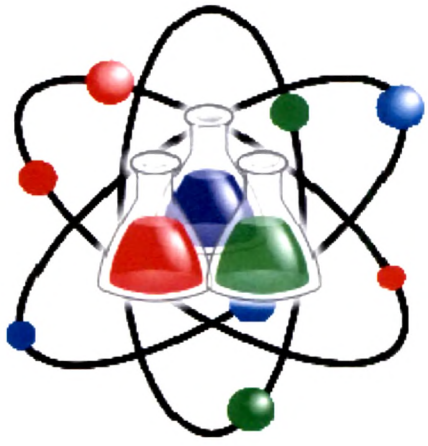
Chapter 4



ANALYTICAL METHODS FOR DRUG ESTIMATION

4. ANALYTICAL METHODS FOR DRUG ESTIMATION

4.1 Solubility studies:

The spontaneous solubility of Etoposide (ET) and Quercetin Dihydrate (QD) was studied in various solvents at room temperature. Drugs (5 mg) were added to 5 ml of solvent in test tubes, shaken on vortex shaker for few min and observed. The observations are compiled in Table 4.1

Table 4.1 Solubility of ET and QD at RT

Solvent	Solubility	
	ET	QD
DMSO	+++	+++
Acetonitrile	++	++
Acetone	++	++
Chloroform	+	+
Ethanol	+	+
Methanol	+	+
PBS pH 7.4	-	-
Distilled Water	-	-

+++ Freely soluble ++ Soluble

\+ Slightly soluble - Practically insoluble

4.2 Estimation of Etoposide and Quercetin Dihydrate in PLGA nanoparticles:

This method of estimation is developed based on the observation that ET and QD in acetonitrile shows strong absorbance in the ultraviolet region of the electromagnetic spectrum. PLGA does not interfere with the drug peak in the same solvent system. Thus calibration curves for both the drugs are constructed in the acetonitrile solvent.

4.2.1 Stock solutions:

Stock solutions of ET and QD were prepared by dissolving 10 mg of ET/QD in acetonitrile with the aid of vortexing for few minutes, until clear solution was obtained and then volume was made upto 10ml with acetonitrile.

4.2.2a Procedure for calibration curve of ET:

Suitable aliquots of the 1000µg/ml stock solution of ET in acetonitrile were pipetted into 10ml volumetric flasks to contain 10,20,40,60,80,100,120 µg/ml. The volume was made up

with the solvent, the contents shaken well and the absorbance measured at 283.5nm using a Shimadzu UV-1700 Pharmaspec UV-Visible spectrophotometer with quartz cells of 10mm path length against the solvent acetonitrile as a blank. The above procedure was carried out in triplicates. Mean absorbance values are shown in Table 4.2a. The calibration curve was drawn by plotting the absorbances versus concentration of ET (Figure 4.2) and linearly regressed.

The absorptivity scan over the wavelength range 200 to 400nm of the solution of ET in acetonitrile is shown in Figure 4.1

The analytical parameters for the estimation of ET in acetonitrile was tabulated in Table 4.3a

4.2.2b Procedure for calibration curve of QD:

Stock solution of QD (1000µg/ml) was further diluted to obtain 100µg/ml solution.

Suitable aliquots of the 100µg/ml stock solution of QD in acetonitrile were pipetted into 10ml volumetric flasks to contain 2, 4, 6, 8, 10, 20, 30µg/ml. The volume was made up with the solvent, the contents shaken well and the absorbance measured at 368.0nm using a Shimadzu UV-1700 Pharmaspec UV-Visible spectrophotometer with quartz cells of 10mm path length against the solvent acetonitrile as a blank. The above procedure was carried out in triplicates. Mean absorbance values are shown in Table 4.2b. The calibration curve was drawn by plotting the absorbances versus concentration of QD (Figure 4.4) and linearly regressed.

The absorptivity scan over the wavelength range 200 to 400nm of the solution of QD in acetonitrile is shown in Figure 4.3

The analytical parameters for the estimation of QD in acetonitrile was tabulated in Table 4.3b

4.2.3 Stability and selectivity:

Changes in absorbance of the solutions of ET and QD in acetonitrile, used for preparing the calibration curve at analytical wavelength, were monitored for a period of 24 hrs. Thus stability of these solutions with time was established.

The selectivity of the method was ascertained by estimating drugs, ET and QD in presence of other constituents of nanoparticles (for eg. Polymer PLGA), at the level, they were included in the nanoparticles.

4.2.4 Accuracy and precision:

Known amounts of ET and QD were subjected to recovery studies using the method described in detail above, in triplicate, to establish the accuracy and precision of the method. The result of analysis for ET and QD was summarized in Table 4.4a and 4.4b respectively.

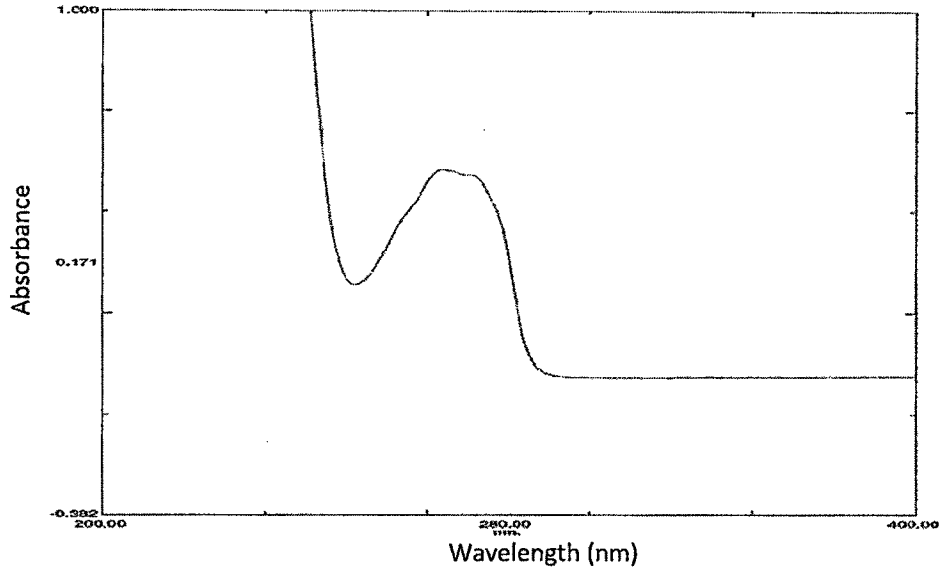


Figure 4.1 Absorptivity Scan of ET in Acetonitrile

Table 4.2a Calibration curve of ET in Acetonitrile

Concentration µg/ml	Mean absorbance	± SE	Regressed values
10	0.074	0.002	0.065
20	0.140	0.003	0.135
40	0.293	0.004	0.275
60	0.431	0.005	0.415
80	0.576	0.008	0.555
100	0.728	0.002	0.695
120	0.887	0.010	0.835

n = 3

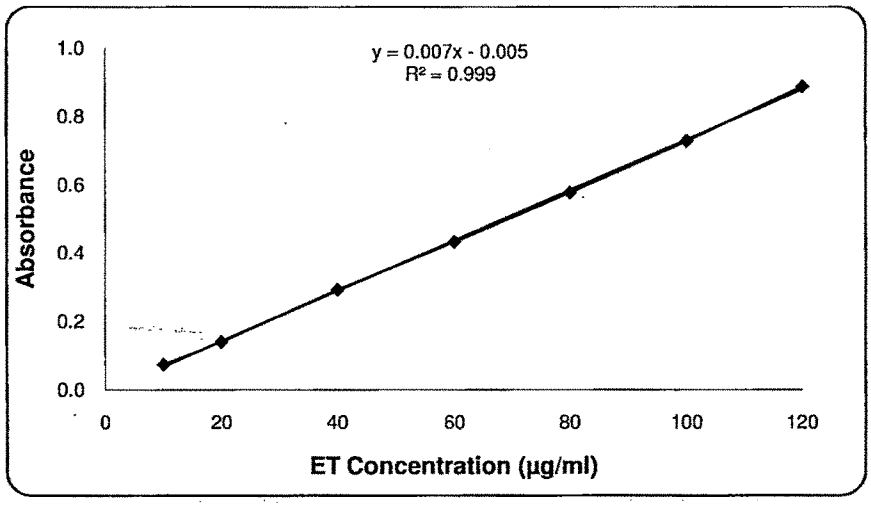


Figure 4.2 Standard curve of ET in Acetonitrile

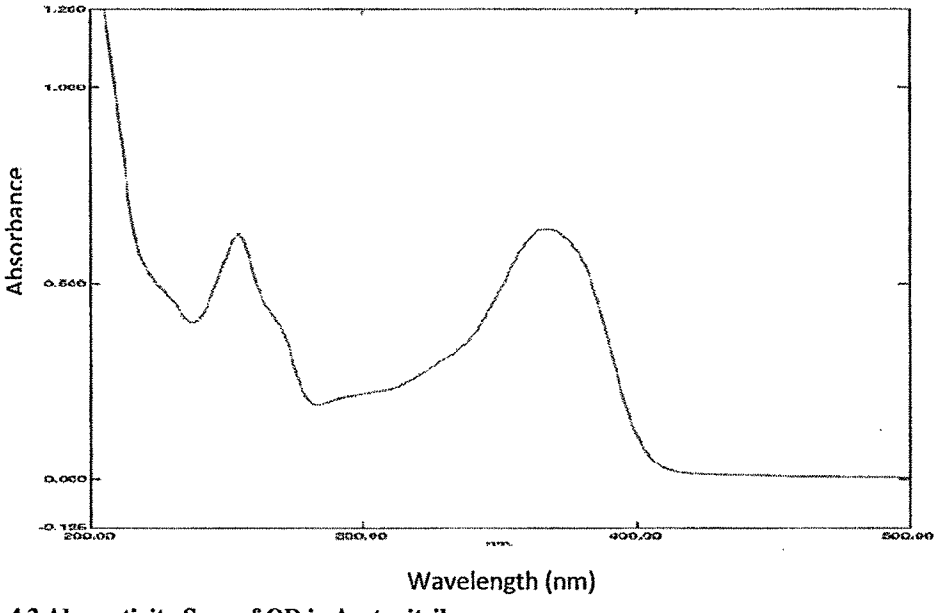


Figure 4.3 Absorptivity Scan of QD in Acetonitrile

Table 4.2b Calibration curve of QD in Acetonitrile

Concentration µg/ml	Mean absorbance	±SE	Regressed values
2	0.122	0.004	0.125
4	0.251	0.007	0.253
6	0.374	0.003	0.381
8	0.536	0.007	0.509
10	0.638	0.005	0.637
20	1.290	0.008	1.277
30	1.931	0.007	1.917

n = 3

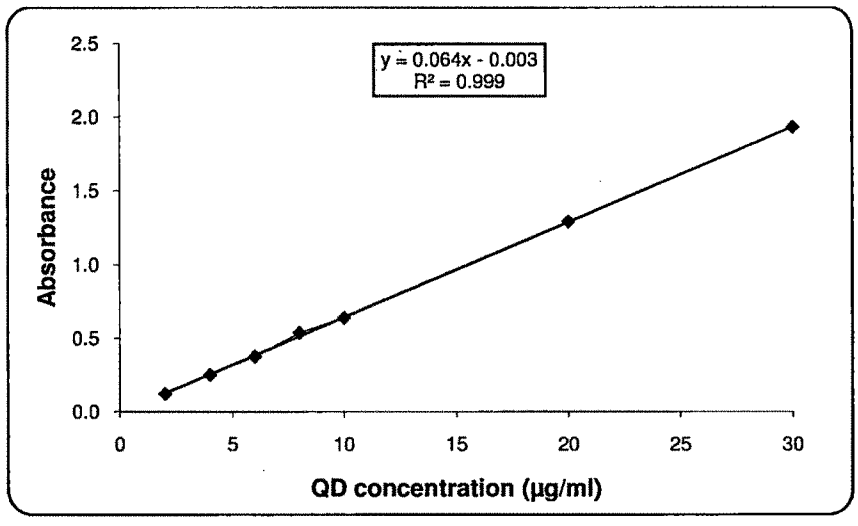


Figure 4.4 Standard curve of QD in Acetonitrile

Table 4.3a Analytical parameters for estimation of ET in acetonitrile

Analytical Parameter	Value
Absorption maxima (nm)	283.5nm
Beer's law limits at 283.5nm (µg/ml)	10 - 120
Apparent molar absorptivity at 283.5nm ($l\ mol^{-1}cm^{-1}$)	4.09×10^3
Regression equation	y = 0.007x - 0.005
Co-rrelation co-efficient	0.999

Table 4.3b Analytical parameters for estimation of QD in acetonitrile

Analytical Parameter	Value
Absorption maxima (nm)	254.0, 368.0
Beer's law limits at 368.0nm (µg/ml)	2 - 30
Apparent molar absorptivity at 368.0nm ($l\ mol^{-1}cm^{-1}$)	21.3×10^3
Regression equation	y = 0.064x – 0.003
Co-rrelation co-efficient	0.999

Table 4.4a Evaluation of accuracy and precision of estimation method of ET in acetonitrile

Added (mg)	Found (mg) Mean	±SD	SE
5	4.81	0. 11	0.064
10	9.43	0.060	0.035
15	14.17	0.235	0.135

n = 3

Table 4.4b Evaluation of accuracy and precision of estimation method of QD in acetonitrile

Added (mg)	Found (mg) Mean	±SD	SE
5	4.43	0.100	0.057
10	9.21	0.336	0.194
15	14.12	0.193	0.112

n = 3

4.3 Estimation of Etoposide and Quercetin Dihydrate in pH 7.4 phosphate buffer:

This method of estimation is developed based on the observation that ET and QD in pH 7.4 phosphate buffer shows strong absorbance in the ultraviolet region of the electromagnetic spectrum.

4.3.1 Preparation of stock solutions:

a) pH 7.4 phosphate buffer was prepared according to the procedure given in the Indian Pharmacopoeia (1996).

b) Stock solution of ET was prepared by dissolving 10mg of ET in 1ml methanol and making up the volume to 10 ml with pH 7.4 phosphate buffer.

c) Stock solution of QD was prepared by dissolving 5mg of QD in 1 ml methnol and making up the volume with pH 7.4 phosphate buffer.

Quercetin exhibits pH dependant solubility and stability profile. Thus to ensure stability and solubility of QD in pH 7.4 buffer, BSA is added to QD solutions at the concentration 0.3mg/ml. (Webb and Ebeler, 2004)

4.3.2a Procedure for calibration curve of ET:

Suitable aliquots of the 1000µg/ml stock solution of ET in pH 7.4 phosphate buffer were pipetted into 10ml volumetric flasks to contain 10,20,40,60,80,100µg/ml. The volume was made up with the buffer, the contents shaken well and the absorbance measured at 283.5nm using a Shimadzu UV-1700 Pharmaspec UV-Visible spectrophotometer with quartz cells of 10mm path length against the buffer as a blank. The above procedure was carried out in triplicates. Mean absorbance values are shown in Table 4.5a. The calibration curve was drawn by plotting the absorbances versus concentration of ET (Figure 4.6) and linearly regressed.

The absorptivity scan over the wavelength range 200 to 400nm of the solution of ET in pH 7.4 phosphate buffer is shown in Figure 4.5

The analytical parameters for the estimation of ET in pH 7.4 phosphate buffer was tabulated in Table 4.6a.

4.3.2b Procedure for calibration curve of QD:

The 7.4 pH phosphate buffer used to prepare QD Standard solutions contain BSA at a concentration of 0.3mg/ml. Stock solution of QD (500µg/ml) was further diluted to obtain 100µg/ml solution. Suitable aliquots of the 100µg/ml stock solution of QD in phosphate buffer were pipetted into 10ml volumetric flasks to contain 5, 10, 15, 20, 25, 30µg/ml. The volume was made up with the buffer, the contents shaken well and the absorbance measured at 377.0nm using a Shimadzu UV-1700 Pharmaspec UV-Visible spectrophotometer with

quartz cells of 10mm path length against the buffer containing BSA as a blank. The above procedure was carried out in triplicates. Mean absorbance values are shown in Table 4.5b. The calibration curve was drawn by plotting the absorbances versus concentration of QD (Figure 4.8) and linearly regressed.

The absorptivity scan over the wavelength range 200 to 400nm of the solution of QD in pH 7.4 buffer is shown in Figure 4.7

The analytical parameters for the estimation of QD in pH 7.4 phosphate buffer was tabulated in Table 4.6b.

4.3.3 Stability:

Changes in absorbance of the solutions of ET in 7.4 pH buffer, used for preparing the calibration curve at analytical wavelength, were monitored for a period of 24 hrs. Thus stability of these solutions with time was established.

Changes in absorbance of 10µg/ml solution of QD in 7.4 pH buffer with BSA and without BSA were monitored in triplicate for a period of 24 hrs (Figure 4.9). The mean absorbance is plotted against time in hrs, thus stabilizing effect of BSA was ensured.

4.3.4 Accuracy and precision:

Known amounts of ET and QD were subjected to recovery studies using the method described in detail above, in triplicate, to establish the accuracy and precision of the method. The result of analysis was summarized in Table 4.7a and 4.7b respectively for ET and QD.

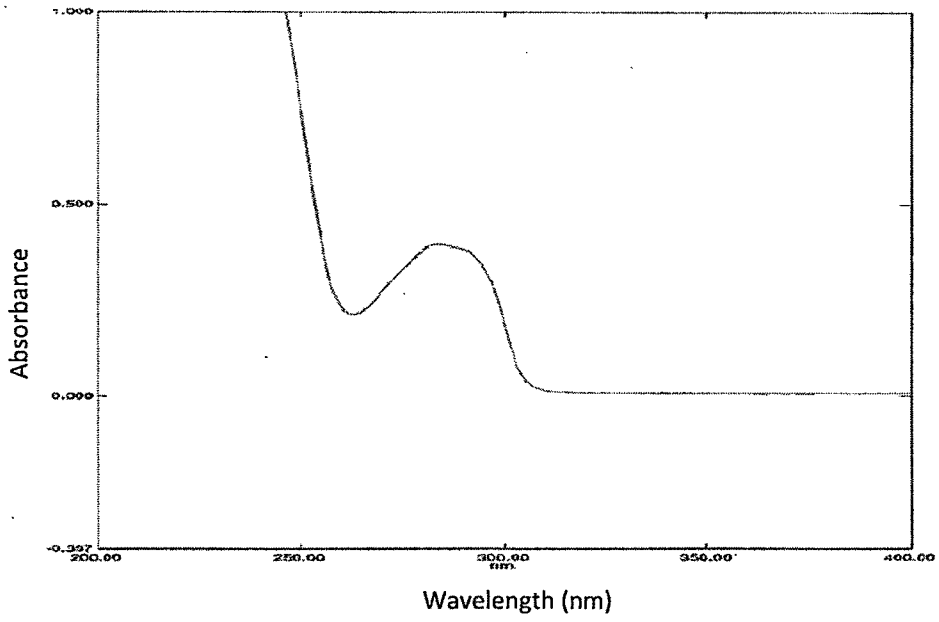


Figure 4.5 Absorptivity Scan of ET in pH 7.4 Phosphate Buffer

Table 4.5a Calibration curve of ET in pH 7.4 phosphate buffer

Concentration µg/ml	Mean absorbance	±SE	Regressed values
10	0.082	0.004	0.071
20	0.142	0.002	0.131
40	0.269	0.003	0.251
60	0.396	0.003	0.371
80	0.541	0.002	0.491
100	0.666	0.005	0.611

n = 3

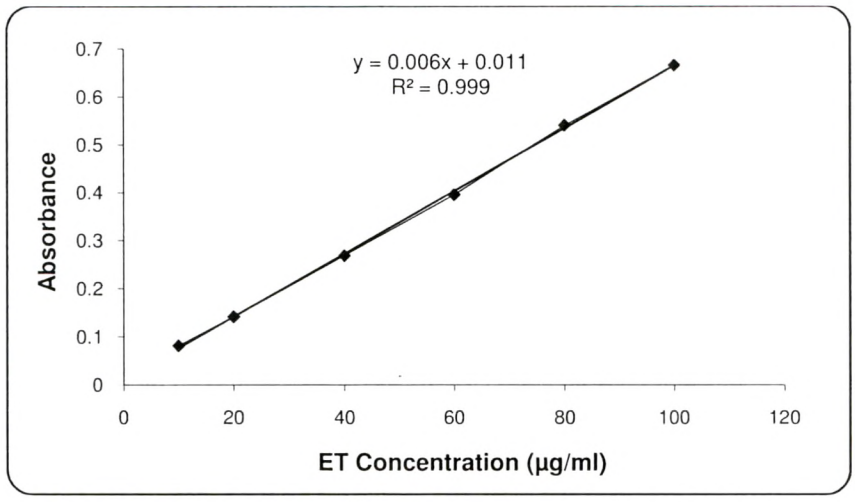


Figure 4.6 Standard curve of ET in pH 7.4 phosphate buffer

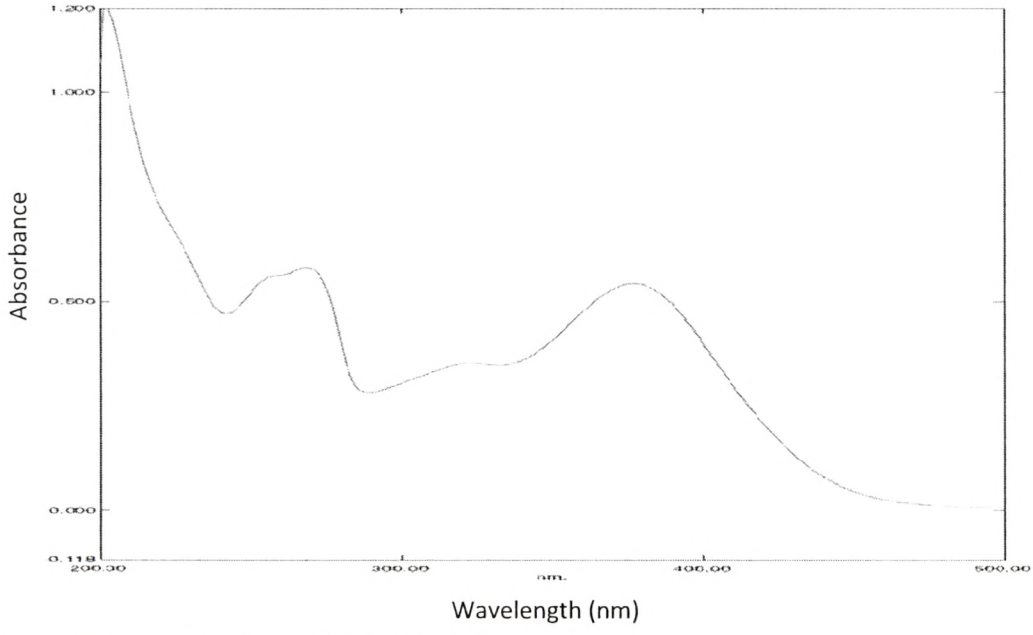


Figure 4.7 Absorptivity Scan of QD in pH 7.4 Phosphate Buffer

Table 4.5b Calibration curve of QD in pH 7.4 phosphate buffer

Concentration µg/ml	Mean absorbance	±SE	Regressed values
5	0.271	0.010	0.268
10	0.543	0.008	0.538
15	0.812	0.008	0.808
20	1.082	0.006	1.078
25	1.349	0.009	1.348
30	1.635	0.010	1.618

n = 3

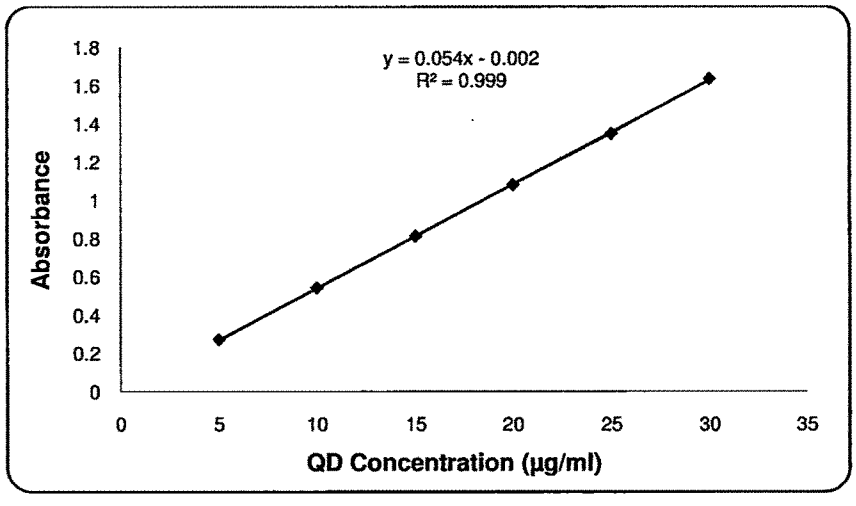


Figure 4.8 Standard curve of QD in pH 7.4 phosphate buffer

Table 4.6a Analytical parameters for estimation of ET in pH 7.4 phosphate buffer

Analytical Parameter	Value
Absorption maxima (nm)	283.5nm
Beer's law limits at 283.5nm (µg/ml)	10 -100
Apparent molar absorptivity at 283.5nm (1 $mol^{-1}cm^{-1}$)	3.6×10^3
Regression equation	y = 0.006x + 0.001
Co-rrelation co-efficient	0.999

Table 4.6b Analytical parameters for estimation of QD in pH 7.4 phosphate buffer

Analytical Parameter	Value
Absorption maxima (nm)	268.0, 323.0, 377.0
Beer's law limits at 377.0 nm (µg/ml)	5 -30
Apparent molar absorptivity at 377.0 nm (1 $mol^{-1}cm^{-1}$)	18.0×10^3
Regression equation	y = 0.054x – 0.002
Co-rrelation co-efficient	0.999

Table 4.7a Evaluation of accuracy and precision of estimation method of ET in pH 7.4 phosphate buffer

Added (mg)	Found (mg)Mean	±SD	SE
5	4.44	0.160	0.093
10	9.52	0.135	0.078
15	14.72	0.151	0.087

Table 4.7b Evaluation of accuracy and precision of estimation method of QD in pH 7.4 phosphate buffer

Added (mg)	Found (mg)Mean	±SD	±SE
5	4.17	0.070	0.040
10	8.81	0.153	0.088
15	14.07	0.174	0.100

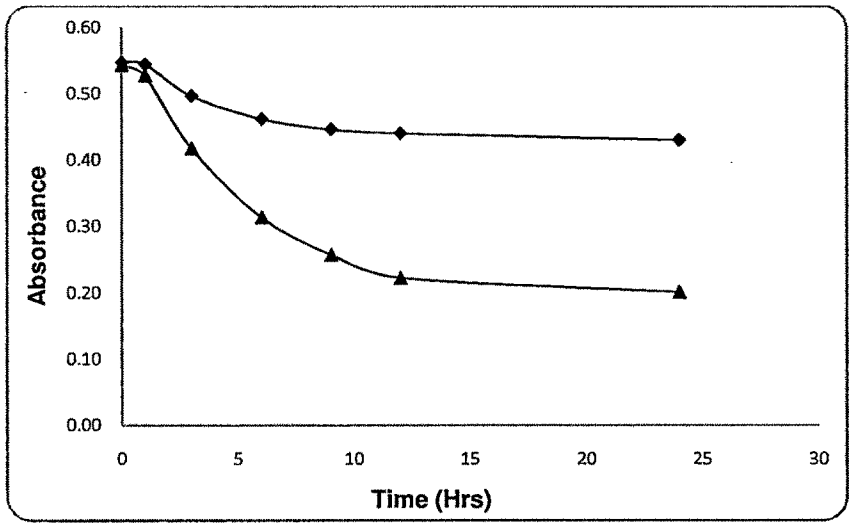


Figure 4.9 Effect of 0.3mg/ml BSA on stability of QD in pH 7.4 Phosphate buffer

(▲) Mean absorbance of 10µg/ml QD in pH 7.4 phosphate buffer

(♦) Mean absorbance of 10µg/ml QD in pH 7.4 phosphate buffer with 0.3mg/ml of BSA

n = 3

4.4 Result and discussion:

4.4.1 Solubility studies:

Solubility profile of ET and QD in different solvents at room temperature (Table 4.1) indicated that the drugs were freely soluble in DMSO and chloroform, soluble in acetone and acetonitrile, slightly soluble in ethanol and methanol, and practically insoluble in PBS pH 7.4 and distilled water.

4.2 Estimation of ET and QD in PLGA nanoparticles:

4.2a Estimation of ET in PLGA nanoparticles:

ET in acetonitrile yields a characteristic curve when scanned in UV- Visible wavelength range between 200 to 400 nm, even in presence of other constituents of nanopaticles such as PLGA. The scan (Figure 4.1) shows absorption maxima at 283.5nm. The absrorptivity of ET at 283.5nm was 4.09×10^3 mol^{-1} cm^{-1}, and found to be satisfactory for the purpose.

No interference of the additives was observed in the wavelength range of estimation. Thus 283.5 was selected as analytical wavelength and used for the further studies.

Regression analysis of the experimental data was carried out and the absorbance values along with regressed values are shown in Table 4.2a.

Table 4.3a summarises the analytical parameters for estimation of ET in acetonitrile.

The graph of absorbance versus concentration was shown in Figure 4.2. The regression equation was found to be $y = 0.007x - 0.005$

A correlation coefficient of 0.999 indicates that the absorbance and concentration of the drug were linearly related and Beer's law was found to be obeyed in the range of 10 to 120µg/ml. (Table 4.2a). The low variability of the data is supported by the low standard error values of mean absorbance values of the solutions used for preparing the calibration curve. The value of the slope (0.007) indicates high sensitivity of the method which is also reflected by the high absorptivity of the compound (4.09×10^3 mol^{-1} cm^{-1}).

The stability of the ET in acetonitrile was ascertained over a period of 24 hrs. Analysis of mean absorbance values of the solutions of different concentrations at various time intervals revealed that there was no significant difference between the readings. From this, it is concluded that ET is stable over the determined period in acetonitrile.

Estimation of ET was carried out in the presence of other constituents of nanoparticles, such as PLGA at the approximate levels at which they are present in the formulations. None of the materials interfered in the estimation of the ET using the above method. This method was used to determine the content of ET in PLGA nanoparticles.

The results of the recovery studies on known amounts of ET, in triplicate, are presented in Table 4.4a and were used to ascertain the accuracy and precision of the method. Low SE values indicate low variability between each data point. Precision of the method was ascertained from SD values. The overall data supports the precision of the method of estimation of ET in acetonitrile and concludes no interference of PLGA with the absorbance of ET and also indicates the accuracy of the method.

4.2b Estimation of QD in PLGA nanoparticles

QD in acetonitrile yields a characteristic curve when scanned in UV- Visible wavelength range between 200 to 500 nm, even in presence of other constituents of nanopaticles such as PLGA. The scan (Figure 4.3) shows absorption maxima at 254.0 and 368.0nm. The absrorptivity of QD at 368.0nm was 21.3×10^3 mol^{-1} cm^{-1}. No interference of the additives was observed in the wavelength range of estimation. Thus 368.0nm was selected as analytical wavelength and used for the further studies.

Regression analysis of the experimental data was carried out and the absorbance values along with regressed values are shown in Table 4.2b.

Table 4.3b summarises the analytical parameters for estimation of QD in acetonitrile.

The graph of absorbance versus concentration was shown in Figure 4.4. The regression equation was found to be $y = 0.064x - 0.003$.

A correlation coefficient of 0.999 indicates that the absorbance and concentration of the drug were linearly related and Beer's law was found to be obeyed in the range of 2 to 30µg/ml. (Table 4.2b). The low variability of the data is supported by the low standard error values of mean absorbance values of the solutions used for preparing the calibration curve. The value of the slope (0.064) indicates moderate sensitivity of the method which is also reflected by the high absorptivity of the compound (21.3×10^3 mol^{-1} cm^{-1}).

The stability of the QD in acetonitrile was ascertained over a period of 24 hrs. Analysis of mean absorbance values of the solutions of different concentrations at various time intervals revealed that there was no significant difference between the readings. From this, it is concluded that QD is stable over the determined period in aceonitrile.

Estimation of QD was carried out in the presence of other constituents of nanoparticles, such as PLGA at the approximate levels at which they are present in the formulations. None of the materials interfered in the estimation of the QD using the above method. This method was used to determine the content of QD in PLGA nanoparticles.

The results of the recovery studies on known amounts of QD, in triplicate, are presented in Table 4.4b and were used to ascertain the accuracy and precision of the method. Low SE

values indicate low variability between each data point. Precision of the method was ascertained from SD values. The overall data supports the precision of the method of estimation of QD in acetonitrile and concludes no interference of PLGA with the absorbance of QD and also indicates the accuracy of the method.

4.3 Estimation of ET and QD in pH 7.4 phosphate buffer

4.3a Estimation of ET in pH 7.4 phosphate buffer

ET in pH 7.4 phosphate buffer yields a characteristic curve when scanned in UV- Visible wavelength range between 200 to 400 nm. The scan (Figure 4.5) shows absorption maxima at 283.5nm. The absrorptivity of ET at 283.5nm was 3.6×10^3 mol^{-1} cm^{-1} and found to be satisfactory. The analytical wavelength 283.5nm was selected because it avoids any possible interference of the additives in the ultraviolet range during estimation.

Regression analysis of the experimental data was carried out and the absorbance values along with regressed values are shown in Table 4.5a.

Table 4.6a summarises the analytical parameters for estimation of ET in pH 7.4 phosphate buffer.

The graph of absorbance versus concentration was shown in Figure 4.6. The regression equation was found to be $y = 0.006x + 0.011$

A correlation coefficient of 0.999 indicates that the absorbance and concentration of the drug were linearly related and Beer's law was found to be obeyed in the range of 10 to 100µg/ml. (Table 4.5a). The low variability of the data is supported by the low standard error values of mean absorbance values of the solutions used for preparing the calibration curve. The value of the slope (0.006) indicates moderate sensitivity of the method which is also reflected by the high absorptivity of the compound (3.6×10^3 mol^{-1} cm^{-1}).

The stability of the ET in pH 7.4 phosphate buffer was ascertained over a period of 24 hrs. Analysis of mean absorbance values of the solutions of different concentrations at various time intervals revealed that there was no significant difference between the readings. From this, it is concluded that ET is stable over the determined period in pH 7.4 phosphate buffer.

The results of the recovery studies on known amounts of ET, in triplicate, are presented in Table 4.7a and were used to ascertain the accuracy and precision of the method. Low SE values indicate low variability between each data point. Precision of the method was ascertained from SD values. The overall data supports the precision and accuracy of the method of estimation of ET in pH 7.4 phosphate buffer. This method of estimation was used to obtain the release profile of ET from PLGA nanoparticles using dialysis technique.

4.3a Estimation of QD in pH 7.4 phosphate buffer

QD in pH 7.4 phosphate buffer yields a characteristic curve when scanned in UV- Visible wavelength range between 200 to 500 nm. The scan (Figure 4.7) shows absorption maxima at 377.0, 323.0 and 268.0nm. The absrorptivity of QD at 377.0nm was 18.0×10^3 mol^{-1} cm^{-1} and found to be very good. Thus the analytical wavelength was selected at 377.0nm.

Regression analysis of the experimental data was carried out and the absorbance values along with regressed values are shown in Table 4.5b.

Table 4.6b summarises the analytical parameters for estimation of QD in pH 7.4 phosphate buffer.

The graph of absorbance versus concentration was shown in Figure 4.8. The regression equation was found to be $y = 0.054x - 0.002$

A correlation coefficient of 0.999 indicates that the absorbance and concentration of the drug were linearly related and Beer's law was found to be obeyed in the range of 5 to 30µg/ml. (Table 4.5b). The low variability of the data is supported by the low standard error values of mean absorbance values of the solutions used for preparing the calibration curve. The value of the slope (0.054) indicates moderate sensitivity of the method which is also reflected by the high absorptivity of the compound (18.0×10^3 mol^{-1} cm^{-1}).

The stability of the QD in pH 7.4 phosphate buffer was studied over a period of 24 hrs. Analysis of mean absorbance values of the solutions of different concentrations at various time intervals revealed that there was significant difference between the readings. This is due to the oxidative degradation of QD in aqueous medium. Quercetin exhibits pH dependant solubility and stability. Webb and Ebeler (2004) studied the effects of BSA on the solution stability of quercetin by monitoring the changes in the absorbance under varying solution conditions. As evidenced by the loss in absorbance, quercetin was unstable at pH 7 and was stabilized by BSA. Up to pH 7.5, quercetin was relatively stabilised by the presence of BSA, which was also observed in our experiment

(Figure 4.9) but at higher pH i.e. at pH 8, DTT is effective in stabilizing quercetin. Thus we used BSA at recommended concentration (3mg/ml) to stabilize the QD solution in pH 7.4 pH phosphate buffer. The graph of absorbance of QD in pH 7.4 phosphate buffer with and without BSA (0.3mg/ml) versus time was shown in Figure 4.9. Our results are in agreement with those mentioned above (Webb and Ebler, 2004)

Such solubility and stability behaviour was exhibited by most of the flavonols.

Under certain conditions, such as high ionic strength, low solvent strength, high concentrations of solute, low pH, or an appropriate combination of these, the soluble aggregate will tend to precipitate out of solution, thus causing loss in absorbance. The flavonols, appear to be susceptible to degradation in aqueous solution, presumably by auto-oxidation under aerobic conditions. These mechanisms were counteracted by BSA.

The basis for this BSA effect is not known. BSA may act by binding flavonoids and preventing aggregation and/or precipitation, but, if this is the case, the binding mechanism is unclear. The literature indicates (somewhat unconvincingly) that BSA binds the anionic form of flavonoids only (Dangles et al., 2001), and, if this is true, BSA may affect the apparent stability of flavonoids by binding the anionic form, shifting the acid–base equilibrium towards deprotonation, thereby decreasing the tendency to aggregate. Alternatively, flavonoids may simply bind BSA weakly, creating an equilibrium pool of both bound and free forms of quercetin, thus lowering the concentration of free flavonoids and slowing aggregation. BSA may also act as an antioxidant in this system (Webb and Ebeler, 2004).

Hence pH 7.4 phosphate buffer was selected as a medium in *in-vitro* release studies. This selection of medium is also supported by the work of Song et al. (2008).

The results of the recovery studies on known amounts of QD, in triplicate, are presented in Table 4.7b and were used to ascertain the accuracy and precision of the method. Low SE values indicate low variability between each data point. Precision of the method was ascertained from SD values. The overall data supports the precision and accuracy of the method of estimation of QD in pH 7.4 phosphate buffer.

This method of estimation was used to obtain the release profile of QD from PLGA nanoparticles in pH 7.4 phosphate buffer with BSA using dialysis technique.

4.4 Conclusions:

Analytical methods for the estimation of ET and QD were successfully established. The quantitative evaluation of ET and QD in PLGA nanoparticles and in pH 7.4 phosphate buffer for in vitro release study was performed using UV spectroscopic methods. The methods were validated by carrying out the stability, selectivity, accuracy and precision studies for the method.

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