Chapter 3

Analytical Method Development

3.1 Materials

Docetaxel Trihydrate (DTX) was obtained as gift sample from Sun Pharma Advanced Research Centre (SPARC), Vadodara, India. Vinorelbine Tartrate (VBT) was obtained as gift sample from Cipla Limited, Mumbai, India. Triethylamine was purchased from S.D. Fine-Chem Limited, Mumbai, India. Chloroform, Methanol and Acetonitrile were of HPLC grade and purchased from Merck Chemicals, Mumbai, India. Monosodium phosphate monohydrate and Disodium phosphate heptahydrate were purchased from HB chemicals, Vadodara, India. Double distilled water used in the study was filtered using 0.22-µm nylon filter (Nylon N66 membrane filters 47 mm, Rankem, India). All the other solvents and reagents used were of analytical grade.

3.2 Analytical Methods for Estimation of DTX

3.2.1 Estimation of DTX by UV-Visible Spectrophotometer

Standard stock solution (100µg/mL) was prepared by dissolving 5 mg of DTX in 50 ml methanol. The UV-visible spectrophotometric method of analysis was developed by obtaining spectra of DTX solution (10µg/mL) in the ultraviolet range 200-400 nm to determine its λmax. Further dilutions were made from stock solution and calibration curve was plotted between 2 to 18 µg/mL. The above procedure was repeated three times. The interference between the human serum albumin (HSA) and the drug was also studied by UV. The absorbance of 10 µg/mL DTX solution, HSA solution and the mixture of DTX and HSA solution were taken at λmax of drug by UV-Visible Spectrophotometer.

3.2.2 Estimation of DTX by Reverse Phase-HPLC (RP-HPLC)

Instrument

HPLC: Shimadzu LC-20AT

Detector: UV-visible detector

Chromatographic Conditions

Column: Kromasil C18 column (150 x 4.6 mm x 5 µm)

Detection wavelength: 229 nm

Flow rate: 1 mL/min

Run time: 10 min

Injection volume: 20 µl (Rheodyne 7725 injector valve with fixed loop at 20 µl)

Diluent: Methanol

Mobile Phase Preparation

Solution A: Acetonitrile

Solution B: Water

Mix solution A and B in ratio of 60:40

Method

Stock solution of Docetaxel (100 µg/ml) was prepared by dissolving 5 mg of drug in 50 ml methanol. Suitable aliquots of the stock solution were pipetted into 1.5 ml microcentrifuge tubes and the volume was made up to 1 ml with methanol to give final concentrations of 1-100 µg/ml. 20 µl of these solutions was injected in HPLC and analyzed using above mentioned parameters. The samples were analyzed in triplicate.

Additionally for estimation of drug in diffusion media calibration curve was developed using RP-HPLC. As the DTX is not completely soluble in PBS 7.4 so phosphate buffer along with methanol in a ratio of 7:3 was used.

3.2.3 Analytical Method Validation

The method was validated for accuracy, precision, linearity, detection limit and quantitation limit.

3.2.3.1 Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations of analyte in sample (1). Linearity of a light absorption determination should be examined to ensure that Beer’s law operates over the range of interest. Least square regression method was used to determine the regression coefficient, r^2 and the equation for the best fitting line. The method can said to be linear for estimation of drug if r^2 is near to 1.

3.2.3.2 Accuracy

Accuracy refers to the closeness of an individual observation or mean of the observations to true value (2). The “true” value is the result which would be observed in absence of error. Accuracy of the assay is defined as the percentage of the agreement between the measured value and the true value as follows (3).The determined mean value of the obtained data should lie within 2 % of the true value for analytical methods and 15% of the true value for bio-analytical method for acceptable accuracy. Accuracy was expressed as the percentage relative error and calculated by using following formula:

Accuracy (% Relative error) = (True value – Measured value/True value)*100

3.2.3.3 Precision

It refers to the extent of variability of a group of measurements observed under similar conditions. Precision provides an indication of random errors and is generally subdivided into two cases: repeatability and reproducibility, which were determined by calculating RSD (Relative standard deviation) or CV (Coefficient of variation) of inter-day and intra-day determinations (4). One of the common ways of expressing the variability, which takes into account its relative magnitude, is the ratio of standard deviation (SD) and mean. This ratio, often expressed as a percentage, is called the coefficient of variation (CV) or the relative standard deviation (RSD). For pharmaceuticals the precision value should be better than 2%.

3.2.3.4 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

3.2.3.5 Detection Limit (DL) & Quantitation Limit (QL)

The DL of an individual analytical procedure is the lowest concentration of analyte in a sample which can be detected but not necessarily quantitated as an exact value, while, the QL is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. As per guidelines the DL and QL of the developed analytical methods were determined by using calibration standards. DL and QL were calculated as 3.3 r/S and 10 r/S, respectively, where S is the slope of the calibration curve and r is the standard deviation of y-intercept of regression equation.

3.3 Results and Discussion

3.3.1 Estimation of DTX by UV-Visible Spectrophotometer

DTX in methanol yields characteristic curve when scanned in UV wavelength range and absorption maxima was found at 229 nm shown in **Figure 3.1**. The linearity was observed between absorbance and concentration of DTX in the range of 2-18 µg/mL. Data are given in **Table 3.1** and the standard curve is shown in **Figure 3.2**.

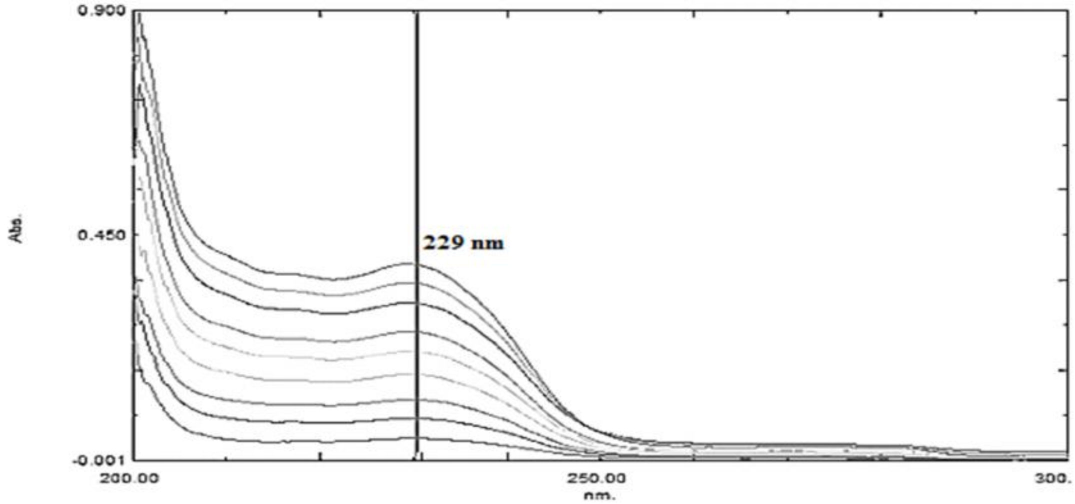


Figure 3. 1 UV Spectrum of DTX in Methanol

Table 3. 1 Calibration Data for DTX in Methanol by UV spectroscopy

Sr. No.	Concentration (µg/mL)	Mean Absorbance ± SD*	% RSD
1.	2	0.041±0.0006	1.561
2.	4	0.080±0.0015	1.875
3.	6	0.121±0.002	1.653
4.	8	0.161±0.003	1.863
5.	10	0.201±0.004	1.990
6.	12	0.239±0.004	1.674
7.	14	0.281±0.004	1.423
8.	16	0.322±0.005	1.553
9.	18	0.361±0.006	1.662

* The experiment was performed in triplicate (n=3)

The regression analysis of the plot using the method of least squares was made to evaluate the intercept, slope and correlation coefficient (r^2). The high value of correlation coefficient of the regression equation and the negligible value of intercept confirm the linearity of calibration plot. Parameters indicating linearity for the developed UV spectrometric method of analysis for DTX are shown in **Table 3.2.**

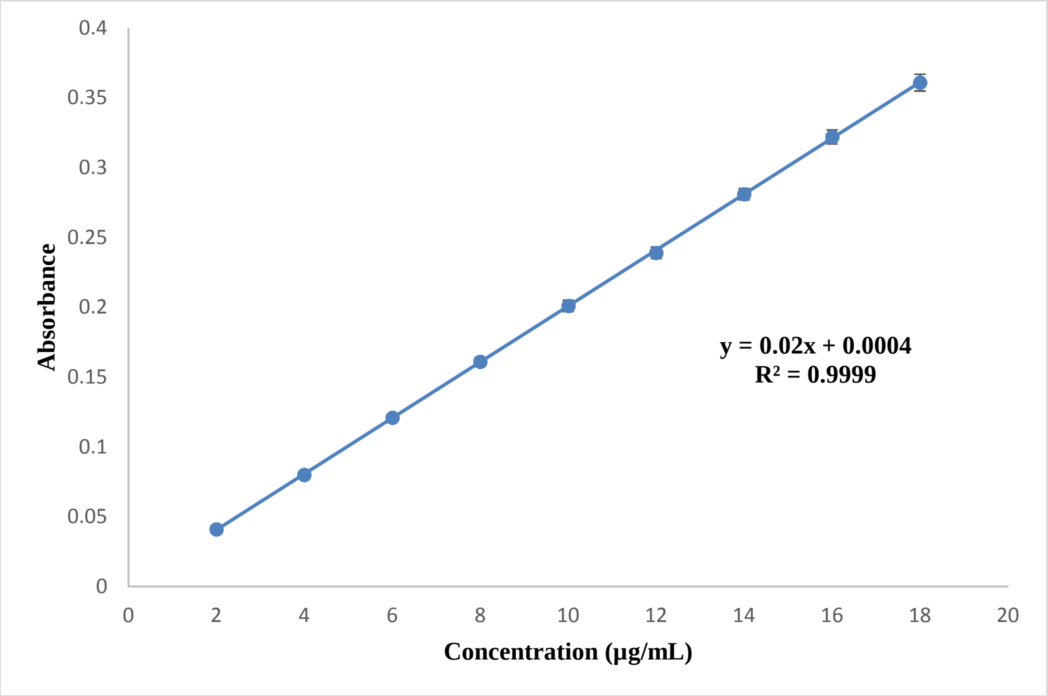


Figure 3. 2 Standard Plot of DTX in Methanol by UV spectroscopy

Table 3. 2 Parameters for estimation of DTX in Methanol by UV spectroscopy

Parameters	**Results**
λ_{max}	229.0 nm
Linearity range	2-18 µg/mL
Regression equation	y= 0.020x + 0.0004
Correlation coefficient (r^2)	0.9999

The interference between the human serum albumin (HSA) and the drug was observed at 229 nm. Due to interference, the absorbance of drug in the presence of HSA was found to be increase shown in **Figure 3.3.**

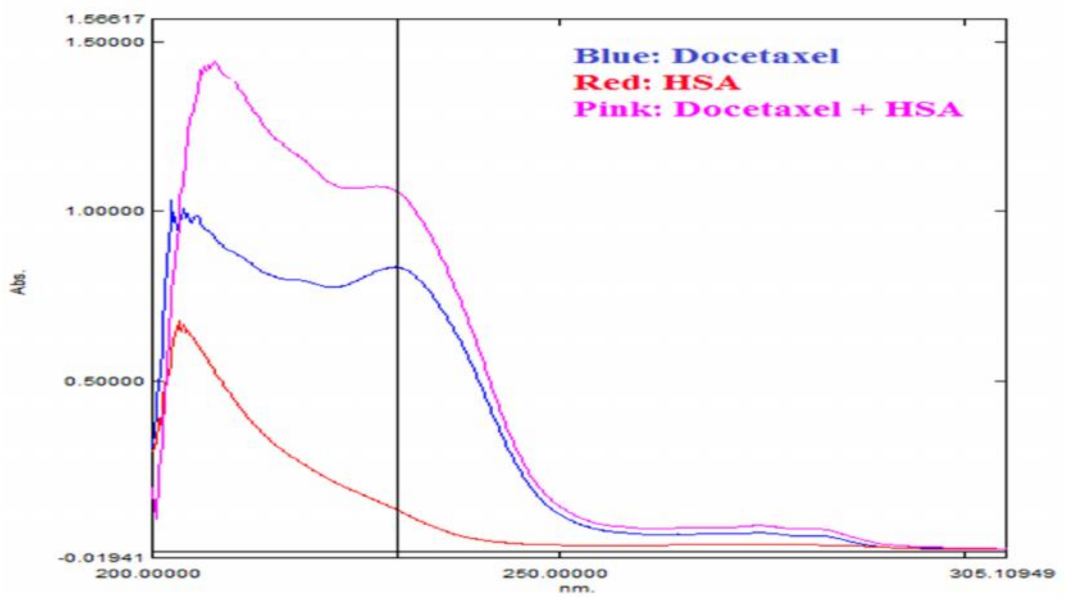


Figure 3. 3 UV spectrum of DTX, HSA and mixture of DTX and HSA

Further the first derivative spectrum was taken to nullify the interference and absorbance of drug in the presence of HSA but same results was observed in first derivative spectrum shown in **Figure 3.4**. So, HPLC method was selected for further estimation of drug.

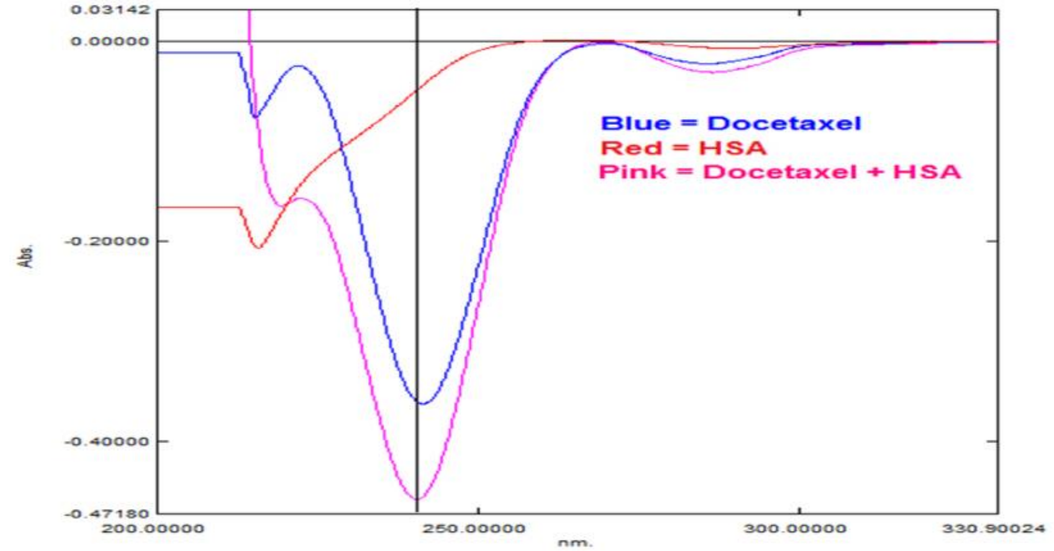


Figure 3. 4 First derivative spectrum of DTX, HSA and mixture of DTX and HSA

3.3.2 Estimation of DTX in Formulation by RP-HPLC

The retention time of DTX was found 4.7 min. The chromatogram of DTX in methanol is shown in **Figure 3.5** and data given in **Table 3.3.**

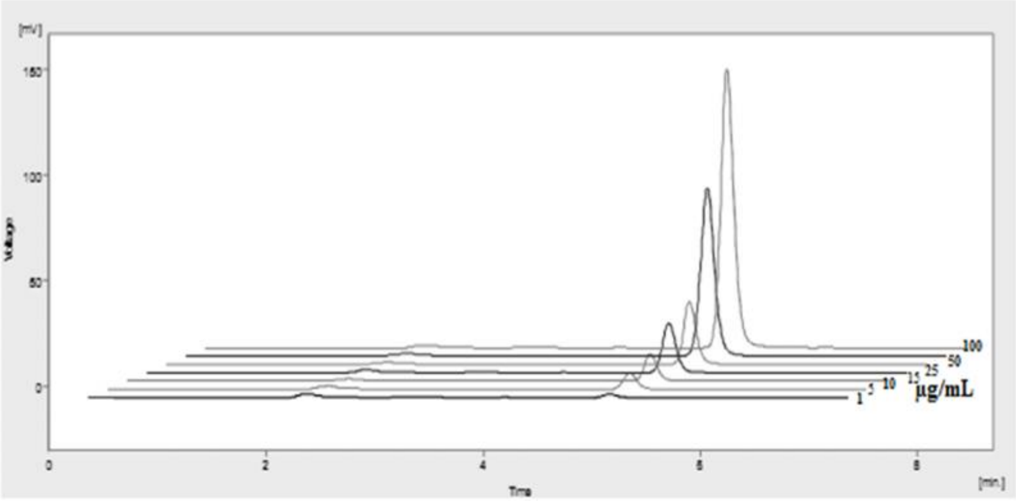


Figure 3. 5 Chromatogram of DTX at λmax of 229 nm

Table 3. 3 Calibration Data for DTX by RP-HPLC

Sr. No	**Concentration (µg/mL)**	**Mean Area (mV.s)± SD***	**% RSD**
1.	1	12.47±0.21	1.684
2.	5	64.14±1.10	1.715
3.	10	138.12±2.57	1.861
4.	15	202.18±3.59	1.776
5.	25	310.16±4.76	1.535
6.	50	645.74±7.12	1.103
7.	100	1297.81±8.91	0.687

* The experiment was performed in triplicate (n=3)

The parameters for calibration plot of DTX by RP-HPLC given in **Table 3.4** was fitted into a linear equation y=12.946x + 0.5404 with correlation coefficient of r^2=0.9997, which indicated the linearity of the plot shown in **Figure 3.6.**

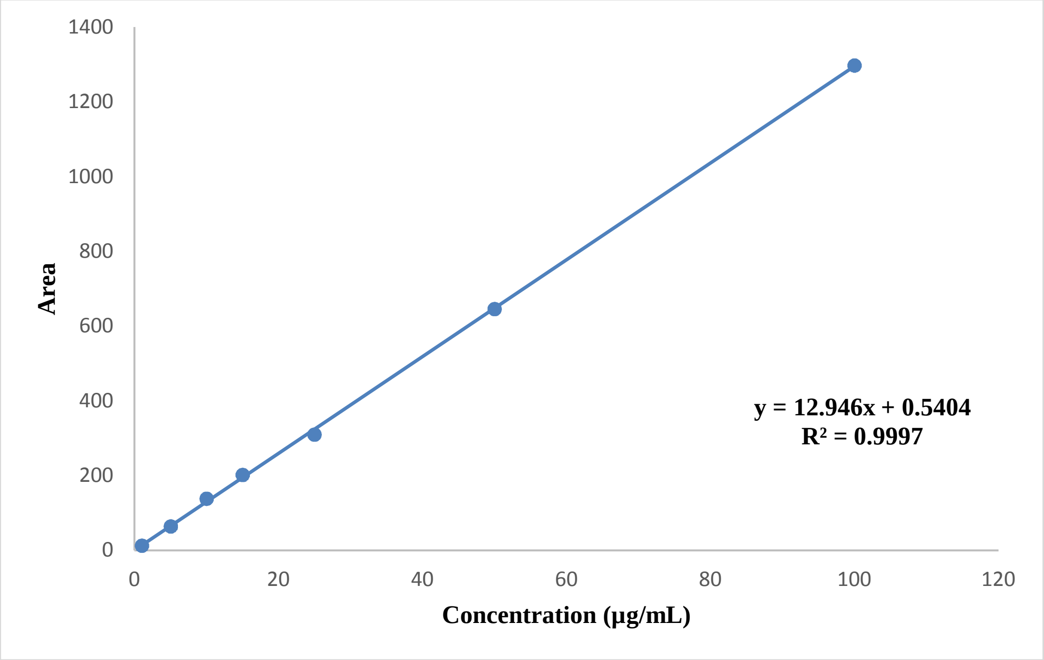


Figure 3. 6 Standard Plot of DTX by RP-HPLC

Table 3. 4 Parameters for Estimation of DTX by RP-HPLC

Parameters	Results
λmax	229 nm
Linearity range	1-50 µg/mL
Regression equation	Y=12.946x + 0.5404
Correlation coefficient (r^2)	0.9997
Retention time	4.7 min
Detection Limit (DL)	0.2791µg/mL
Quantification Limit (QL)	0.8459 µg/mL

Intraday and Interday precision and accuracy data for estimation of DTX to determine entrapment efficiency in formulation by RP-HPLC is given in **Table 3.5 and Table 3.6.** The less than 2 % coefficient of variance (CV) values indicates the precision of method. No significant difference between the amounts of drug added (True) and observed concentration at all the concentration levels tested was noticed indicating accuracy of the method (5, 6).

Table 3. 5 Intraday precision and accuracy for estimation of DTX by RP-HPLC

Standard Concentration (µg/ml)		Precision (% CV)[a]	Accuracy (% Relative error)[b]
Actual	**Observed**		
5	5.04±0.035	0.694	- 0.80
10	10.05±0.049	0.487	-0.50
15	15.08±0.071	0.470	-0.53

Table 3. 6 Interday precision and accuracy for estimation of DTX by RP-HPLC

Standard Concentration (µg/ml)		Precision (% CV)[a]	Accuracy (% Relative error)[b]
Actual	**Observed**		
5	4.98±0.042	0.843	0.40
10	9.97±0.054	0.541	0.30
15	15.06±0.079	0.524	-0.40

[a] Precision (% CV)= (Standard deviation/Mean concentration)*100

[b] Accuracy(% Relative error) = (Actual value – Observed value/Actual value)*100

3.3.3 Estimation of DTX in Diffusion Media by RP-HPLC

The retention time of DTX was found 4.7 min. The values for the standard plot of DTX in PBS pH 7.4: Methanol (7:3) is given **Table 3.7.**

Table 3. 7 Calibration Data for estimation of DTX in diffusion media by RP-HPLC

Sr. No	Concentration (µg/ml)	Mean Area (mV.s)± SD*	% RSD
1.	1	13.51±0.26	1.925
2.	5	67.84±1.07	1.577
3.	10	141.12±2.81	1.991
4.	15	207.21±3.96	1.911
5.	25	335.38±6.47	1.929
6.	50	647.47±8.92	1.378
7.	100	1302.41±9.12	0.700

* The experiment was performed in triplicate (n=3)

The parameters for calibration plot of DTX by RP-HPLC given in **Table 3.8** was fitted into a linear equation Y=12.946x + 6.8809 with correlation coefficient of r^2=0.9999, which indicated the linearity of the plot shown in **Figure 3.7.**

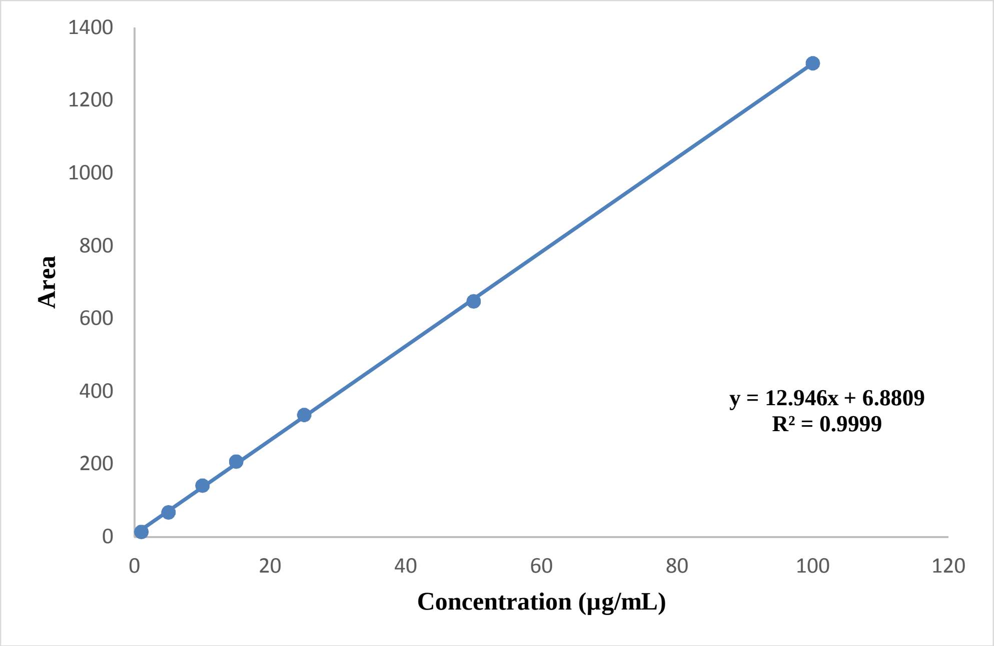


Figure 3. 7 Standard plot for estimation of DTX in diffusion media by RP-HPLC

Table 3. 8 Parameters for Estimation of DTX in diffusion media by RP-HPLC

Parameters	Results
λmax	229 nm
Linearity range	1-50 µg/mL
Regression equation	Y=12.946x + 6.8809
Correlation coefficient (r^2)	0.9999
Retention time	4.7 min
Detection Limit (DL)	0.3004 µg/mL
Quantification Limit (QL)	0.9104 µg/mL

Intraday and Interday precision and accuracy data for estimation of DTX in diffusion media by RP-HPLC is given **Table 3.9** and **Table 3.10.** The less than 2% CV value indicates the precision of method. No significant difference between the amounts of drug added (actual) and observed concentration at all the concentration levels tested was noticed indicating accuracy of the method (5, 6).

Table 3. 9 Intraday precision and accuracy for estimation of DTX by RP-HPLC

Standard Concentration (µg/ml)		Precision (% CV)[a]	Accuracy (% Relative error)[b]
Actual	**Observed**		
5	5.04±0.036	0.712	-0.8
15	15.06±0.054	0.358	-0.4
25	25.04±0.096	0.383	-0.1

Table 3. 10 Interday precision and accuracy for estimation of DTX by RP-HPLC

Standard Concentration (µg/ml)		Precision (% CV)[a]	Accuracy (% Relative error)[b]
Actual	**Observed**		
5	4.99±0.042	0.841	0.2
15	15.08±0.038	0.251	-0.5
25	24.94±0.078	0.312	0.2

[a]Precision (% CV) = (Standard deviation/Mean concentration)*100

[b]Accuracy(% Relative error) = (Actual value – Observed value/Actual value)*100

3.4 Analytical Methods for Estimation of VBT

3.4.1 Estimation of VBT by UV-Visible Spectrophotometer

Standard stock solution (100 µg/mL) was prepared by dissolving 5 mg of VBT in 50 ml water. The UV-visible spectrophotometric method of analysis was developed by scanning solution of VBT (50 µg/mL) in the ultraviolet range 200-400 nm to determine its λmax. Further dilutions were made from stock solution and calibration curve was plotted between 5 to 70 µg/mL. The above procedure was repeated thrice. The interference between the human serum albumin (HSA) and the drug was also studied. The absorbance of 50 µg/mL VBT solution, HSA solution and the mixture of VBT and HSA solution were taken at 271.2 nm in UV-Visible Spectrophotometer.

3.4.2 Estimation of VBT by RP-HPLC

Instrument:

HPLC: Shimadzu LC-20AT

Detector: UV-visible detector

Chromatographic Conditions:

Column: Kromasil C18 column (150 x 4.6 mm x 5 µm)

Detection wavelength: 271.2 nm

Flow rate: 1 mL/min

Run time: 10 min

Injection volume: 20 µl (Rheodyne 7725 injector valve with fixed loop at 20 µl)

Diluent: Mobile phase

Mobile Phase Preparation

Solution A: Acetonitrile

Solution B: 50 mM phosphate buffer solution containing 1% triethylamine (the pH of aqueous phase was adjusted to 4.0 with H_3PO_4)

Mix solution A and B in ratio of 60:40

Method

Stock solution of VBT (100 µg/ml) was prepared by dissolving 5 mg of drug in 50 ml diluent. Suitable aliquots of the stock solution were pipetted into 1.5 ml microcentrifuge tubes and the volume was made up to 1 ml with acetonitrile to give final concentrations of 1-100 µg/ml. 20 µl of these solutions was injected in HPLC and analyzed using above mentioned parameters. The readings were recorded in triplicate.

3.4.3 Analytical Method Validation

The method was validated for accuracy, precision, linearity, detection limit and quantification limit as per mentioned in **Section 3.2.3.**

3.5 Results and Discussion

3.5.1 Estimation of VBT by UV-Visible Spectrophotometer

VBT in water yields characteristic curve when scanned in UV wavelength range and absorption maxima was found at 271.2 nm shown in **Figure 3.8**. The linearity was observed in the range of 5-70 µg/mL of DTX concentration. The data for calibration plot is given in **Table 3.11** and calibration curve shown in **Figure 3.9**. The regression analysis of the plot using the method of least squares was made to evaluate the intercept, slope and correlation coefficient (r^2). The high value of correlation coefficient (greater than 0.999) of the regression equation and the negligible value of intercept confirm the linearity of calibration plot. Parameters indicating linearity for the developed UV spectrometric method of analysis for VBT are shown in **Table 3.12.**

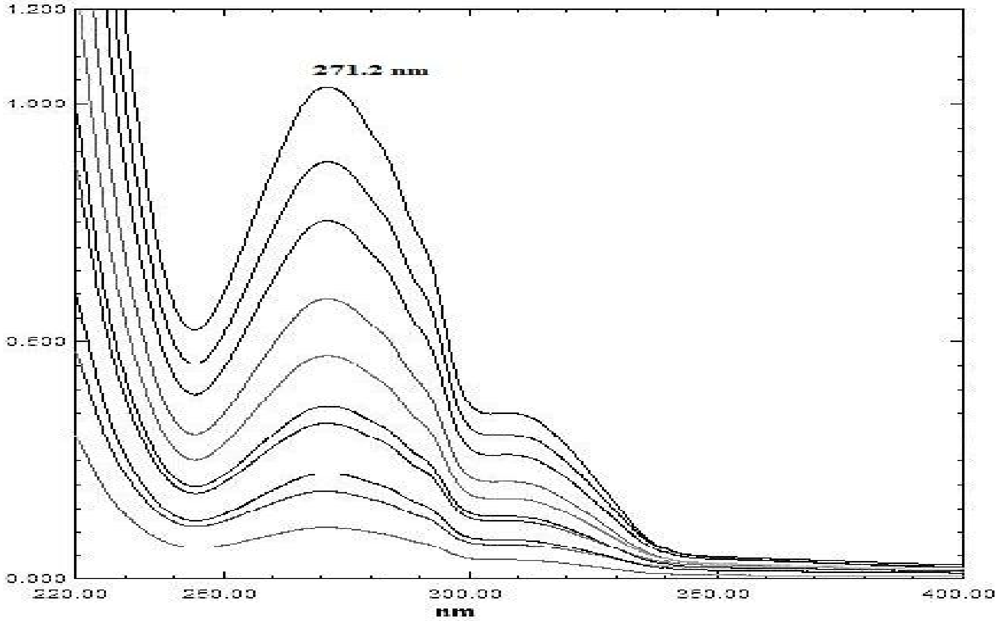


Figure 3. 8 UV Spectrum of VBT in Water

Table 3. 11 Calibration Data for VBT in Water by UV spectroscopy

Sr. No	Concentration (µg/mL)	Mean Absorbance ± SD*	% RSD
1.	5	0.097±0.001	1.031
2.	10	0.185±0.003	1.622
3.	15	0.271±0.005	1.845
4.	20	0.346±0.006	1.734
5.	30	0.519±0.008	1.541
6.	40	0.702±0.009	1.282
7.	50	0.862±0.016	1.856
8.	60	1.011±0.019	1.879
9.	70	1.171±0.021	1.793

* The experiment was performed in triplicate (n=3)

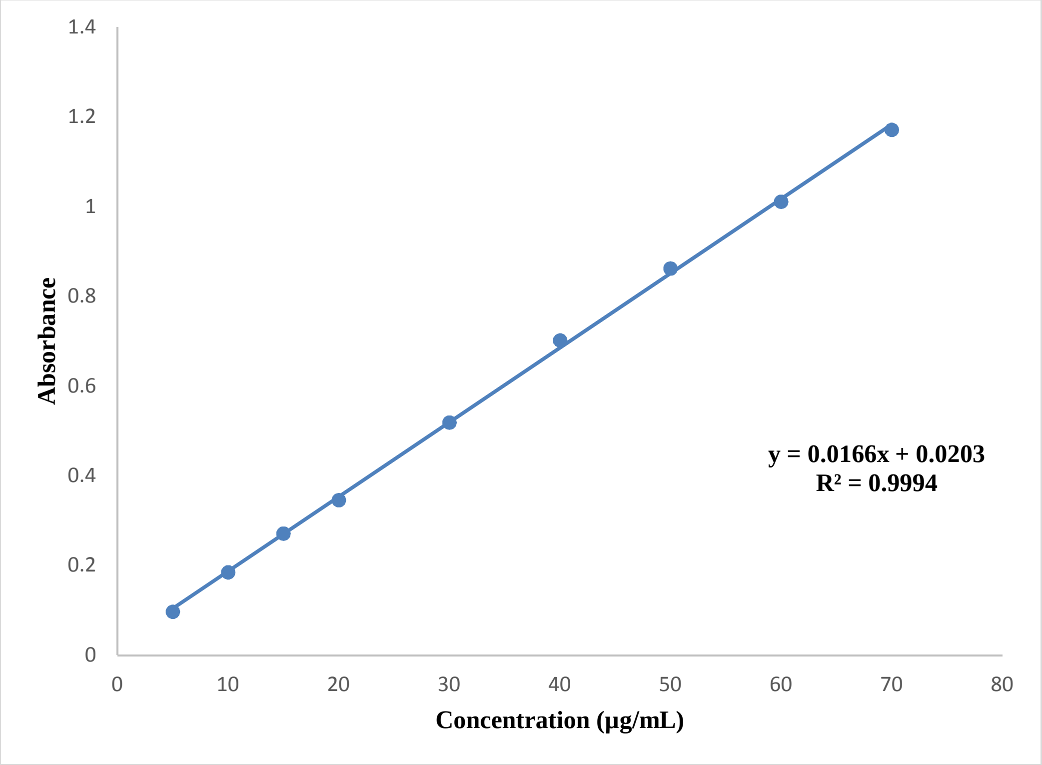


Figure 3. 9 Standard Plot of VBT in Water by UV spectroscopy

Table 3. 12 Parameters for estimation of VBT in Water by UV spectroscopy

Parameters	**Results**
λmax	271.2 nm
Linearity range	5-70 µg/mL
Regression equation	y = 0.0166x + 0.0203
Correlation coefficient (r^2)	0.9994

The interference between the human serum albumin (HSA) and the drug was observed at 229 nm. In case of interference, the absorbance of drug in the presence of HSA was found to be increased shown in **Figure 3.10**. Further the first derivative spectrum was taken to nullify the interference and absorbance of drug in the presence of HSA but same results was observed in first derivative spectrum shown in **Figure 3.11**. So, HPLC method was selected further for the estimation of drug.

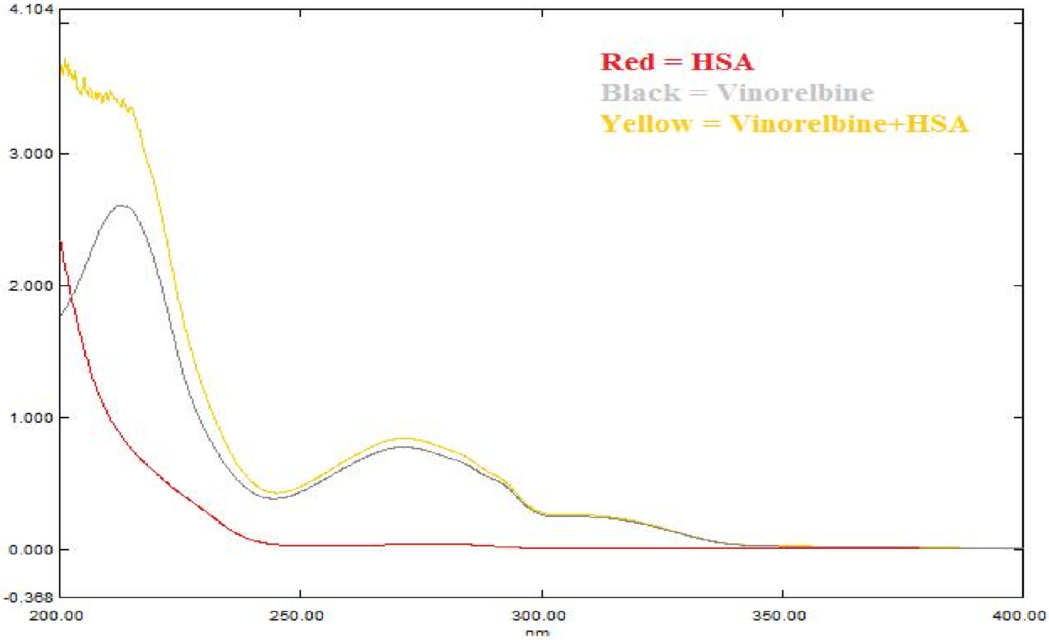


Figure 3. 10 UV spectrum of VBT, HSA and mixture of VBT and HSA

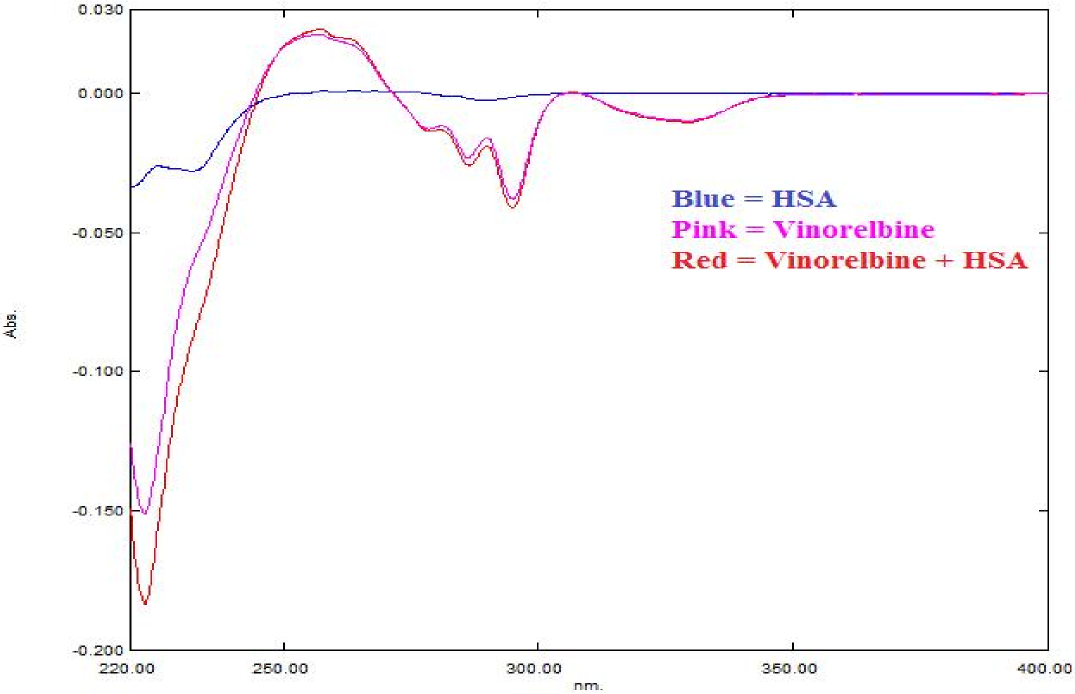


Figure 3. 11 First derivative spectrum of VBT, HSA and mixture of VBT and HSA

3.5.2 Estimation of VBT in Formulation by RP-HPLC

The retention time of VBT was found 3.9 min. The chromatogram of VBT in water is shown in **Figure 3.10** and data given in **Table 3.13.**

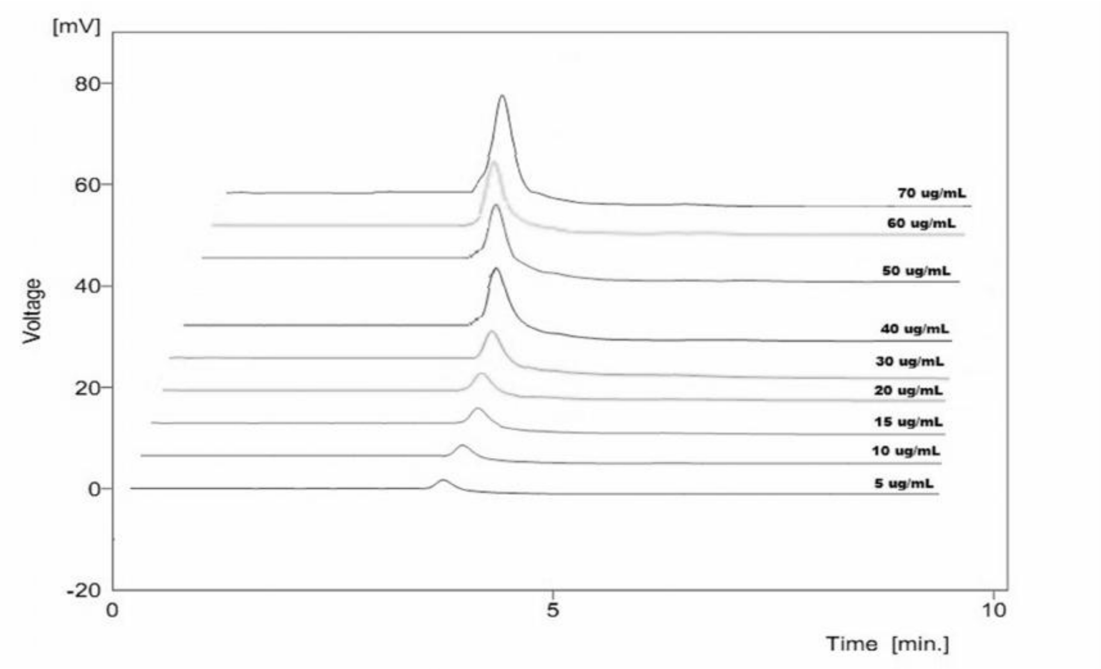


Figure 3. 12 Chromatogram of VBT at λmax of 271.2 nm

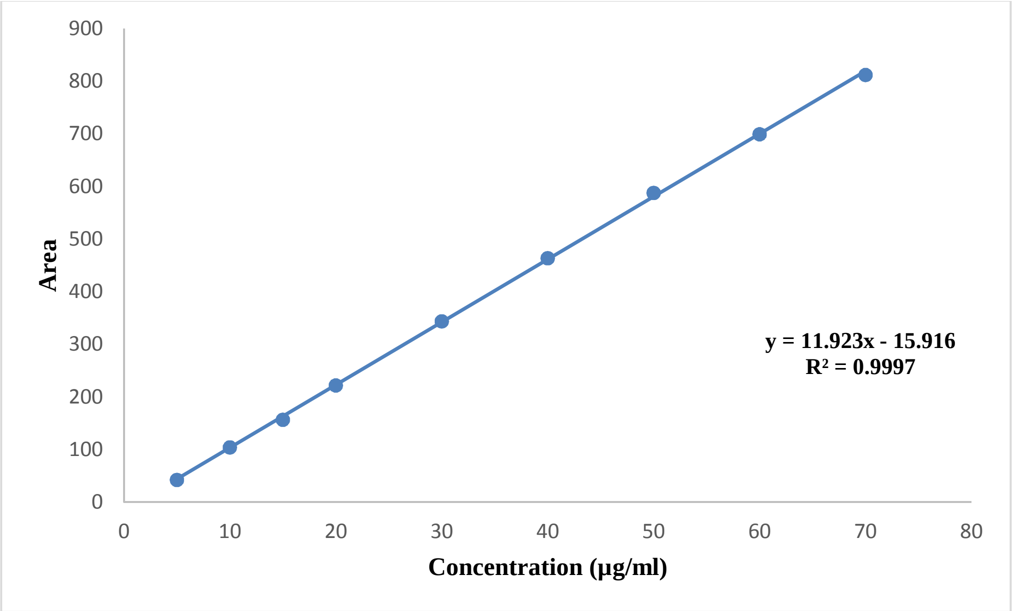


Figure 3. 13 Standard Plot of VBT by RP-HPLC

Table 3. 13 Calibration Data for VBT by RP-HPLC

Sr. No	Concentration (µg/mL)	Mean Area (mV.s)± SD*	% RSD
1.	5	42.67±0.71	1.664
2.	10	104.64±2.04	1.950
3.	15	156.81±2.65	1.690
4.	20	222.46±4.14	1.861
5.	30	344.14±6.33	1.839
6.	40	463.86±7.34	1.582
7.	50	587.75±8.87	1.509
8.	60	699.21±7.43	1.063
9.	70	812.12±6.21	0.765

* The experiment was performed in triplicate (n=3)

The parameters for calibration plot of VBT by RP-HPLC given in **Table 3.14** was fitted into a linear equation y=11.923x - 15.916 with correlation coefficient of r^2=0.9997, which indicated the linearity of the plot shown in **Figure 3.11.**

Table 3. 14 Parameters for Estimation of VBT by RP-HPLC

Parameters	Results
λmax	271.2 nm
Linearity range	5-70 µg/mL
Regression equation	Y=11.923x - 15.916
Correlation coefficient (r^2)	0.9997
Retention time	3.9 min
Detection Limit (DL)	0.8092µg/mL
Quantification Limit (QL)	2.4522µg/mL

Intraday and Interday precision and accuracy data estimation of VBT to in formulation by RP-HPLC is given **Table 3.15 and Table 3.16.** The less than 2 % CV value indicates precision of the method. No significant difference between the amounts of drug added (actual) and observed concentration at all the concentration levels tested was noticed indicating accuracy of the method (5, 6).

Table 3. 15 Intraday precision and accuracy data for estimation of VBT by RP-HPLC

Standard Concentration (µg/ml)		Precision (% CV)[a]	Accuracy (%Relative error)[b]
Actual	Observed		
10	10.07±0.056	0.523	-0.70
20	20.09±0.081	0.403	-0.45
50	50.14±0.126	0.251	-0.28

Table 3. 16 Interday precision and accuracy data for estimation of VBT by RP-HPLC

Standard Concentration (µg/ml)		Precision (% CV)[a]	Accuracy (%Relative error)[b]
Actual	Observed		
10	10.04±0.061	0.607	-0.4
20	19.98±0.087	0.435	0.1
50	50.08±0.132	0.263	-0.1

[a] Precision (% CV) = (Standard deviation/Mean concentration)*100

[b] Accuracy(% Relative error) = (Actual value – Observed value/Actual value)*100

3.6 Analytical Methods for Estimation of DTX and VBT in Plasma

3.6.1 Estimation of DTX in plasma by RP-HPLC.

Instrument

HPLC: Shimadzu LC-20AT

Detector: SPD-20A UV-Visible Detector (Shimadzu Prominence Detector)

Chromatographic Conditions

Column: Kromasil C18 column (250 x 4.6 mm x 10 µm)

Detection wavelength: 229 nm

Flow rate: 1 mL/min

Run time: 10 min

Injection volume: 20 µl (Rheodyne 7725 injector valve with fixed loop at 20 µl)

Diluent: Methanol

Mobile Phase Preparation

Solution A: Methanol

Solution B: Water

Mix solution A and B in ratio of 80:20

Method

Stock solution of Docetaxel (100 µg/ml) was prepared by dissolving 5 mg of drug in 50 ml methanol. Suitable aliquots of the stock solution were pipetted into 1.5 ml microcentrifuge tubes and the volume was made up to 100µl with plasma to give the final concentration of 0.1, 0.2. 0.5, 1, 2, 3, 4, 5 µg/ml of DTX. Drug was extracted from plasma by protein precipitation method using suitable organic solvent i.e. methanol and centrifuged at 5000 rpm for 15 min. 20 µl of supernatant was injected in HPLC and analyzed using above mentioned parameters. The samples were analyzed in triplicate.

3.6.2 Estimation of VBT in plasma by RP-HPLC

Instrument:

HPLC: Shimadzu LC-20AT

Detector: SPD-20A UV-Visible Detector (Shimadzu Prominence Detector)

Chromatographic Conditions:

Column: Kromasil C18 column (150 x 4.6 mm x 5 µm)

Detection wavelength: 271.2 nm

Flow rate: 1 mL/min

Run time: 10 min

Injection volume: 20 µl (Rheodyne 7725 injector valve with fixed loop at 20 µl)

Diluent: Mobile phase

Mobile Phase Preparation

Solution A: Acetonitrile

Solution B: Aqueous 50 mM triethyl ammonium acetate, pH 5.5

Mix solution A and B in ratio of 42:58

Method

Stock solution of VBT (100 µg/ml) was prepared by dissolving 5 mg of drug in 50 ml diluent. Suitable aliquots of the stock solution were pipetted into 1.5 ml microcentrifuge tubes and the volume was made up to 100µl with plasma to give the final concentration of 0.1, 0.2. 0.5, 1, 2, 3, 4, 5 µg/ml of VBT. Drug was extracted from plasma using suitable organic solvent i.e. acetonitrile. 20 µl this solution was injected in HPLC and analyzed using above mentioned parameters. The samples were analyzed in triplicate.

3.6.3 Analytical Method Validation

The method was validated for accuracy, precision, linearity, detection limit and quantification limit as per mentioned in **Section 3.2.3.**

3.7 Results and Discussion

3.7.1 Estimation of DTX in Plasma by RP-HPLC

The retention time of DTX was found 4.8 min.

Table 3. 17 Calibration Data for DTX in Plasma by RP-HPLC

Sr. No	Concentration (µg/mL)	Mean Area ± SD* (µV.s)
1.	0.1	21175 ± 200
2.	0.2	35256 ± 367
3.	0.5	63085 ± 415
4.	1	100226 ± 563
5.	2	163354 ± 677
6.	3	232465 ± 691
7.	4	275742 ± 748
8.	5	336532 ± 804

* The experiment was performed in triplicate (n=3)

The parameters for calibration plot of DTX by RP-HPLC given in **Table 3.17** was fitted into a linear equation y = 63320x + 28422 with correlation coefficient of r^2=0.9931, which indicated the linearity of the plot shown in **Figure 3.14.**

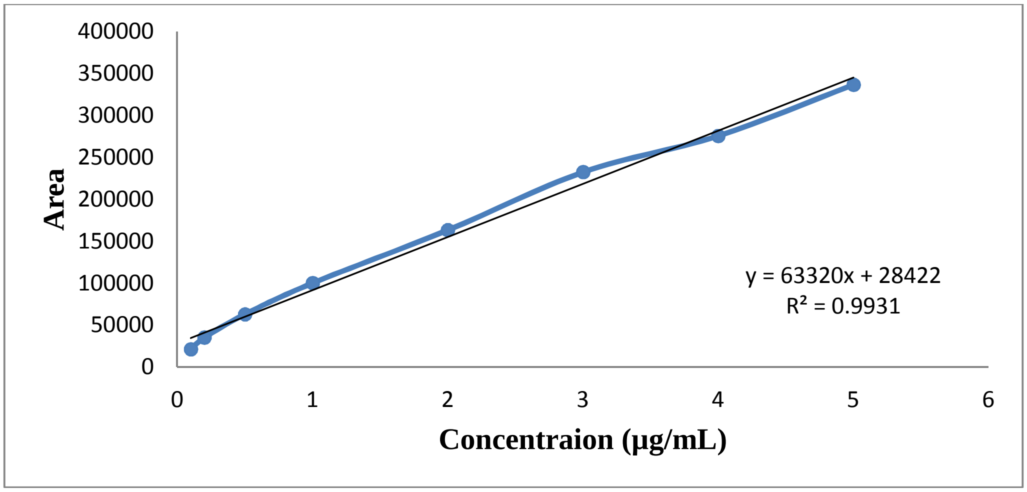


Figure 3. 14 Standard Plot of DTX in Plasma by RP-HPLC

Table 3. 18 Parameters for Estimation of DTX in Plasma by RP-HPLC

Parameters	Results
λmax	229 nm
Linearity range	0.1-5 µg/mL
Regression equation	Y= 63320x + 28422
Correlation coefficient (r^2)	0.9931
Retention time	4.8 min

Intraday and Interday precision and accuracy data for estimation of DTX to determine entrapment efficiency in formulation by RP-HPLC is given in **Table 3.19** and **Table 3.20.** The less than 2 % coefficient of variance (CV) values indicates the precision of method. No significant difference between the amounts of drug added (True) and observed concentration at all the concentration levels tested was noticed indicating accuracy of the method (5, 6).

Table 3. 19 Intraday precision and accuracy data for estimation of DTX in plasma by RP-HPLC

Standard Concentration (µg/ml)		Precision (% CV)[a]	Accuracy (%Relative error)[b]
Actual	**Observed**		
1	1.04±0.019	1.82	-4.00
2	2.03±0.034	1.67	-1.50
3	3.05±0.056	1.83	-1.66

Table 3. 20 Interday precision and accuracy data for estimation of DTX in plasma by RP-HPLC

Standard Concentration (µg/ml)		Precision (% CV)[a]	Accuracy (%Relative error)[b]
Actual	**Observed**		
1	1.03±0.018	1.74	-3.00
2	2.03±0.035	1.72	-1.50
3	3.04±0.057	1.87	-1.33

[a] Precision (% CV) = (Standard deviation/Mean concentration)*100

[b] Accuracy (% Relative error) = (Actual value – Observed value/Actual value)*100

3.7.2 Estimation of VBT in Plasma by RP-HPLC

The retention time of VBT was found 3.9 min.

Table 3. 21 Calibration Data for VBT in Plasma by RP-HPLC

Sr. No	Concentration (µg/mL)	Mean Area ± SD* (µV.s)
1.	0.1	17231 ± 330
2.	0.2	31312 ± 564
3.	0.5	58986 ± 538
4.	1	95265 ± 730
5.	2	154846 ± 806
6.	3	215478 ± 968
7.	4	268113 ± 1070
8.	5	328675 ± 851

* The experiment was performed in triplicate (n=3)

The parameters for calibration plot of VBT by RP-HPLC given in **Table 3.21** was fitted into a linear equation y = 62010x + 23768 with correlation coefficient of r^2 = 0.9955, which indicated the linearity of the plot shown in **Figure 3.15.**

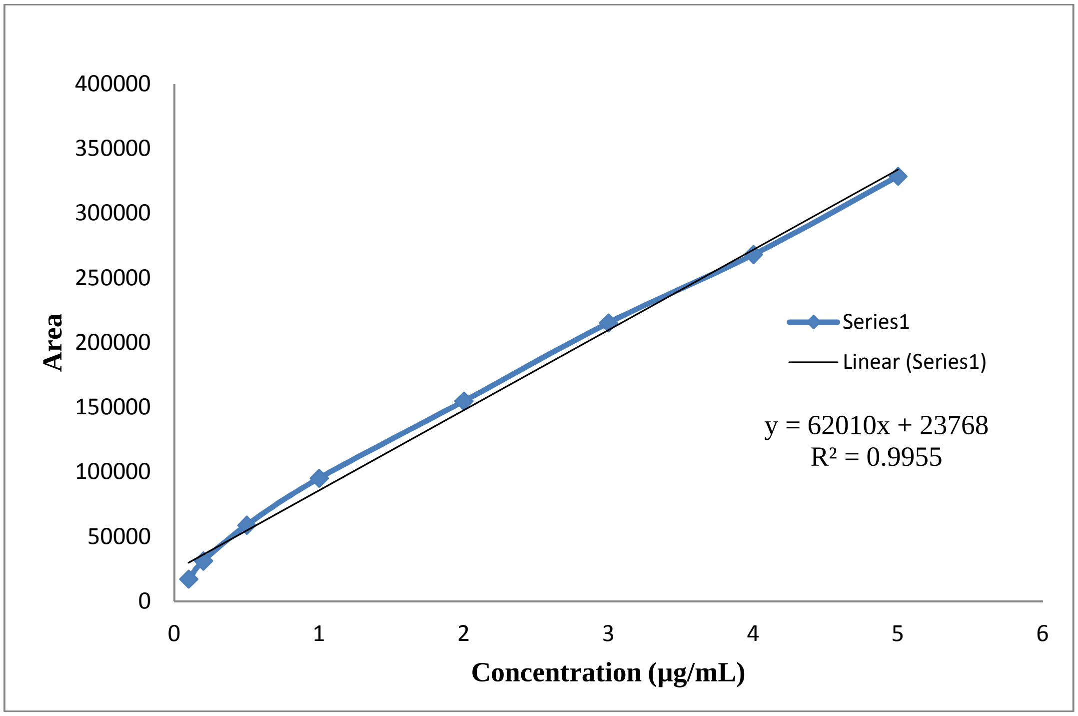


Figure 3. 15 Standard Plot of VBT in Plasma by RP-HPLC

Table 3. 22 Parameters for Estimation of VBT in Plasma by RP-HPLC

Parameters	Results
λmax	271.2 nm
Linearity range	0.1-5 µg/mL
Regression equation	Y= 62010x + 23768
Correlation coefficient (r^2)	0.9955
Retention time	4.1 min

Intraday and Interday precision and accuracy data for estimation of VBT to determine entrapment efficiency in formulation by RP-HPLC is given in **Table 3.23** and **Table 3.24.** The value of coefficient of variance (CV) less than 2 % indicates the precision of method. No significant difference between the amounts of drug added (True) and observed concentration at all the concentration levels tested was noticed indicating accuracy of the method (5, 6).

Table 3. 23 Intraday precision and accuracy data for estimation of VBT in plasma by RP-HPLC

Standard Concentration (µg/ml)		**Precision**	**Accuracy**
Actual	**Observed**	**(% CV)[a]**	**(%Relative error)[b]**
1	1.03±0.020	1.94	-3.00
3	3.05±0.054	1.70	-1.66
5	5.08±0.087	1.71	-1.60

Table 3. 24 Interday precision and accuracy data for estimation of VBT in plasma by RP-HPLC

Standard Concentration (µg/ml)		**Precision**	**Accuracy**
Actual	**Observed**	**(% CV)[a]**	**(%Relative error)[b]**
1	1.02±0.019	1.86	-2.00
3	3.04±0.057	1.87	-1.33
5	5.09±0.092	1.80	-1.80

[a] Precision (% CV) = (Standard deviation/Mean concentration)*100

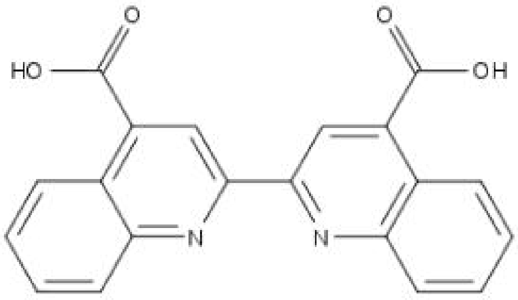
[b] Accuracy (% Relative error) = (Actual value – Observed value/Actual value)*100

3.8 Estimation of Proteins (BCA protein estimation method)

Principle: Protein assay based on bicinchoninic acid (BCA) is a most sensitive and detergent compatible method for the colorimetric detection and quantitation of total protein. This method is a combination of the well-known biuret reaction, the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium and the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) with reagent containing bicinchoninic acid (7). The purple colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm.

BCA-Protein Reaction:

1. Protein (peptide bonds) + Cu^{2+} tetradentate = Cu^{1+} complex
2. Cu^{1+} + 2 Bicinchoninic Acid = BCA-Cu^{1+} complex (purple coloured at 562 nm)



Bicinchoninic acid [2-(4-carboxyquinolin-2-yl) quinoline-4-carboxylic acid]

Figure 3.12 Structure of bicinchoninic acid

Preparation of standard BSA solution

The standard calibration curve of BSA was prepared as per the standard protocol provided along with BCA protein estimation kit (Bangalore Genei, Bangalore).

Preparation of the BCA working reagent (BWR): To prepare BWR mixed 50 parts of Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartarate in 0.1 M sodium hydroxide) with 1 part of Reagent B (copper sulphate solution). When Reagent A is initially added to Reagent B, turbidity is observed that quickly disappears upon mixing to yield a clear-green BWR. Prepared sufficient volume of BWR based upon the number of tests to be done.

Protocol (Sample to BWR ratio = 1:10):

1. Pipetted 0.2 ml of each standard or unknown sample into appropriately labeled test tubes. The 0.2 ml of the diluent was used as blank.
2. Added 2.0 ml of the BCA working reagent (BWR) to each tube and mixed well.

3. Incubated all the tubes at the selected temperature and time, Enhanced Protocol: 60°C for 30 minutes (Standard Assay range = 6.25-500µg/ml)
4. After incubation, cooled all tubes to room temperature (RT).
5. Measured the absorbance at 562 nm (A-562) of each tube against water as reference.
6. Subtracted the average A-562 reading of the blanks from the A-562 reading for each standard or unknown sample.
7. Prepared a standard curve by plotting the average blank corrected A-562 reading for each BSA standard against its concentration in µg/ml.

3.9 Results and Discussion

Estimation of bovine serum albumin (BSA) by bicinchoninic acid (BCA) method

Estimation of protein was performed using bovine serum albumin as standard protein. Cu^{1+} BCA complex shows absorbance at 562 nm with correlation coefficient of 0.997 and the slope of the regressed line was 0.008 which indicates that absorbance and concentration of protein are linear. Calibration data for BSA by UV given in **Table 3.25** and curve shown in **Figure 3.16.** This method was used for estimation of non desolvated protein (albumin) in nanoparticles prepared by desolvation method and quantification of isolated recombinant protein (scFv-EGFR).

Table 3. 25 Calibration Data for BSA by UV at 562 nm.

Sr. No	Concentration (µg/mL)	Actual Absorbance	Blank Corrected Absorbance
1.	0	0.170	0.000
2.	12.5	0.215	0.045
3.	25	0.226	0.056
4.	50	0.249	0.079
5.	100	0.296	0.126
6.	150	0.330	0.160
7.	250	0.412	0.242
8.	500	0.607	0.437
9.	750	0.812	0.642
10.	1000	0.997	0.827

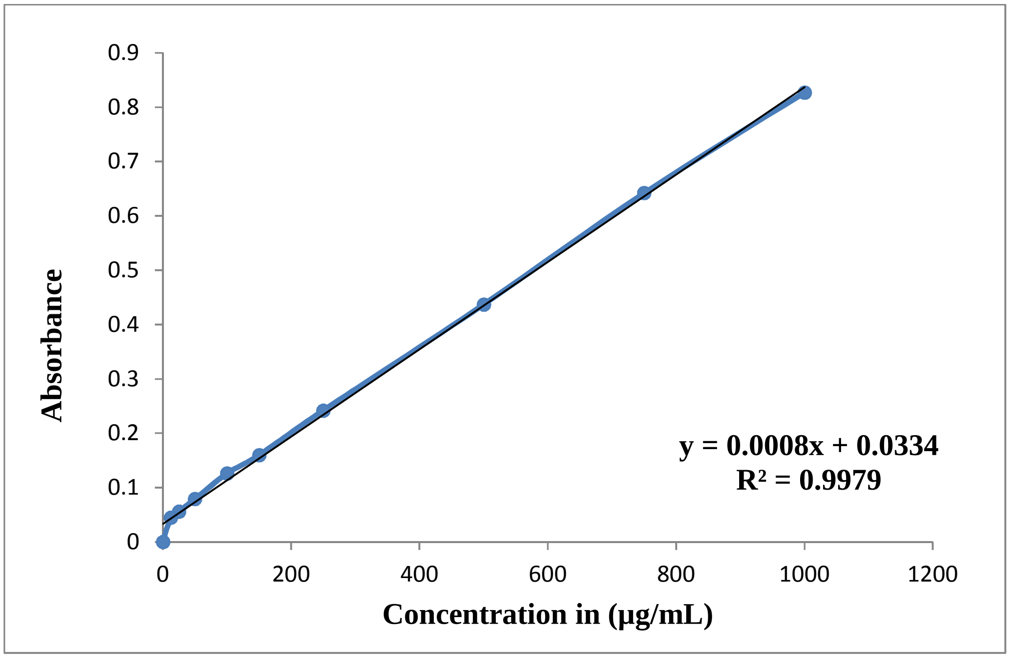


Figure 3. 16 Standard Plot of BSA by UV

3.10 References

1. Hubert PH, Chiap P, Crommen J, Boulanger B, Chapuzet E, Mercier N, et al. Validation of the quantitative analytical procedures. Harmonization of the steps. STP Pharma practice. 2003;13:101-38.
2. Bolton S, Swarbrick J, editors. Basic definitions and concepts 3rd ed. New York: Marcel Dekker; 1990.
3. Merodia M, Mirshahi T, Mirshahi M. Development of a sensitive method for the determination of ganciclovir by reversed phase high-performance liquid chromatography. J Chromatogr. 2000;870(1-2):159-67
4. Indian Pharmacopoeia. Indian Pharmacopoeial Commission, Ghaziabad, 2007; 2: 697-700.
5. Guidance for industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), May 2001.
6. Boulanger B, Dewe W, Chiap P, Crommen J, PH. H. An analysis of the SFSTP guide on validation of bioanalytical methods: progress and limitations. J Pharm Biomed Anal. 2003;32:753-65.
7. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985;150:76-85.