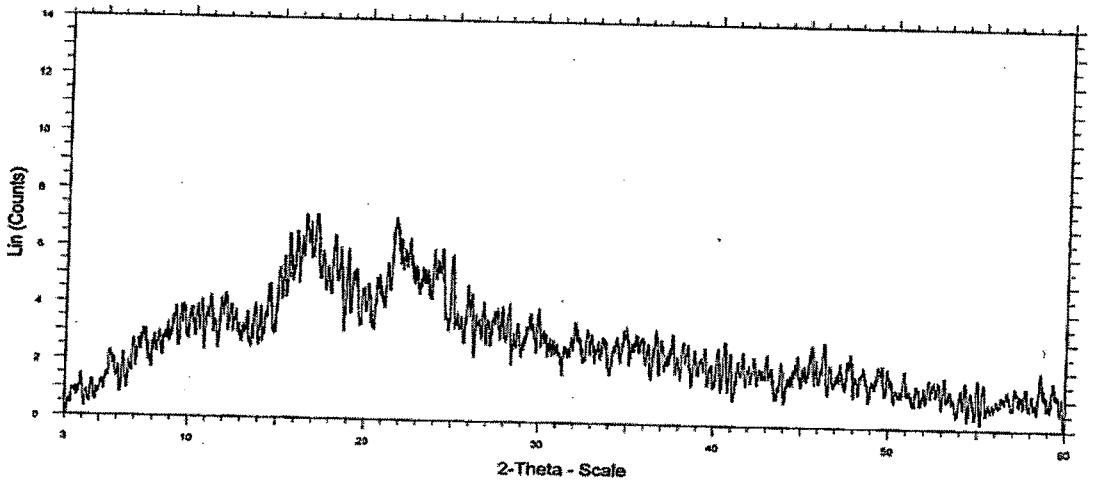

Figure 4.3.2.5.9. X-Ray diffraction pattern of γ-CD - Tadalafil physical mixture (3:1).

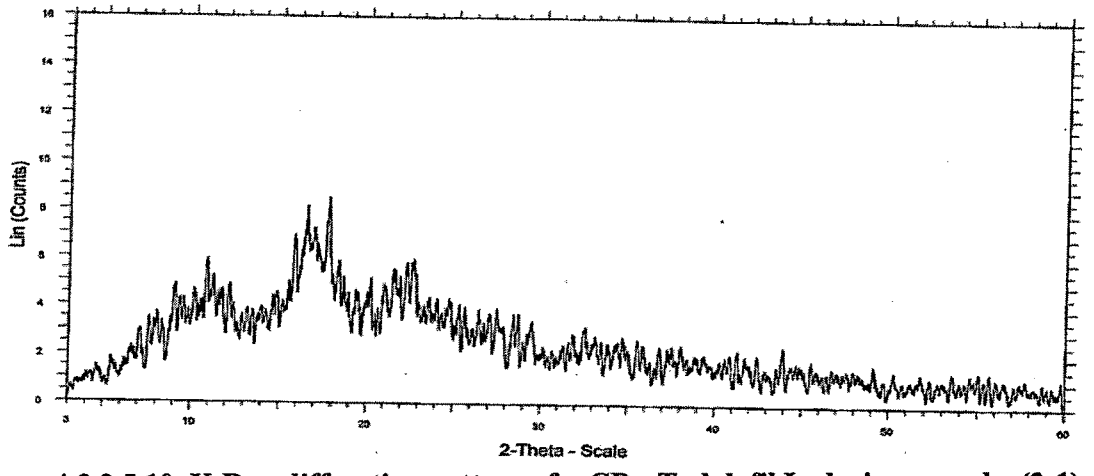

Figure 4.3.2.5.10. X-Ray diffraction pattern of γ-CD - Tadalafil Inclusion complex(2:1).

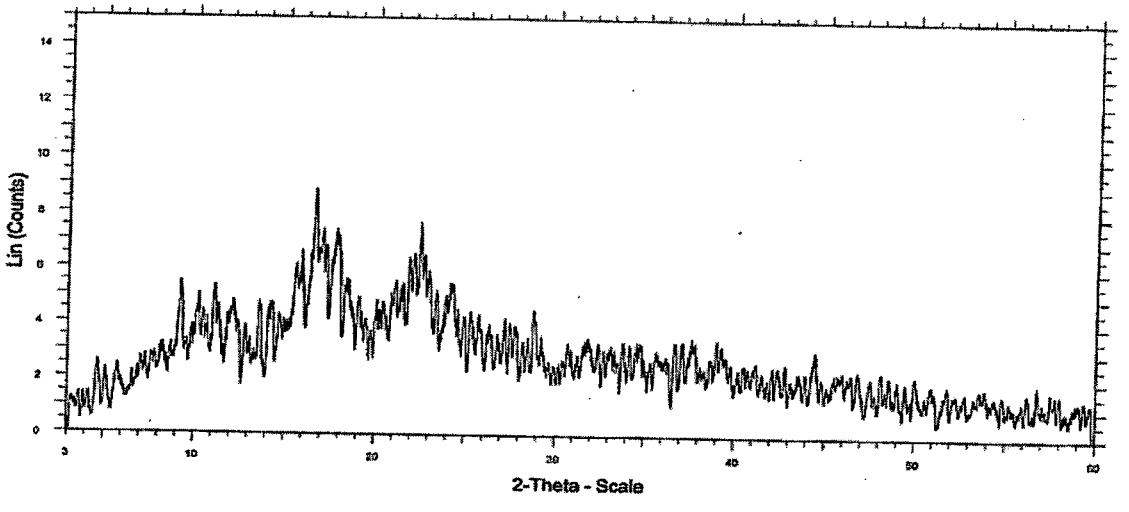

Figure 4.3.2.5.11. X-Ray diffraction pattern of γ-CD - Tadalafil Inclusion complex(3:1).

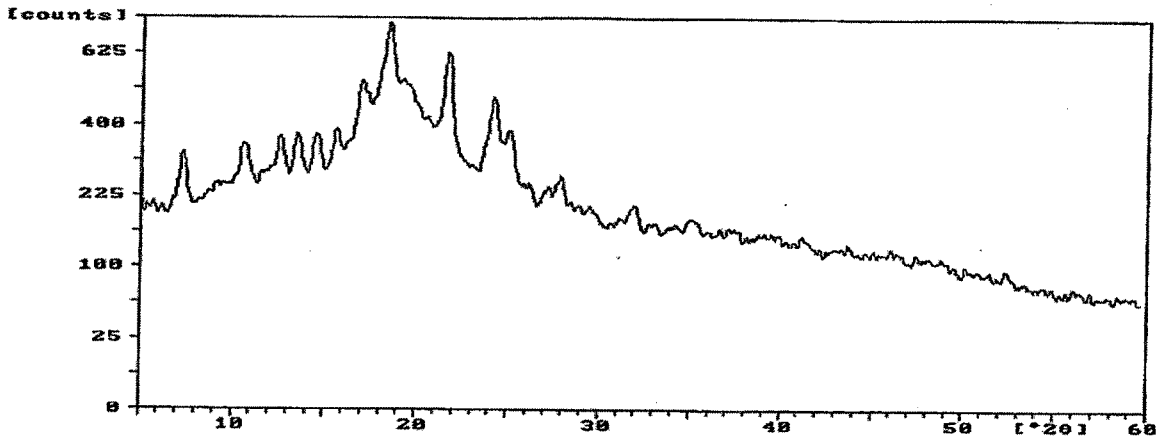

Figure 4.3.2.5.12. X-Ray diffraction pattern of HP-β-CD - Tadalafil physical mixture (3:1).

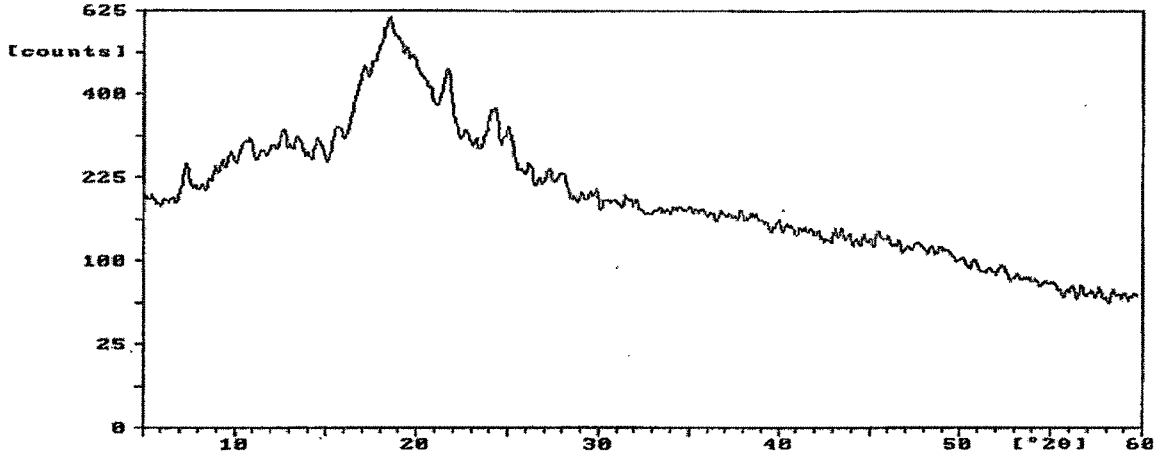

Figure 4.3.2.5.13. X-Ray diffraction pattern of HP-β-CD - Tadalafil Inclusion complex (2:1).

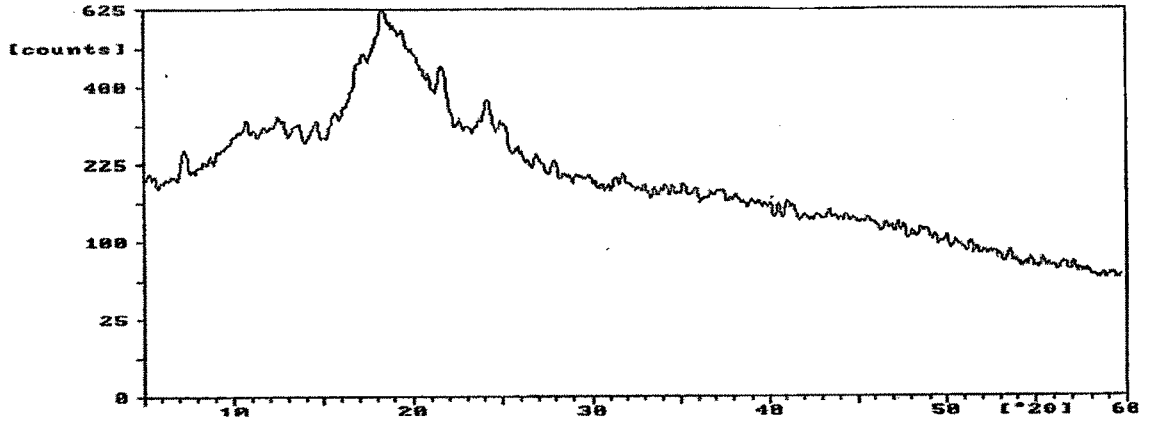

Figure 4.3.2.5.14. X-Ray diffraction pattern of HP-β-CD - Tadalafil Inclusion complex (3:1).

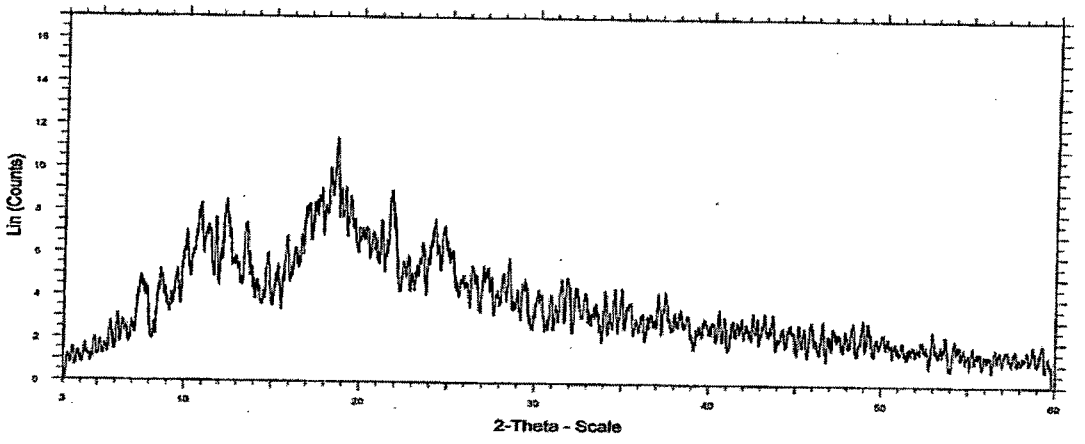

Figure 4.3.2.5.15. X-Ray diffraction pattern of DM-β-CD - Tadalafil physical mixture (3:1).

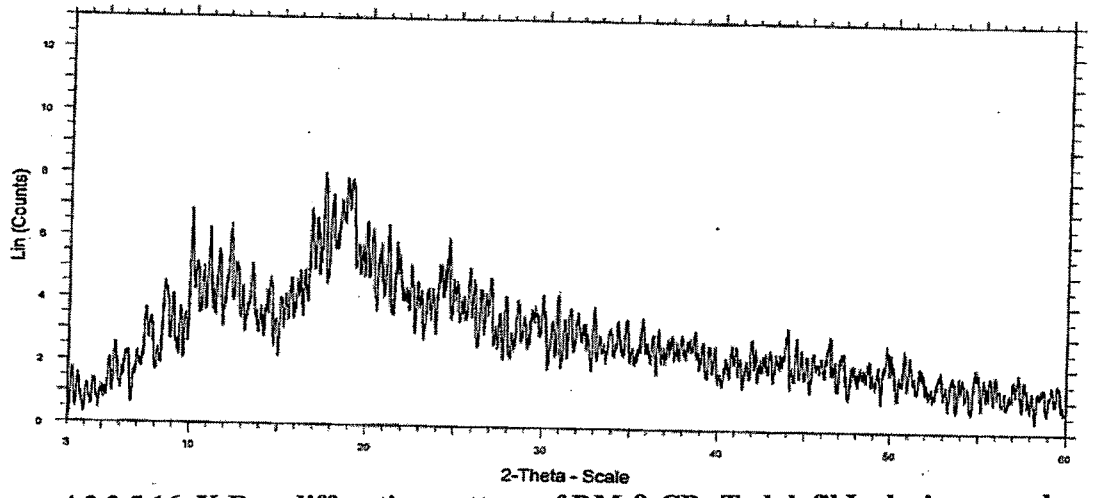

Figure 4.3.2.5.16. X-Ray diffraction pattern of DM-β-CD -Tadalafil Inclusion complex (2:1).

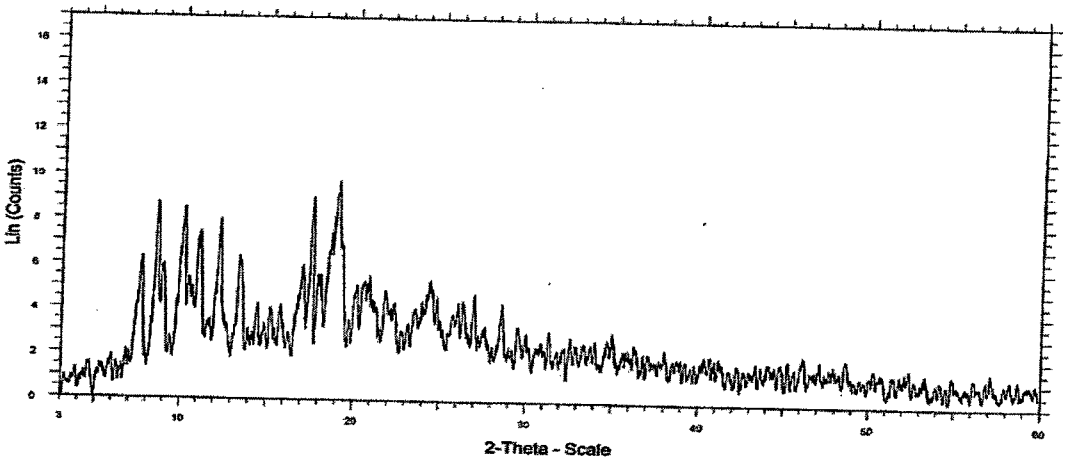

Figure 4.3.2.5.17. X-Ray diffraction pattern of DM-β-CD - Tadalafil Inclusion complex (3:1).

4.3.3. Dissolution profile for Tadalafil-cyclodextrins inclusion complex, Physical mixtures and Marketed formulation (Tadora-20).

Dissolution rate studies were performed in (HCl buffer pH1.2, Distilled water and phosphate buffer pH 6.4, 900 ml)[5] at 37 ± 0.2°C, using USP XXIII[6] apparatus (Electrolab, India Programmable tablet dissolution test apparatus USP XXI/XXII, TDT-06P)) with a basket rotating (Apparatus I) at 50 rpm but the best results were found in HCl buffer pH 1.2 Hence, the further study was continued using HCl buffer pH 1.2 as a dissolution medium. Physical mixtures and Inclusion complexes, each containing 20 mg of Tadalafil were filled into empty hard gelatin capsules shell and subjected to dissolution study. At different time intervals (10.0, 20.0, 30.0, 40.0, 50.0, 60.0,75.00,90.00, 105.0 and 120.0 min), 5 ml of samples withdrawn were filtered (Whatman filter paper No. 41) and spectrophotometrically assayed for drug content at 284.5 nm as mentioned in, Section 4.1.1.4.4. The experiments were run in triplicate and the mean cumulative % drug diffused along with SD are shown in Table 4.3.3.2. to Table 4.3.3.4, Table 4.3.3.6. to 4.3.3.8., Table 4.3.3.10. to 4.3.3.12 and Table 4.3.3.14. to 4.3.3.16.respectively for different Tadalafil-CD inclusion complexes. The graphs are shown in Figure 4.3.3.1, 4.3.3.3., 4.3.3.5. and 4.3.3.7 respectively. The % Dissolution Efficiency ($DE_{120\ min.}$) values based on the dissolution data along with SD were calculated as per Khan method[12] and time taken for 50% drug dissolved ($T_{50\ \%}$ value) was identified from the dissolution profiles. All this data are shown in Table 4.3.3.5., 4.3.3.9., 4.3.3.13. and 4.3.3.17. Comparison data of $DE_{120\ min.}$ study and $T_{50\%}$ study are shown in Figure 4.3.3.2., 4.3.3.4., 343.3.6., and 4.3.3.8 respectively. The release kinetics of diffusion was studied by calculating the regression coefficient for zero order, first order, and Higuchi's equations. The regression coefficients for the different Tadalafil-CDs inclusion complexes, Physical mixtures, Marketed formulation (Tadora-20) and the Tadalafil are recorded in Table 4.3.3.18. Finally the result were compared with the dissolution profile of Marketed formulation (Tadora-20) (Tadoa-20, Tablet containing 20 mg of Tadalafil) and Tadalafil(20 mg) (Table 4.3.3.1.)[7-11].

Table 4.3.3.1. Dissolution data of Pure Tadalafil and it's Marketed formulation (Tadora-20) in HCl buffer pH 1.2.

Time in Min.	% Drug release of Pure Tadalafil[#]	% Drug release of Marketed formulation (Tadora-20) (Tadora - 20)[#]
0	0.00 ± 1.22	0.00 ± 0.88
10	2.85 ± 1.88	3.33 ± 1.45
20	5.27 ± 1.02	7.54 ± 1.99
30	7.96 ± 0.45	13.31 ± 2.10
40	10.36 ± 2.11	19.67 ± 1.90
50	12.63 ± 1.93	26.88 ± 2.35
60	14.93 ± 1.88	32.66 ± 1.45
75	15.07 ± 0.54	35.49 ± 1.66
90	16.26 ± 0.25	39.54 ± 1.68
105	16.98 ± 0.82	43.37 ± 2.14
120	17.06 ± 1.11	44.33 ± 0.62

[#] All values are represented as mean ± SD (±n=3).

Table 4.3.3.2. Dissolution data of Tadalafil -β-CD PHYSICAL MIXTURE in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil-β-CD Physical mixture(1:1)[#]	% Drug release of Tadalafil-β-CD Physical mixture (1:2)[#]	% Drug release of Tadalafil-β-CD Physical mixture (1:3)[#]
0	0.00 ± 0.15	0.00 ± 0.49	0.00 ± 0.86
10	2.38 ± 1.22	5.15 ±0.82	5.69 ± 0.46
20	5.69 ± 1.67	11.21 ±1.72	16.66 ± 1.32
30	9.28 ± 1.92	17.27 ± 1.88	30.45 ± 1.45
40	15.46 ± 2.43	23.11 ± 1.92	45.75 ± 2.02
50	26.76 ± 2.11	29.89 ± 2.82	55.92 ± 2.43
60	39.48 ± 1.89	40.11 ± 2.47	61.47 ± 1.72
75	48.83 ± 1. 45	54.47 ± 2.11	63.93 ± 2.82
90	55.33 ± 2.65	60.12 ± 1.99	68.38 ± 1.57
105	57.27 ± 2.33	65.25 ± 3.01	72.22 ± 2.43
120	59.58 ± 2.61	68.92 ± 2.55	75.36 ± 3.11

[#] All values are represented as mean ± SD (±n=3).

Table 4.3.3.3.Dissolution data of Tadalafil – β-CD Inclusion complex by CO-PRECIPITTION METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil β-CD Inclusion complex (1:1)[#]	% Drug release of Tadalafil β-CD Inclusion complex (1:2)[#]	% Drug release of Tadalafil β-CD Inclusion complex (1:3)[#]
0	0.00 ± 0.11	0.00 ± 0.57	0.00 ± 0.45
10	5.35 ± 1.41	6.36 ± 0.45	9.36 ± 0.64
20	9.23 ± 2.43	11.63 ± 0.99	17.4 ± 0.81
30	16.45 ± 1.62	19.58 ± 1.23	31.34 ± 1.25
40	29.43 ± 1.25	33.34 ± 1.56	45.55 ± 1.63
50	36.56 ± 2.31	45.31 ± 1.86	59.89 ± 1.69
60	49.59 ± 1.65	56.36 ± 1.37	68.65 ± 1.09
75	56.38 ± 1.92	60.5 ± 1.46	73.39 ± 1.36
90	61.92 ±1.88	65.55 ± 1.67	79.53 ± 1.57
105	63.9 ± 2.22	71.64 ± 1.89	83.68 ± 1.84
120	67.65 ± 2.01	74.98 ± 2.53	86.72 ± 1.99

[#] All values are represented as mean ± SD (±n=3).

Table 4.3.3.4. Dissolution data of Tadalafil–β-CD Inclusion complex by KNEADING METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil β-CD Inclusion complex (1:1)[#]	% Drug release of Tadalafil β-CD Inclusion complex (1:2)[#]	% Drug release of Tadalafil β-CD Inclusion complex (1:3)[#]
0	0.00 ± 0.88	0.00 ± 1.21	**0.00 ± 2.11**
10	5.32 ± 0.28	6.82 ± 1.49	**11.32 ± 1.41**
20	11.26 ± 1.82	13.45 ± 1.99	**24.45 ± 3.21**
30	19.57 ± 2.49	21.83 ± 2.36	**39.66 ± 3.42**
40	33.26 ± 2.99	35.93 ± 1.56	**51.82 ± 2.64**
50	45.55 ± 3.22	49.92 ± 1.11	**59.59 ± 2.83**
60	58.96 ± 3.09	63.33 ± 2.11	**66.35 ±2.91**
75	64.44 ± 2.58	71.48 ± 2.39	**72.73 ± 3.46**
90	68.16 ± 2.89	75.36 ± 2.69	**81.8 ± 2.82**
105	74.84 ± 2.79	78.68 ± 2.83	**85.26 ± 2.57**
120	78.32 ± 2.76	83.72 ± 3.33	**90.38 ± 1.25**

[#] All values are represented as mean ± SD (±n=3).

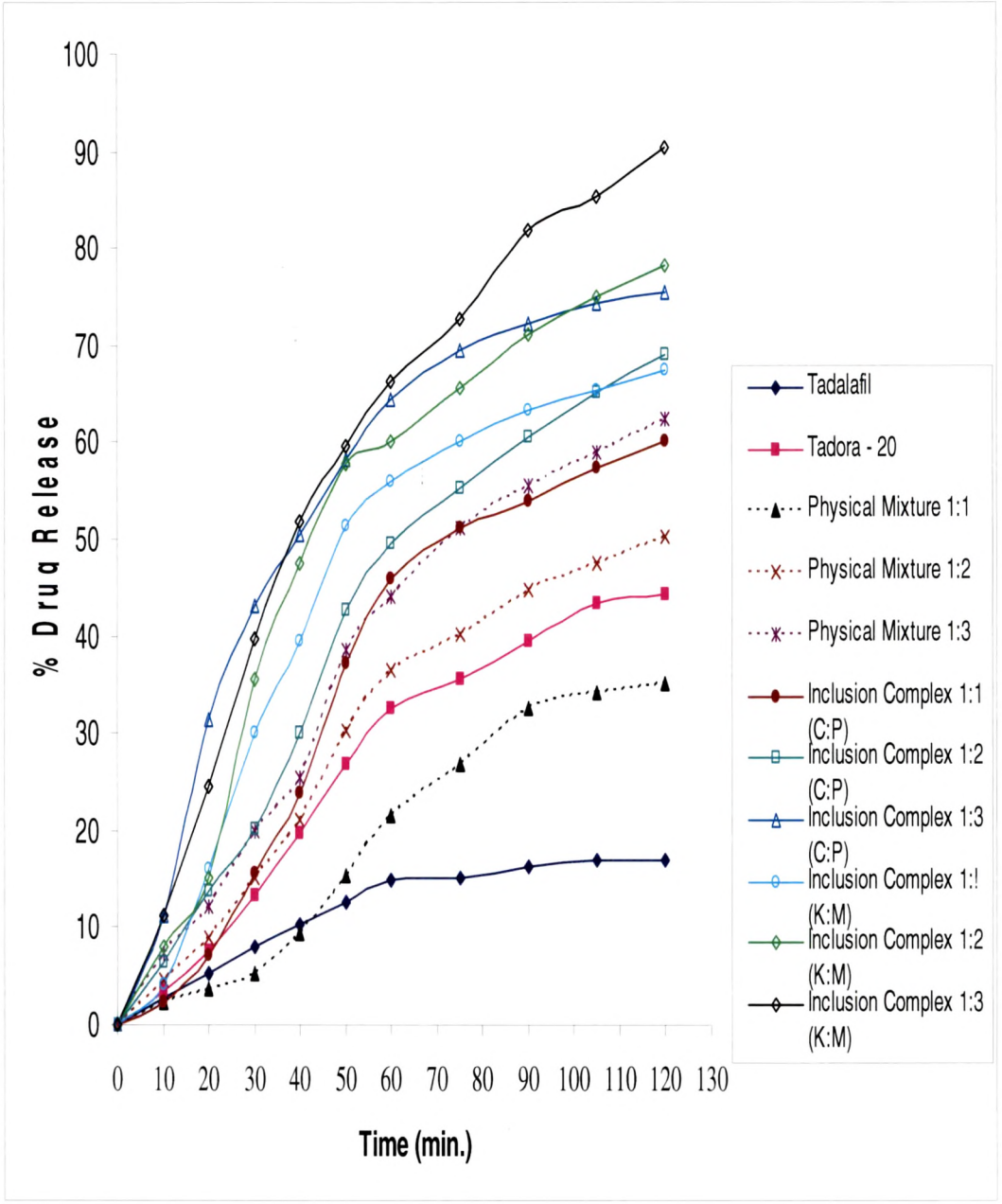

Figure 4.3.3.1. Cumulative % drug released from Tadalafil–β-CD inclusion complexes, Physical mixtures, Marketed formulation (Tadora-20) and Pure Drug at different time intervals.

Table 4.3.3.5. Dissolution efficiency and time required for 50% drug dissolved ($T_{50\,\%}$)for Tadalafil, Marketed formulation (Tadora-20), Tadalafil-β-CD Physical mixtures and Inclusion complexes.

Type	Dissolution efficiency for 120 min (%)		Time required for 50% drug dissolved (Min)	
	Mean ± SD[#]	%RSD	Mean ± SD[#]	%RSD
Tadalafil	11.916 ± 0.98	1.00	351.69 ± 0.74	0.85
Tadora – 20	26.8668 ± 1.91	1.72	135.34 ± 0.50	0.96
Physical Mixture 1:1	32.9791 ± 1.48	1.97	76.79 ± 0.61	1.03
Physical Mixture 1:2	38.1847 ± 1.79	1.51	68.84 ± 0.23	0.87
Physical Mixture 1:3	49.5518 ± 0.96	2.00	43.71 ± 0.73	1.18
Inclusion Complex 1:1(C:P)	40.2537 ± 1.95	1.97	60.3 ± 0.82	0.69
Inclusion Complex 1:2(C:P)	44.9533 ± 1.78	1.83	55.17 ± 0.52	1.07
Inclusion Complex 1:3(C:P)	55.9533 ± 1.79	1.81	43.9 ± 0.67	1.12
Inclusion Complex 1:1(K:M)	46.5466 ± 0.97	1.87	54.88 ± 0.70	1.59
Inclusion Complex 1:2(K:M)	50.6818 ± 0.88	0.89	50.08 ± 0.92	1.32
Inclusion Complex 1:3(K:M)	**64.7289 ± 1.33**	1.04	**30.2 ± 0.70**	0.70

[#] All values are represented as mean ± SD (±n=3).

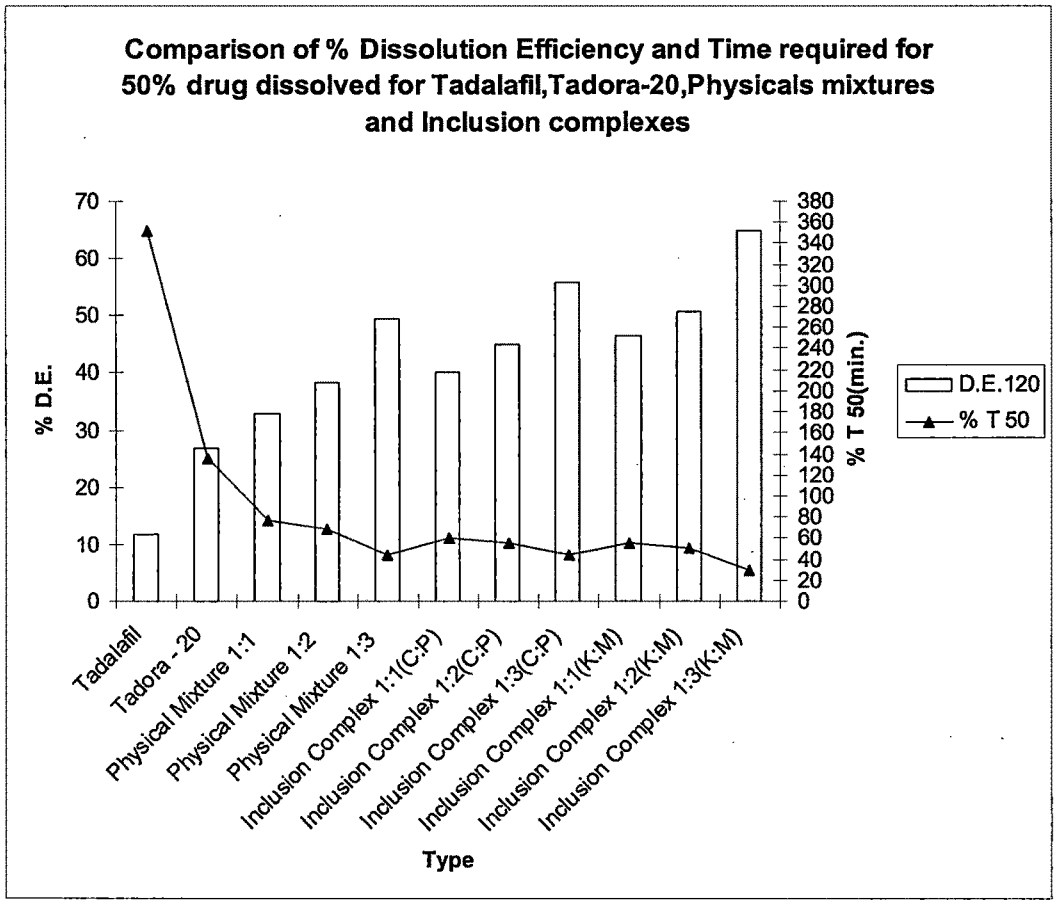

Figure 4.3.3.2. Comparison of % Dissolution efficiency and time required for 50 % drug dissolved for Tadalafil, Marketed formulation (Tadora-20), Tadalafil-β-CD physical mixtures and inclusion complexes.

Table 4.3.3.6. Dissolution data of Tadalafil – γ-CD Inclusion complex by PHYSICAL MIXTURE in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil – γ-CD Physical mixture (1:1)[#]	% Drug release of Tadalafil – γ-CD Physical mixture (1:2)[#]	% Drug release of Tadalafil – γ-CD Physical mixture (1:3)[#]
0	0.00 ± 1.89	0.00 ± 1.44	0.00 ± 1.49
10	2.96 ± 1.35	2.32 ± 1.38	2.34 ± 1.43
20	4.21 ± 1.93	3.87 ± 1.55	5.62 ± 1.82
30	7.35 ± 1.23	6.29 ± 1.55	8.17 ± 1.56
40	10.04 ± 0.66	9.57 ± 2.83	11.45 ± 2.65
50	12.87 ± 1.36	12.84 ± 1.28	15.73 ± 2.39
60	15.01 ± 1.72	15.98 ± 1.93	17.86 ± 2.58
75	17.98 ± 1.83	17.26 ± 2.93	20.14 ± 1.83
90	18.84 ± 1.22	18.54 ± 3.05	22.00 ± 1.96
105	19.14 ± 1.68	19.82 ± 2.22	23.14 ± 2.69
120	19.84 ± 2.42	20.82 ± 1.86	24.14 ± 1.88

[#] All values are represented as mean ± SD (±n=3).

Table 4.3.3.7. Dissolution data of Tadalafil – γ-CD Inclusion complex by CO-PRECIPITATION METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil – γ-CD Inclusion complex (1:1)[#]	% Drug release of Tadalafil –γ-CD Inclusion complex (1:2)[#]	% Drug release of Tadalafil – γ-CD Inclusion complex (1:3)[#]
0	0.00 ± 2.11	0.00 ± 1.11	0.00 ± 2.46
10	3.88 ± 1.63	2.34 ± 2.54	2.53 ± 1.34
20	7.84 ± 3.20	5.48 ± 1.89	6.50 ± 2.64
30	10.68 ± 3.69	11.03 ± 1.45	12.05 ± 3.45
40	12.92 ± 2.62	15.73 ± 2.54	17.88 ± 3.11
50	15.62 ± 3.76	21.42 ± 2.56	22.02 ± 2.43
60	17.45 ± 2.51	23.83 ± 2.83	24.44 ± 2.24
75	20.45 ± 2.03	25.67 ± 1.67	26.72 ± 2.83
90	22.00 ± 2.15	27.19 ± 3.35	27.99 ± 2.47
105	23.56 ± 2.84	28.16 ± 2.25	28.83 ± 2.81
120	24.56 ± 2.82	28.44 ± 2.73	29.83 ± 1.93

[#] All values are represented as mean ± SD (±n=3).

Table 4.3.3.8. Dissolution data of Tadalafil – γ-CD KNEADING METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil – γ-CD Inclusion complex (1:1)[#]	% Drug release of Tadalafil – γ-CD Inclusion complex (1:2)[#]	% Drug release of Tadalafil – γ-CD Inclusion complex (1:3)[#]
0	0.00 ± 2.42	0.00 ± 1.32	**0.00 ± 2.21**
10	2.89 ± 1.91	4.76 ± 2.54	**4.85 ± 1.57**
20	3.97 ± 1.65	7.28 ± 1.56	**10.74 ± 2.73**
30	5.44 ±2.58	10.75 ± 2.73	**17.67 ± 3.45**
40	8.66 ± 3.46	15.52 ± 2.49	**25.90 ± 2.34**
50	11.01 ± 2.45	19.43 ± 2.94	**36.46 ± 2.56**
60	15.95 ±3.82	22.89 ± 2.58	**41.12 ± 3.86**
75	18.62 ± 2.82	24.42 ± 1.11	**43.68 ± 2.89**
90	19.73 ± 1.93	26.53 ± 1.62	**44.92 ± 1.58**
105	21.97 ± 2.43	27.19 ± 1.45	**45.42 ± 2.47**
120	25.39 ±3.56	28.16 ± 2.83	**46.14 ± 1.35**

[#] All values are represented as mean ± SD (±n=3).

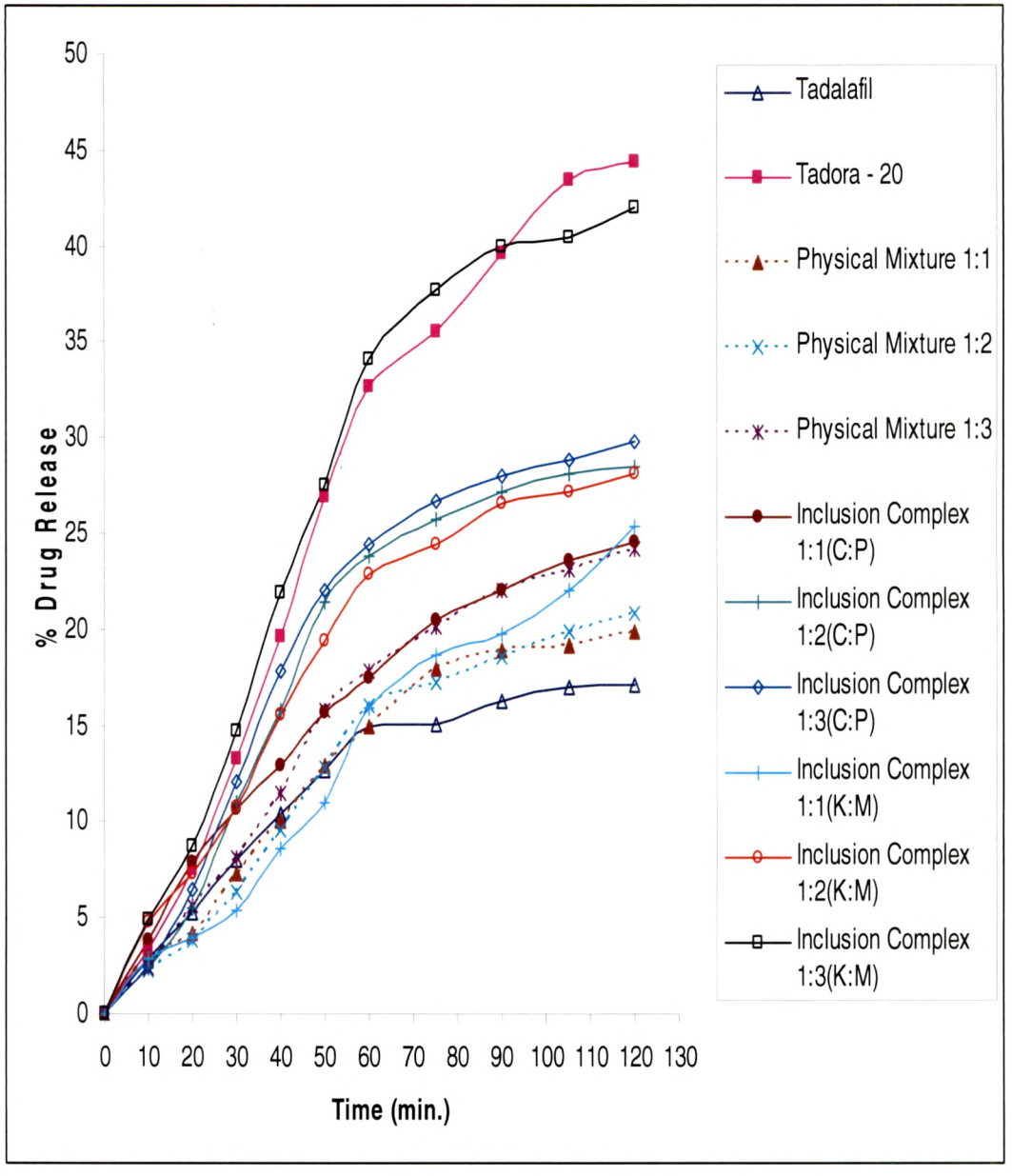

Figure 4.3.3.3. Cumulative % drug released from Tadalafil–γ-CD inclusion complexes, Physical mixtures, Marketed formulation (Tadora-20) and Pure Drug at different time intervals.

Table 4.3.3.9. Dissolution efficiency and time required for 50% drug dissolved ($T_{50\,\%}$) for Tadalafil, Marketed formulation (Tadora-20), Tadalafil-γ-CD Physical mixtures and Inclusion complexes.

Type	Dissolution efficiency for 120 min (%)		Time required for 50% drug dissolved (Min)	
	Mean ± SD[#]	%RSD	Mean ± SD[#]	%RSD
Tadalafil	11.916 ± 1.29	1.00	351.69 ± 1.45	2.50
Tadora - 20	26.8668 ± 1.84	1.72	135.34 ± 1.57	0.69
Physical Mixture 1:1	12.1771 ± 1.49	1.97	302.41 ± 0.83	1.07
Physical Mixture 1:2	12.8258 ± 1.10	1.51	288.18 ± 1.84	1.12
Physical Mixture 1:3	15.1383 ± 1.53	2.00	248.29 ± 1.96	1.59
Inclusion Complex 1:1(C:P)	15.8489 ± 1.16	1.97	244.29 ± 1.06	1.32
Inclusion Complex 1:2(C:P)	19.0539 ± 1.55	1.83	210.97 ± 1.33	0.70
Inclusion Complex 1:3(C:P)	19.9343 ± 1.46	1.81	201.13 ± 1.67	1.34
Inclusion Complex 1:1(K:M)	13.4225 ± 0.46	1.66	236.31 ± 0.67	0.67
Inclusion Complex 1:2(K:M)	18.7235 ± 1.38	1.81	213.06 ± 0.82	1.35
Inclusion Complex 1:3(K:M)	27.3728 ± 0.88	1.27	144.61 ± 1.11	1.69

[#] All values are represented as mean ± SD (±n=3).

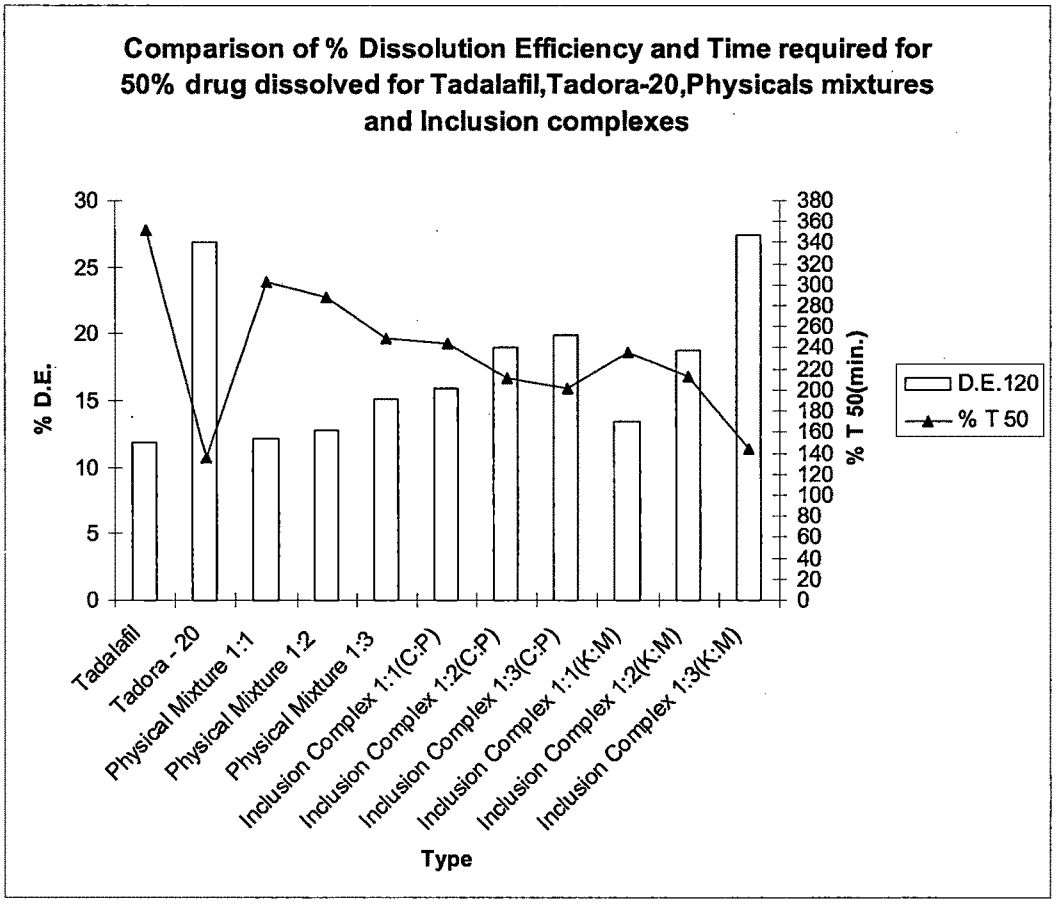

Figure 4.3.3.4. Comparison of % Dissolution efficiency and time required for 50 % drug dissolved for Tadalafil, Marketed formulation (Tadora-20), Tadalafil-γ-CD physical mixtures and inclusion complexes.

Table 4.3.3.10. Dissolution data of Tadalafil–HP-β-CD PHYSICAL MIXTURE in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil – HP-β-CD Physical mixture (1:1)[#]	% Drug release of Tadalafil – HP-β-CD Physical mixture (1:2)[#]	% Drug release of Tadalafil – HP-β-CD Physical mixture (1:3)[#]
0	0.00 ± 1.21	0.00 ± 1.88	0.00 ± 1.35
10	2.22 ± 1.45	4.64 ± 1.36	7.27 ± 1.56
20	3.61 ± 1.23	8.92 ± 1.90	12.22 ± 1.92
30	5.27 ± 1.47	15.25 ± 2.01	19.97 ± 2.22
40	9.39 ± 1.99	21.11 ± 2.36	25.5 ± 1.38
50	15.28 ± 1.57	30.26 ± 1.57	38.58 ± 1.01
60	21.51 ± 1.82	36.46 ± 1.77	44.08 ± 0.11
75	26.88 ± 2.11	40.04 ± 1.45	51.11 ± 0.54
90	32.56 ± 2.31	44.82 ± 1.72	55.45 ± 0.31
105	34.21 ± 1.81	47.57 ± 1.82	59.00 ± 0.46
120	35.11 ± 2.11	50.31 ± 1.92	62.44 ± 1.58

[#] All values are represented as mean ± SD (±n=3).

Table 4.3.3.11. Dissolution data of Tadalafil–HP-β-CD Inclusion complex by CO-PRECIPITATION METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil – HP-β-CD Inclusion complex (1:1)[#]	% Drug release of Tadalafil – HP-β-CD Inclusion complex (1:2)[#]	% Drug release of Tadalafil – HP-β-CD Inclusion complex (1:3)[#]
0	0.00 ± 3.11	0.00 ± 2.34	0.00 ± 2.52
10	2.32 ± 2.82	6.43 ± 2.31	11.21 ± 3.67
20	7.21 ± 2.36	13.75 ± 3.69	31.33 ± 1.45
30	15.63 ± 2.56	20.11 ± 2.83	43.11 ± 1.38
40	23.87 ± 1.73	29.99 ± 1.67	50.55 ± 2.11
50	37.27 ± 3.3	42.58 ± 3.91	58.36 ± 3.45
60	45.97 ± 2.71	49.49 ± 2.88	64.44 ± 2.56
75	51.11 ± 2.4	55.35 ± 1.58	69.44 ± 3.62
90	54.00 ± 1.77	60.66 ± 3.56	72.30 ± 2.88
105	57.37 ± 1.58	65.05 ± 2.74	74.33 ± 2.67
120	60.00 ± 2.54	69.09 ± 3.22	75.45 ± 3.25

[#] All values are represented as mean ± SD (±n=3).

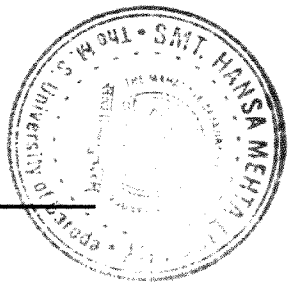

Table 4.3.3.12. Dissolution data of Tadalafil–HP-β-CD Inclusion complex by KNEADING METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil – HP-β-CD Inclusion complex (1:1)#	% Drug release of Tadalafil – HP-β-CD Inclusion complex (1:2)#	% Drug release of Tadalafil – HP-β-CD Inclusion complex (1:3)#
0	0.00 ± 1.15	0.00 ± 1.21	**0.00 ± 0.22**
10	4.21 ± 2.35	8.11 ± 1.45	**11.4 ± 1.12**
20	16.1 ± 1.88	15.11 ± 1.55	**25.35 ± 3.26**
30	30 ± 1.72	35.66 ± 2.43	**39.57 ± 3.74**
40	39.45 ± 1.48	47.57 ± 2.75	**50.01 ± 2.59**
50	51.31 ± 1.96	57.77 ± 3.76	**69.39 ± 2.5**
60	56.06 ± 1.09	60.09 ± 2.54	**79.17 ± 1.78**
75	60.12 ± 1.45	65.55 ± 3.22	**81.77 ± 1.81**
90	63.20 ± 2.34	71.11 ± 2.48	**83.30 ± 2.8**
105	65.32 ± 1.76	75.00 ± 3.58	**85.30 ± 3.35**
120	67.35 ± 2.11	78.12 ± 1.21	**86.95 ± 1.88**

All values are represented as mean ± SD (±n=3).

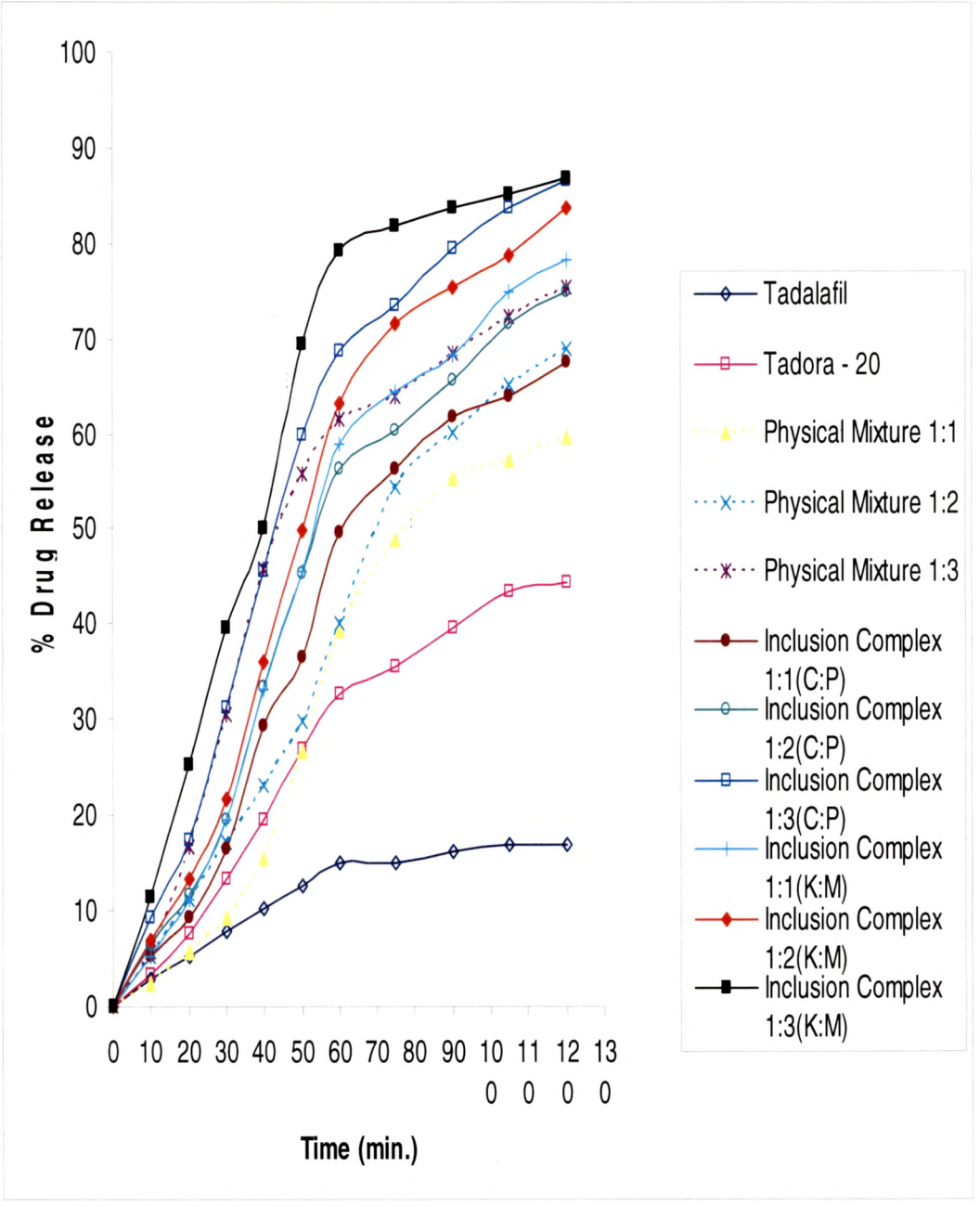

Figure 4.3.3.5. Cumulative % drug released from Tadalafil–HP-β-CD inclusion complexes, Physical mixtures, Marketed formulation (Tadora-20) and Pure Drug at different time intervals.

Table 4.3.3.13. Dissolution efficiency and time required for 50% drug dissolved ($T_{50\ \%}$) for Tadalafil, Marketed formulation (Tadora-20), Tadalafil-HP-β-CD Physical mixtures and Inclusion complexes.

Type	Dissolution efficiency for 120 min (%)		Time required for 50% drug dissolved (Min)	
	Mean ± SD[#]	%RSD	Mean ± SD[#]	%RSD
Tadalafil	11.916 ± 0.98	1.87	351.69 ± 1.63	1.29
Tadora - 20	26.8668 ± 1.61	0.89	135.34 ± 0.52	1.86
Physical Mixture 1:1	19.22 ± 1.91	1.04	170.89 ± 0.67	1.49
Physical Mixture 1:2	30.1777 ± 1.48	0.85	116.93 ± 0.70	1.10
Physical Mixture 1:3	37.8175 ± 1.95	0.96	73.73 ± 0.99	1.53
Inclusion Complex 1:1(C:P)	36.0402 ± 1.93	1.03	73.73 ± 1.70	1.16
Inclusion Complex 1:2(C:P)	41.5108 ± 1.78	0.87	60.61 ± 1.63	1.55
Inclusion Complex 1:3(C:P)	54.6502 ± 1.79	1.18	39.56 ± 1.26	1.46
Inclusion Complex 1:1(K:M)	45.3847 ± 0.74	1.27	48.72 ± 1.36	0.88
Inclusion Complex 1:2(K:M)	51.2843 ± 0.50	1.55	42.04 ± 0.84	1.56
Inclusion Complex 1:3(K:M)	61.1625 ±	1.61	39.99 ± 0.55	0.91

[#] All values are represented as mean ± SD (±n=3).

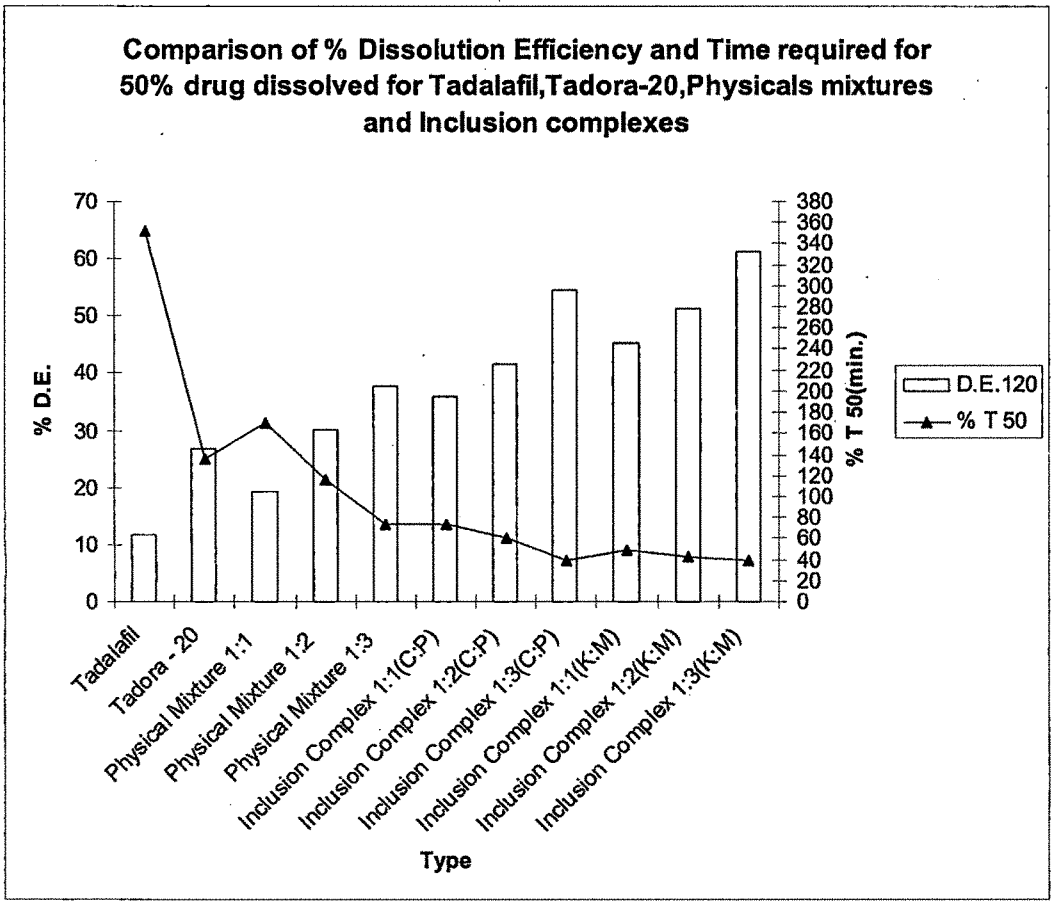

Figure 4.3.3.6. Comparison of % Dissolution efficiency and time required for 50 % drug dissolved for Tadalafil, Marketed formulation (Tadora-20), Tadalafil-HP-β-CD physical mixtures and inclusion complexes.

Table 4.3.3.14. Dissolution data of Tadalafil–DM-β-CD Inclusion complex by PHYSICAL MIXTURE in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil DM–β-CD Physical mixture (1:1)[#]	% Drug release of Tadalafil DM–β-CD Physical mixture (1:2)[#]	% Drug release of Tadalafil DM– β-CD Physical mixture (1:3)[#]
0	0.00 ± 0.21	0.00 ± 1.41	0.00 ± 2.34
10	1.27 ± 0.81	2.01 ± 2.65	2.83 ± 1.46
20	3.60 ± 1.73	4.40 ± 0.11	5.53 ± 2.55
30	6.87 ± 1.92	7.82 ± 1.34	11.81 ± 3.11
40	11.15 ± 2.89	10.09 ± 2.32	15.36 ± 2.73
50	14.71 ± 2.55	13.51 ± 3.21	19.91 ± 1.68
60	16.12 ± 3.43	17.92 ± 2.42	21.47 ± 3.53
75	18.54 ± 2.89	19.48 ± 2.57	23.16 ± 1.56
90	19.26 ± 3.52	21.89 ± 3.21	26.85 ± 1.89
105	20.12 ± 2.56	22.17 ± 2.76	27.72 ± 3.32
120	20.82 ± 3.34	23.17 ± 2.51	28.72 ± 1.01

[#] All values are represented as mean ± SD (±n=3).

Table 4.3.3.15. Dissolution data of Tadalafil–DM-β-CD Inclusion complex by CO-PRECIPITATION METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil DM–β-CD Inclusion complex (1:1)#	% Drug release of Tadalafil DM–β-CD Inclusion complex (1:2)#	% Drug release of Tadalafil DM– β-CD Inclusion complex (1:3)#
0	0.00 ± 1.56	0.00 ± 1.56	0.00 ± 2.32
10	2.83 ± 2.22	3.17 ± 3.22	2.99 ± 2.89
20	5.11 ± 3.26	5.39 ± 1.58	5.69 ± 1.11
30	8.25 ± 2.52	8.81 ± 1.88	11.52 ± 0.56
40	11.67 ±3.57	12.64 ± 1.56	17.80 ± 1.83
50	15.22 ± 1.88	16.30 ± 2.62	25.49 ± 0.89
60	20.33 ± 3.23	21.72 ± 3.89	28.04 ± 2.34
75	23.88 ± 2.45	25.99 ± 3.56	30.46 ± 3.21
90	26.85 ± 1.61	27.27 ± 2.82	32.87 ± 2.65
105	28.13 ± 2.82	29.24 ± 3.11	33.85 ± 1.28
120	29.13 ± 1.58	30.10 ± 2.99	34.15 ± 0.89

All values are represented as mean ± SD (±n=3).

Table 4.3.3.16. Dissolution data of Tadalafil–DM-β-CD KNEADING METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil DM– β-CD Inclusion Complex (1:1)#	% Drug release of Tadalafil DM–β-CD Inclusion Complex (1:2)#	% Drug release of Tadalafil DM– β-CD Inclusion Complex (1:3)#
0	0.00 ± 1.89	0.00 ± 0.56	**0.00 ± 1.11**
10	1.14 ± 2.56	7.09 ± 1.8	**5.03 ± 2.83**
20	3.70 ± 1.65	16.38 ± 1.83	**11.70 ± 1.89**
30	6.67 ± 1.89	22.20 ± 2.67	**21.52 ± 2.56**
40	11.27 ± 2.35	24.83 ± 3.21	**35.26 ± 3.43**
50	15.46 ± 2.43	29.55 ± 2.72	**48.54 ± 1.46**
60	20.68 ± 1.67	30.80 ± 3.56	**54.89 ± 2.33**
75	22.76 ± 3.33	34.95 ± 1.65	**58.36 ± 3.48**
90	24.14 ± 2.83	36.20 ± 2.89	**60.05 ± 2.61**
105	25.67 ± 1.47	37.31 ± 3.11	**62.27 ± 1.53**
120	26.50 ± 3.04	39.25 ± 2.85	**63.46 ± 1.34**

All values are represented as mean ± SD (±n=3).

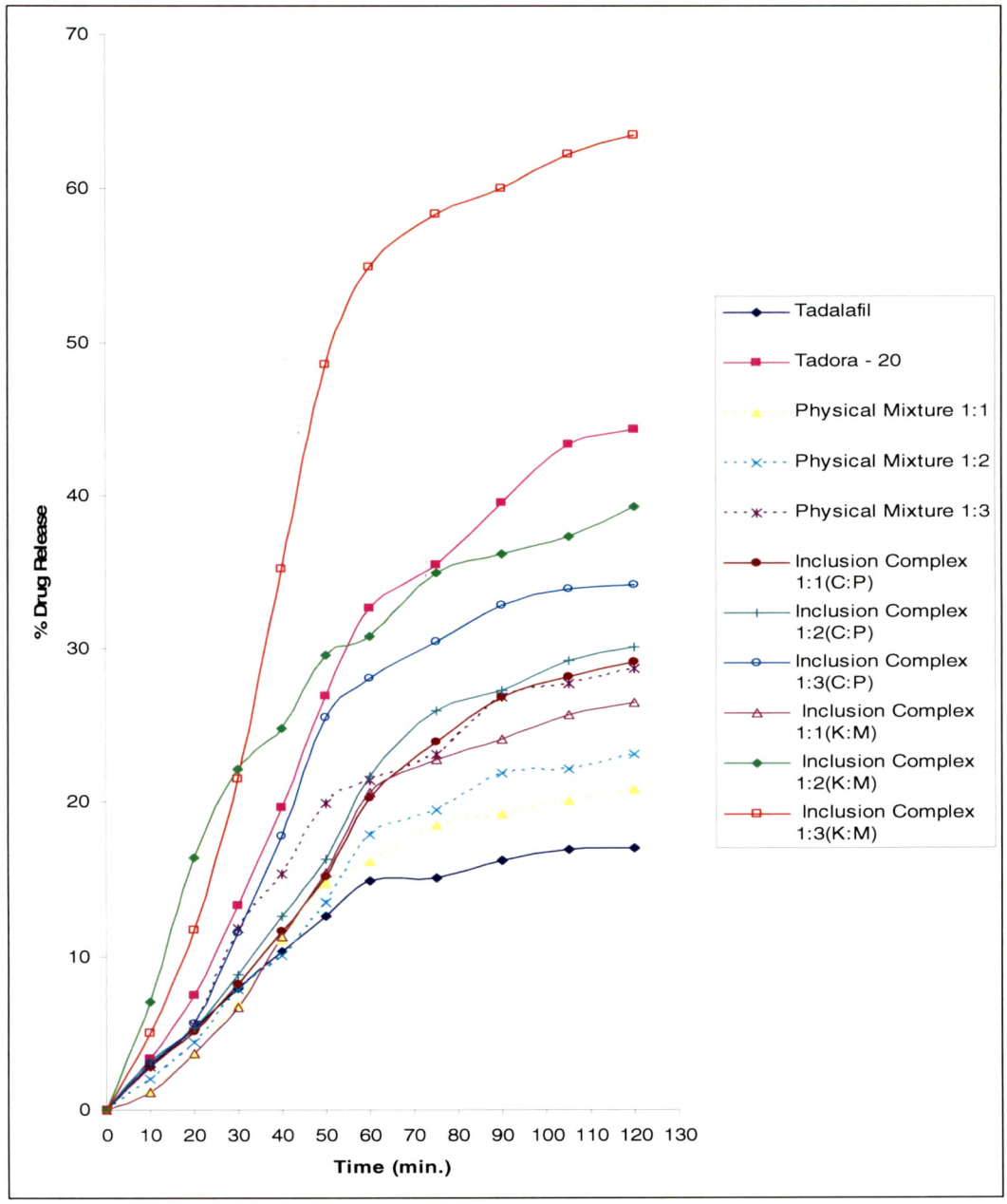

Figure 4.3.3.7. Cumulative % drug released from Tadalafil–DM-β-CD inclusion complexes, Physical mixtures, Marketed formulation (Tadora-20) and Pure Drug at different time intervals.

Table 4.3.3.17. Dissolution efficiency and time required for 50% drug dissolved ($T_{50\%}$) for Tadalafil, Marketed formulation (Tadora-20), Tadalafil-β-CD Physical mixtures and Inclusion complexes.

Type	Dissolution efficiency for 120 min (%)		Time required for 50% drug dissolved (Min)	
	Mean ± SD#	%RSD	Mean ± SD#	%RSD
Tadalafil	11.916 ± 1.87	1.88	351.69 ± 1.29	1.96
Tadora – 20	26.8668 ± 1.28	1.13	135.34 ± 1.07	1.24
Physical Mixture 1:1	13.3537 ± 1.01	1.45	288.18 ± 1.21	1.44
Physical Mixture 1:2	14.4097 ± 0.82	0.82	258.95 ± 1.85	1.56
Physical Mixture 1:3	18.3677 ± 0.93	0.67	208.91 ± 1.20	1.73
Inclusion Complex 1:1(C:P)	17.3858 ± 0.64	1.32	205.97 ± 1.19	0.84
Inclusion Complex 1:2(C:P)	18.3154 ± 0.88	0.94	199.33 ± 0.97	0.39
Inclusion Complex 1:3(C:P)	22.4943 ± 1.19	1.54	175.69 ± 1.35	1.29
Inclusion Complex 1:1(K:M)	16.0683 ±1.09	1.48	226.41 ± 1.46	1.56
Inclusion Complex 1:2(K:M)	27.5564 ± 1.51	2.05	152.86 ± 1.71	1.67
Inclusion Complex 1:3(K:M)	42.4397 ± 0.75	1.88	51.5 ± 1.27	1.43

All values are represented as mean ± SD (±n=3).

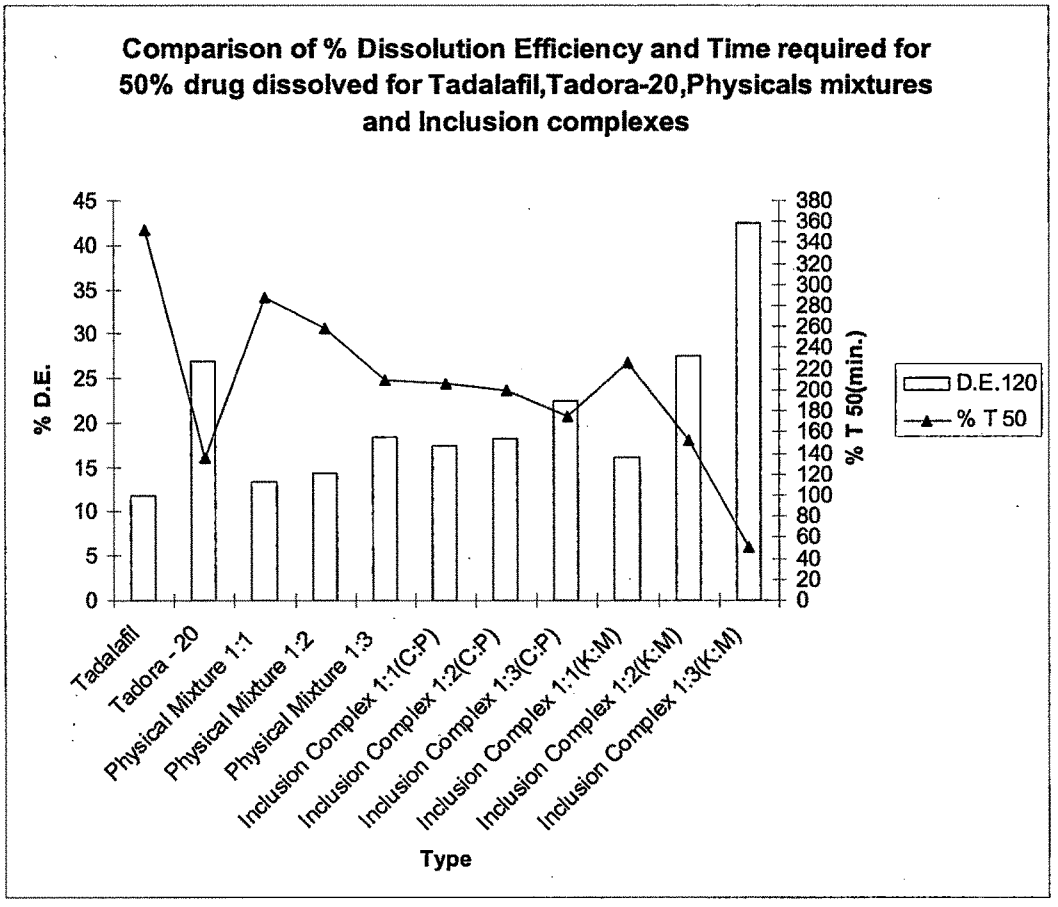

Figure 4.3.3.8. Comparison of % Dissolution efficiency and time required for 50 % drug dissolved for Tadalafil, Marketed formulation (Tadora-20), Tadalafil-DM-β-CD physical mixtures and inclusion complexes.

Table 4.3.3.18. Regression coefficients of Tadalafil–CDs inclusion complexes, Physical mixtures, Marketed formulation (Tadora-20) and Pure Drug

Formulations		Zero-order equation	First-order equation	Higuchi's equation
		r^2	r^2	r^2
Tadalafil		0.8766	0.6909	0.9594
Tadora– 20		0.9495	0.7389	0.9222
Tadalafil – β-CD Physical mixture	**1:1**	0.9872	0.827	**0.8375**
	1:2	0.9601	0.7615	**0.8891**
	1:3	0.8986	0.6217	**0.9276**
Tadalafil – β-CD Inclusion complex (Co-precipitation)	**1:1**	0.9606	0.7346	**0.8987**
	1:2	0.9548	0.7048	**0.9104**
	1:3	0.9358	0.6299	**0.9346**
Tadalafil – β-CD Inclusion complex (Kneading method)	**1:1**	0.9549	0.7188	**0.9015**
	1:2	0.9501	0.6881	**0.9046**
	1:3	0.8692	0.5203	**0.9554**
Tadalafil – γ-CD Physical mixture	**1:1**	0.9246	0.7564	**0.9248**
	1:2	0.938	0.7858	**0.9403**
	1:3	0.9384	0.7491	**0.9409**
Tadalafil – γ-CD Inclusion complex (Co-precipitation)	**1:1**	0.9399	0.6862	**0.9215**
	1:2	0.8828	0.7073	**0.9297**
	1:3	0.8805	0.6871	**0.9785**
Tadalafil – γ-CD Inclusion complex (Kneading method)	**1:1**	0.9785	0.845	**0.899**
	1:2	0.9101	0.6839	**0.9298**
	1:3	0.9109	0.6892	**0.9518**
Tadalafil –HP- β-CD	**1:1**	0.9632	0.8678	**0.8403**

Physical mixture	**1:2**	0.9525	0.7249	**0.9243**
	1:3	0.9529	0.6879	**0.9304**
Tadalafil – HP-β-CD Inclusion complex (Co-precipitation)	**1:1**	0.9513	0.7411	**0.8886**
	1:2	0.9278	0.6889	**0.9284**
	1:3	0.8349	0.5131	**0.9625**
Tadalafil – HP-β-CD Inclusion complex (Kneading method)	**1:1**	0.8853	0.6218	**0.9292**
	1:2	0.8705	0.6094	**0.9343**
	1:3	0.8397	0.5608	**0.9326**
Tadalafil –DM- β-CD Physical mixture	**1:1**	0.9595	0.7414	**0.9158**
	1:2	0.9394	0.7759	**0.9224**
	1:3	0.9277	0.7145	**0.9411**
Tadalafil – DM-β-CD Inclusion complex (Co-precipitation)	**1:1**	0.9795	0.7953	**0.9132**
	1:2	0.9713	0.7793	**0.9154**
	1:3	0.9245	0.7193	**0.9118**
Tadalafil – DM-β-CD Inclusion complex (Kneading method)	**1:1**	0.9504	0.7748	**0.893**
	1:2	0.9021	0.5497	**0.905**
	1:3	0.8721	0.6618	**0.9731**

It can be concluded from the above studies that Tadalafil-β-CD inclusion complex having a ratio 1:3 prepared by kneading method showed maximum incorporation of Tadalafil. This fact was supported by its FTIR, DSC and XRD study. Hence, it was selected further for chemical stability study.

4.3.4. Chemical Stability (Drug retention study) of Tadalafil-cyclodextrins Inclusion complex.

Tadalafil-β-CD Inclusion complex having a ratio 1:3 prepared by kneading method was subjected to accelerated temperature and stress conditions.The Inclusion complex was analyzed for chemical stability. Approximately 5 gms of the formulation was filled in USP type III glass vial and sealed using VP6 crimp on spray pump fitted with 10 µm actuator.

The accelerated stability was performed at 30° C ± 2° C / 65% ± 5% relative humidity (RH) and 40° C ± 2° C / 75% ± 5% RH as per ICH guideline (Photostbility testing for new drug substances and products- Q1A (R2)). The duration of stability was 6 months and samples were withdrawn at predetermined time intervals after 1 month, 2 months, 3 months and 6 months consider as a test samples. The method is well described in ICH guidelines Q1A (R2) and Q1B. Withdrawn samples were frequently compared with *in vitro* dissolution profiles of a test product before and after stability study as per the SUPAC IR guidelines which helps in assures similarity in the product performance and bioequivalence. The similarity factor f_2 was calculated using the equation proposed by various scientist[13,14], in which initial dissolution data considered as a reference values.

$$f_2 = 50LOG\{[1+\frac{1}{n}\sum_{t=1} n(R_t - T_t)^2]^{-0.5} \times 100\}$$

Where R_t and T_t are the percentage of drug dissolved for Reference and Test samples at each time point. An f_2 value between 50 and 100 suggests the dissolution profiles are consider as similar.

Table 4.3.4.1. Dissolution data for stability study of Tadalafil – β-CD Inclusion complex (1:3) prepared by KNEADING METHOD in HCl buffer pH 1.2.

Time in Min.	% Drug release of Tadalafil β-CD Inclusion complex (1:3)# (Reference)	% Drug release of Tadalafil β-CD Inclusion complex (1:3)# after 1 month stability	% Drug release of Tadalafil β-CD Inclusion complex (1:3)# after 2 months stability	% Drug release of Tadalafil β-CD Inclusion complex (1:3)# after 4 months stability	% Drug release of Tadalafil β-CD Inclusion complex (1:3)# after 6 months stability
0	0.00 ± 2.11	0.00 ± 1.5	0.00 ± 2.15	0.00 ± 1.35	0.00 ± 2.63
10	11.32 ± 1.41	10.05 ± 2.55	9.25 ± 2.45	7.35 ± 2.86	5.67 ± 7.27
20	24.45 ± 3.21	23.66 ± 2.25	21.78 ± 3.53	18.75 ± 1.55	16.15 ± 6.58
30	39.66 ± 3.42	37.25 ± 1.95	35.55 ± 2.88	32.67 ± 3.78	30.89 ± 5.55
40	51.82 ± 2.64	49.55 ± 1.45	47.57 ± 3.65	45.60 ± 4.82	43.33 ± 3.57
50	59.59 ± 2.83	58.37 ± 2.35	55.35 ± 2.98	52.11 ±5.05	50.44 ± 4.58
60	66.35 ±2.91	63.31 ± 2.55	64.59 ± 4.15	63.37 ± 7.21	62.11 ± 5.45
75	72.73 ± 3.46	72.17 ± 3.81	71.38 ± 3.45	69.45 ± 6.43	67.23 ± 6.73
90	81.8 ± 2.82	81.29 ± 2.87	80.87 ± 4.15	79.29 ± 3.63	78.58 ± 4.56
105	85.26 ± 2.57	84.94 ± 3.09	84.22 ± 2.65	83.33 ± 4.56	81.77 ± 3.89
120	90.38 ± 1.25	90.03 ± 2.35	89.39 ± 3.85	88.38 ± 5.87	86.91 ± 6.23

All values are represented as mean ± SD (±n=3).

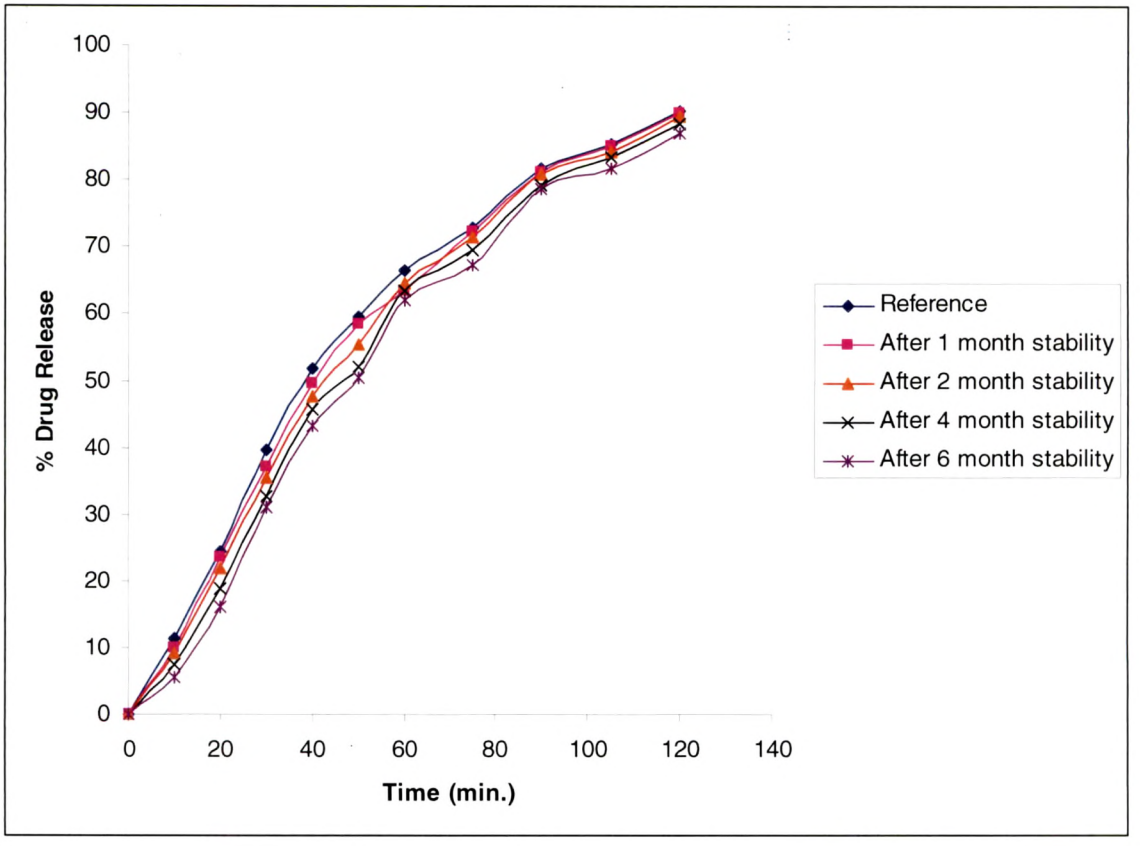

Figure : 4.3.4.1. Cumulative % drug released from Tadalafil-β-CD inclusion complex(1:3) by Kneading method for Stability study at different time intervals.

Table :4.3.4.2. Similarity factor (f_2) and Student's T-test values for the stability profile of Tadalafil-β-CD inclusion complex(1:3) by Kneading method for 6 months.

Batch	F_2	T stat	T cri
Reference batch compared with storage batch for 6 months.	74.60	0.4169	2.0859

4.3.5. Results and Discussion.

Tadalafil-CDs Inclusion complexes were successfully prepared using different methods like Kneading method and Co- precipitation method. The complexing agents selected were β-CD, γ-CD, HP-β-CD and DM-β-CD according to their suitability for oral administration.

Solubility study is the preliminary study to identify the interaction between drug and CD in solution because it gives not only the solubilizing ability of the host molecules but also the stability constant of the complexes by analysis of the solubility curve.

The phase solubility profiles for the complex formation between Tadalafil and CDs in aqueous solution (HCl buffer pH 1.2, water and Phosphate buffer pH 6.8) at 37° C are shown in figure 4.3.2.1.1, 4.3.2.1.2 and 4.3.2.1.3.[15] .The primary study indicated that Tadalafil is chemically stable in water for at least 7 days at 37° C. The extremely low solubility of Tadalafil (0.98 ± 0.004 µg/mL in water at 37° C) was increased in a concentration depending manner by adding CDs. The data obtained by this study, showed enhanced solubility of Tadalafil in β-CD containing aqueous solution (HCl buffer pH 1.2) shown the A_P type of isotherm having a regression values (r^2) < 0.99, which was having a positive deviation from linearity indicating a continuous increase in the stoichiometry of the complex, that is the original 1:1 complex tends to associated with further guest, forming 2:3 compositions etc,. As can be seen, the solubility of the Tadalafil increased linearly with the increase of γ-CD, HP-β-CD and DM-β-CD concentration, which giving rise to A_L - type solubility diagrams with strictly linear ascent with a regression values (r^2) > 0.99[16]. This linear Tadalafil-CDs correlation, with a slope of less than 1, suggested the formation of 1:1 (mol/mol) Tadalafil-CDs inclusion complexes at the different pH values. The calculated stability constant values are shown in Table 4.3.2.1.1. These results indicated that Tadalafil-CDs complex (1:1 molar ratio) are sufficiently stable in HCl buffer pH 1.2 and the values of stability constants were more than 100 M^{-1}.Values of stability constants were less than 100 M^{-1} when the another aqueous solutions (water and phosphate buffer pH 6.8) were used for stability study, which indicated that the inclusion complexes of Tadalafil-CDs were not stable. As per results shown in Table 4.3.2.1.1., highest solubility (39.49 ± 2.47 µg/mL) of Tadalafil and stability constant (351.88 ± 21M^{-1}) of Tadalafil-β-CD inclusion complex (1:1 molar ratio) were found in HCl buffer pH 1.2. This may be due to the real contribution of β-CD on the increased solubility of Tadalafil, which was almost completely un-ionized at acidic pH.

According to the solubility studies data, suitable ratios 1:1, 2:1 and 3:1 of CDs along

with Tadalafil were selected to form inclusion complexes.

The inclusion efficiency data of Tadalafil-CDs complexes were recorded in Table 4.3.2.2.1., 4.3.2.2.2, 4.3.2.2.3. and 4.3.2.2.4. The % inclusion efficiency of 1:3 Tadalafil-CDs inclusion complexes were more than 96.2 ± 0.82 % compared with the other ratios used for inclusion complexes and all physical mixtures having a values in the range of 60.2 ± 0.11 to 91.48 ± 0.70 indicating that Tadalafil was uniformly distributed in all 1:3 inclusion complex whereas, the other ratios used for inclusion complexes and physical mixtures did not show satisfactory drug incorporation.

The prepared physical mixtures, inclusion complexes, pure Tadalafil and excipients were subjected to FTIR, DSC and XRD study for their characterization.

FTIR spectra of Tadalafil, Physical mixture and Inclusion complexes were obtained and interpreted in following manner (Table 4.3.5.1.and Table 4.3.5.2.)

Table 4.3.5.1. Important peaks of FTIR spectra of pure Tadalafil, β-CD, γ-CD, HP-β-CD and DM-β-CD.

	Important Peaks	
Pure Tadalafil	- aromatic C-H stretching peaks -	2855 cm^{-1}
	- N-H stretching peak of pyrazine -	2950 cm^{-1}
	- C=O stretching band -	1850 cm^{-1}
	- C=N stretching peak of tetrazole -	1750 cm^{-1}
	-aromatic C=C stretching -	1677.95 and 1650 cm^{-1}
	- C-N stretching peak -	1499 cm^{-1}
	- C-O stretching band of 1,3 banzo dioxole -	1297 cm^{-1}
Pure β-CD	- intense bands at 3465 - 3247 cm^{-1} corresponding to absorption by hydrogen bonded OH groups.	
Pure γ-CD		
Pure HP-β-CD	- C-H and $-CH_2$ stretching bands - 3000 – 2800 cm^{-1}	
Pure DM-β-CD		

Table 4.3.5.2. Important peaks of prepared Tadalafil-CDs physical mixtures and inclusion complexes.

Tadalafil-CDs (molar ratios)	Physical Mixture Important Peaks	Inclusion Complex Important Peaks
	- peaks of both the Tadalafil	- (right shift of OH stretching peak

Tadalafil-β-CD (1:3 ratio)	and β-CD was observed. - reduction in the peak intensity of Tadalafil. - right shift of OH stretching peak from 3352.05 to 3336.62 cm^{-1}	from 3290 to 3150cm^{-1})* - left shift of CH stretching peak of β-CD from 2923.88 to 2980.60cm^{-1}
Tadalafil-γ-CD (1:3 ratio)	- peaks of both the Tadalafil and γ-CD was observed. - reduction in the peak intensity of Tadalafil. -right shift of OH stretching peak from 3380 to 3338.55cm^{-1}	- (right shift of OH stretching peak from 3340 to 3250cm^{-1})* - left shift of CH stretching peak of γ-CD from 2929.67 to 2950 cm^{-1}
Tadalafil-HP-β-CD (1:3 ratio)	- peaks of both the Tadalafil and HP-β-CD was observed. - reduction in the peak intensity of Tadalafil. - right shift of OH stretching peak from 3380.98 to 3330.84 cm^{-1}	- (right shift of OH stretching peak from 3407.98 to 3350.34 cm^{-1})* - left shift of CH stretching peak of HP-β-CD from 2925.81 to 2950cm^{-1}
Tadalafil-DM-β-CD (1:3 ratio)	- peaks of both the Tadalafil and DM-β-CD was observed. - reduction in the peak intensity of Tadalafil. - right shift of OH stretching peak from 3465.84 to 3446.56 cm^{-1}	- (right shift of OH stretching peak from 3421.48 to 3370 cm^{-1})* - left shift of CH stretching peak of DM-β-CD from 2923.88 to 2960 cm^{-1}

*** results suggested that some of the existing bonds formed between the OH groups were disturbed after the formation of inclusion complexes.**

The DSC thermogram of Tadalafil (Figure 4.3.2.4.5) showed sharp endothermic peak at 307.00°C (ΔH value -453.36 mJ and -266.68J/g, respectively) corresponding to the melting point. The DSC thermogram of β-CD (Figure 4.3.2.4.1.) has large broad endothermic peak between 130.00 °C to 160.00 °C (148.59 °C) (ΔH value -472.42 mJ and -78.74J/g, respectively) that was related to the loss of hydration water of the starting material. β-CD decomposes at about 300.00 °C, so there was no trace of melting peak of β-CD in the chosen temperature range, while no endothermic peak was observed in γ-CD, HP-β-CD and DM-β-

CD (Figure 4.3.2.4.2, 4.3.2.4.3. and 4.3.2.4.4.).

Physical mixture of Tadalafil-β-CD (Figure 4.3.2.4.6) showed small sharp endothermic peak at 297.43 °C corresponding to the Tadalafil (307.00 °C, ΔH value -453.36 mJ and -266.68J/g, respectively),while an inclusion complex of Tadalafil-β-CD showed minute peak at 295.60°C (ΔH value -48.39 mJ and -28.46J/g, respectively) by co-precipitation method (Figure 4.3.2.4.7).**The DSC thermogram of Tadalafil-β-CD inclusion complex prepared by kneading method(Figure 4.3.2.4.8.) did not show any endothermic peak, suggesting that kneading method was suitable method for formation of inclusion complex of Tadalafil.**

Physical mixture of Tadalafil-γ-CD (Figure 4.3.2.4.9.) showed a sharp endothermic peak at 319.92 °C corresponding to the Tadalafil (307.00 °C).The inclusion complexes of Tadalafil-γ-CD prepared by co-precipitation method (Figure 4.3.2.4.10.) showed an endothermic minute peak at 322.90°C. The Tadalafil-γ-CD inclusion complex prepared by kneading method (Figure 4.3.2.4.11.) showed a small endothermic peak at 333.67 °C corresponding to the Tadalafil.

Physical mixture of Tadalafil-HP-β-CD showed (Figure 4.3.2.4.12) a broad endothermic peak at 336.34 °C corresponding to the Tadalafil (307.00 °C), while an inclusion complex of Tadalafil-HP-β-CD showed a minute peak at 294.34 °C by co-precipitation method (Figure 4.3.2.4.13). Observed form the data, DSC thermogram of Tadalafil-HP-β-CD inclusion complex prepared by kneading method (Figure 4.3.2.4.14.) showed a small endothermic peak at 297.69 °C. The drastic change in temperature suggested the maximum incorporation of Tadalafil into the HP-β-CD.

Physical mixture of Tadalafil-DM-β-CD (Figure 4.3.2.4.15.) showed a broad endothermic peak at 345.46 °C corresponding to the Tadalafil (307.00 °C), while an inclusion complex of Tadalafil-DM-β-CD showed a small endothermic peak at (355 °C) by co-precipitation method and (343.92 °C) by kneading method corresponding to the Tadalafil. (Figure 4.3.2.4.16. and 4.3.2.4.17.)

Powder XRD study was used to measure the crystallinity of the formed inclusion complexes. The peak position (angel of diffraction) is an identification tool of a crystal structure, where as the numbers of peaks is a measure of samples crystallinity in a diffractogram[17]. The formation of an amorphous state proves that the drug was dispersed in a molecular state with CD. It was shown by various researchers that the formation of a diffused diffraction pattern, appearance of new peaks, and disappearance of a characteristic peaks of the guest as evidence for the formation of inclusion complexes of a drug with

CDs[18,19,20,21].

The powder X-ray diffraction patterns of pure Tadalafil are represented in Figure 4.3.2.5.5.The diffractogram of Tadalafil exhibited a series of intense peaks at 7.25, 10.60, 12.52,14.53, 21.68, 24.18 and 25.03 °,which were indicative their crystallinity. β-CD (Figure 4.3.2.5.1) exhibited characteristic peaks at 12.74, 21.18 and 22.96° due to its crystalline nature. γ-CD (Figure 4.3.2.5.2.) exhibited characteristic peaks at 4.61, 8.01, 9.09, 14.22,17.81 and 22.56 °. The diffraction peaks were not observed in the spectrum of HP-β-CD (Figure 4.3.2.5.3.) indicating that HP-β-CD was an amorphous compound. DM-β-CD (Figure 4.3.2.5.4) exhibited characteristic peaks at 7.57, 8.51 10.99, 12.16, 13.43, 17.53, and 18.98 °.

The X-ray diffraction patterns of the physical mixture of Tadalafil and β-CD (Figure 4.3.2.5.6) was approximately superimposition of the patterns of the pure Tadalafil and β-CD. The number of peaks was reduced and the peak intensity was also decreased. In contrast to the data observed, inclusion complex of **Tadalafil-β-CD complexes** (Figure 4.3.2.5.7. and 4.3.2.5.8.) **showed a halo pattern, with the disappearance of all the peaks corresponding to the both Tadalafil and β-CD, indicating the incorporation of Tadalafil in to the β-CD by formation of inclusion complex.**

The powder X-ray diffraction pattern of Tadalafil-γ-CD physical mixture (Figure 4.3.2.5.9.) showed that the peaks of Tadalafil were less intense and less in number, suggesting that slight complexation may have occurred in the process of mixing. Whereas X-ray diffraction patterns of Tadalafil-γ-CD inclusion complexes (Figure 4.3.2.5.10. and 4.3.2.5.11.) did not show any prominent peak, as well as less numbers of peaks compared with pure components and the physical mixture.

The X-ray diffraction patterns of physical mixture having Tadalafil and HP-β-CD (Figure 4.3.2.5.12.) was approximately superimposition of the patterns of the pure Tadalafil and HP-β-CD. The number of peaks was reduced and the peak intensity was also decreased. In contrast to the data observed, inclusion complex of Tadalafil-HP-β-CD complexes (Figure 4.3.2.5.13. and 4.3.2.5.14.) showed a halo pattern, with the disappearance of all the peaks corresponding to the both Tadalafil and HP-β-CD, indicated that Tadalafil was completely incorporated in to the cavity of HP-β-CD.

In the case of Tadalafil DM-β-CD physical mixture (Figure 4.3.2.5.15.),intensity of the peaks was reduced compared with pure Tadalafil and DM-β-CD, while in the case of inclusion complexes of Tadalafil- DM-β-CD(Figure 4.3.2.5.16. and 4.3.2.5.17.), diffraction pattern was totally changed compared with physical mixture and pure components. The peak intensity and number of peaks was drastically reduced. This indicated

that the crystallinity of Tadalafil was decreased when the proportion of DM-β-CD in inclusion complex was increased.

It can be concluded from the XRD study that, Drug-CDs inclusion complexes having a ratio of 1:3 showed less numbers of peaks having low intensity. The diffraction pattern for the inclusion complexes of Tadalafil-β-CD, Tadalafil HP-β-CD and Tadalafil-DM-β-CD was changed altogether. These positive effects might be involved in enhancement of dissolution characteristic of Tadalafil.

In vitro dissolution studies were performed to evaluate relative solubility behavior of different formulations of Tadalafil. From the phase solubility study, HCl buffer pH 1.2 was found to be suitable dissolution medium as it showed maximum drug release. Dissolution study were carried for all Tadalafil-CDs physical mixtures in different ratios like(1:1, 1:2 and 1:3) as well as Tadalafil-CDs inclusion complexes prepared in different ratios like (1:1, 1:2 and 1:3) by using co-precipitation and kneading methods and the maximum mean cumulative % drug dissolved ± SD are shown in Table 4.3.5.3.

Table 4.3.5.3.: Maximum % cumulative drug release of all physical mixtures, inclusion complexes, Tadora - 20 and pure Tadalafil.

Method	Tadalafil-CDs Ratios	Maximum % cumulative drug release ± SD			
		Tadalafil-β-CD	Tadalafil-γ-CD	Tadalafil-HP-β-CD	Tadalafil-DM-β-CD
Physical Mixture	1:1	59.58 ± 2.61	19.84 ± 2.42	35.11 ± 2.11	20.82 ± 3.34
	1:2	68.92 ± 2.55	20.82 ± 1.86	50.31 ± 1.92	23.17 ± 2.51
	1:3	75.36 ± 3.11	24.14 ± 1.88	62.44 ± 1.58	28.72 ± 1.01
Co-precipitation Method	1:1	67.65 ± 2.01	24.56 ± 2.82	60.00 ± 2.54	29.13 ± 1.58
	1:2	74.98 ± 2.53	28.44 ± 2.73	69.09 ± 3.22	30.10 ± 2.99
	1:3	86.72 ± 1.99	29.83 ± 1.93	75.45 ± 3.25	34.15 ± 0.89
Kneading Method	1:1	78.32 ± 2.76	25.39 ± 3.56	67.35 ± 2.11	26.50 ± 3.04
	1:2	83.72 ± 3.33	28.16 ± 2.83	78.12 ± 1.21	39.25 ± 2.85
	1:3	**90.38 ± 1.25**	**46.14 ± 1.35**	**86.95 ± 1.88**	**63.46 ± 1.34**
TADORA – 20		44.33 ± 0.62			
Pure Tadalafil		17.06 ± 1.11			

. These are graphically represented in Figure 4.3.3.1, 4.3.3.3., 4.3.3.5. and 4.3.3.7. respectively. It is evident from the data that optimized Tadalafil-CDs inclusion complexes

prepared by kneading method showed better drug release than other inclusion complexes, physical mixtures, marketed formulation (Tadora-20) and drug solution.

Tadalafil-β-CD inclusion complex(1:3 ratio) showed 2.03-fold higher diffusion compared to Tadora-20 and 5.29-fold higher diffusion compared to Tadalafil solution, Tadalafil-γ-CD inclusion complex(1:3 ratio) showed similar % drug diffusion compared to Tadora-20 and 2.70-fold higher diffusion compared to Tadalafil solution. While Tadalafil-HP-β-CD inclusion complex (1:3 ratio) showed 1.96-fold higher diffusion compared to Tadora-20 and 5.09-fold higher diffusion compared to Tadalafil solution and Tadalafil-DM-β-CD inclusion complex (1:3 ratio) showed 1.43-fold higher diffusion compared to Tadora-20 and 3.71-fold higher diffusion compared to Tadalafil solution. (All above complexes are prepared by kneading method).

The regression study was carried out for all inclusion complexes, physical mixtures, Tadora-20 and pure Tadalafil. The graphs of percent drug dissolved vs. time were found to be non-linear, suggesting that the dissolution pattern did not follow zero order kinetics. However, the correlation coefficients indicated that Higuchi's model was found to be the best-fit curve compared with zero order and first order kinetic for all the tested formulations.

The values of DE_{120min} and T_{50} study for all Tadalafil-CDs physical mixtures, inclusion complexes, Tadalafil and marketed formulation (Tadora – 20) are shown in Table 4.3.3.5., 4.3.3.9.,4.3.3.13. and 4.3.3.17. These are graphically represented in Figures 4.3.3.2., 4.3.3.4., 4.3.3.6. and 4.3.3.8. **All this results indicated that inclusion complex of Tadalafil-β-CD in the ratio of 1:3, prepared by kneading method was having highest dissolution efficiency (64.72%) among the all inclusion complexes. Reduction in the time taken for 50 % dissolution (T_{50} value-30.20 min.) indicated that the inclusion complex was having a rapid and higher dissolution rate.** The increase in dissolution of Tadalafil from the inclusion complexes might be attributed to the factors such as a reduction in particle size of the drug in the presence of CDs, increase in the surface area, reduced crystallinity and increase in the solubility of the drug in presence of CDs. As the inclusion complex of Tadalafil-β-CD in the ratio of 1:3, prepared by kneading method was having rapid dissolution rate (less dissolution time) was further selected for stability study.

Stability study was carried out as per ICH Q1A (R2) and SUPAC IR guidelines. The sample was stored at 30° C ± 2° C / 65% ± 5% relative humidity (RH) and 40° C ± 2° C / 75% ± 5% RH for 6 months time duration. The samples were withdrawn at predetermine time intervals upto 6 months and assessed for *in vitro* dissolution study. The dissolution data are

mention in Table 4.3.4.1. and graphically it was represented in Figure 4.3.4.1. As per the result obtained, change of cumulative percentage of drug release after of 6 months was from 4.0 % to 9.0%. The dissolution curve (Fig. 4.3.4.1) also show similar changes in dissolution patterns which indicated that, Tadalafil was stable in the inclusion complex. The results of student's t-test and similarity factor (f_2) show insignificant difference between the dissolution profiles (Table 4.3.4.2). The calculated f_2 values for the batches are higher than 50. Hence, it can be concluded that the there is insignificant change in the formulated product on storage.

It can be concluded from the above studies that Tadalafil-β-CD inclusion complex having a ratio 1:3 prepared by kneading method showed maximum incorporation of Tadalafil. This fact was supported by its FTIR, DSC and XRD study. It also showed higher dissolution rate compared to all other formulations. The dissolution rate and DE_{120} values were increased as the proportion β-CD in inclusion complexes were increased. So it was selected for further *in-vivo* pharmacokinetic study.

4.3.6. References

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