Analytical Method development

3.1 MATERIALS

ETO was a kind gift from Cipla Ltd, Mumbai. Acetonitrile (HPLC & AR grade), hydrochloric acid (HCl) and dichloromethane (HPLC grade) were purchased from S.D. Fine Chem. Ltd., Mumbai. Sodium chloride, disodium hydrogen phosphate, iodine (I_2), potassium iodide (KI) and potassium dihydrogen phosphate were purchased from Spectrochem Ltd, Mumbai. Methanol (AR grade), acetic acid and water of HPLC grade were purchased from Merck, India. Monomethoxy Polyethylene glycol (CH_3O-PEG-OH) of molecular weight 2000 & 5000 and 2, 4, 6 trinitro benzene sulfonic acid (TNBS) [5 % (w/v) in H_2O] were purchased from Sigma Aldrich, Mumbai.

3.2 ANALYTICAL METHOD DEVELOPMENT OF ETO

3.2.1 Analytical method development by UV spectroscopy

3.2.1.1 Calibration curve of ETO in Acetonitrile (ACN) and Phosphate buffer saline (PBS) pH 7.4

Stock solution of ETO was prepared by dissolving 5 mg of accurately weighed ETO in 25 ml of ACN. A serial dilution of ETO was made in the range of 10 to 80 mcg/ml in series of volumetric flask and absorbance of ETO was measured using UV visible spectrophotometer (UV 1700, PharmaSpec, Shimadzu, Japan) at 284 nm. Calibration of ETO in PBS pH 7.4 was performed in similar manner by dissolving 5 mg of accurately weighed ETO in PBS pH 7.4 containing 10 % methanol. The results were expressed as mean ± S. D. and n= 6. The respective calibration curves were plotted as concentration vs. absorbance.

3.2.1.2 Analytical method validation

The above method was validated for accuracy, precision and linearity.

3.2.1.2.1 Accuracy

Accuracy refers to the closeness of an individual observation or mean to true value. The "true" value is the result which would be observed in absence of error. Accuracy of the assay was defined as the percentage of the agreement between the measured value and the true value as follows (Merodia et al., 2000).

$$\text{Accuracy} = \frac{\text{True value - Measured value}}{\text{True value}} \times 100$$

3.2.1.2.2 Precision

It refers to the extent of variability of a group of measurements observed under similar conditions. Precision provides an indication of random errors and is generally subdivided into two cases: repeatability and reproducibility, which were determined by calculating RSD (Relative standard deviation) or CV (Coefficient of variation) of interday and intraday determinations. One of the common ways of expressing the variability, which takes into account its relative magnitude, is the ratio of the standard deviation to the mean (SD/Mean). This ratio, often expressed as a percentage, is called the coefficient of variation (CV) or relative standard deviation (RSD).

3.2.1.2.3 Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample (Hubert et al., 1999; Hubert et al., 2003). Linearity of a light absorption determination should be examined to ensure that Beer's law operates over the range of interest. The calibration curve was built by plotting the drug concentration versus their respective absorbance. The concentrations for unknown samples and validation samples were obtained by using linear regression of the calibration curves.

3.2.1.3 Results and discussion

ETO showed a characteristic spectrum when scanned in the ultraviolet range between 200 to 400 nm. Figure 3.1 shows the characteristics spectrum of ETO in ACN at concentration of 20 µg/ml while Figure 3.2 & 3.3 represents the regressed calibration curve of ETO in acetonitrile and PBS pH 7.4 respectively. In both the solvent, the absorption maxima were found at 284 nm and this wavelength was chosen as analytical wavelength. Table 3.1 and 3.2 shows the measured absorption at different concentrations. Correlation coefficient for developed method was found to be 0.9995 in ACN and 0.9994 in PBS pH 7.4, signifying a linear relationship existed between absorbance and concentration of the drug.

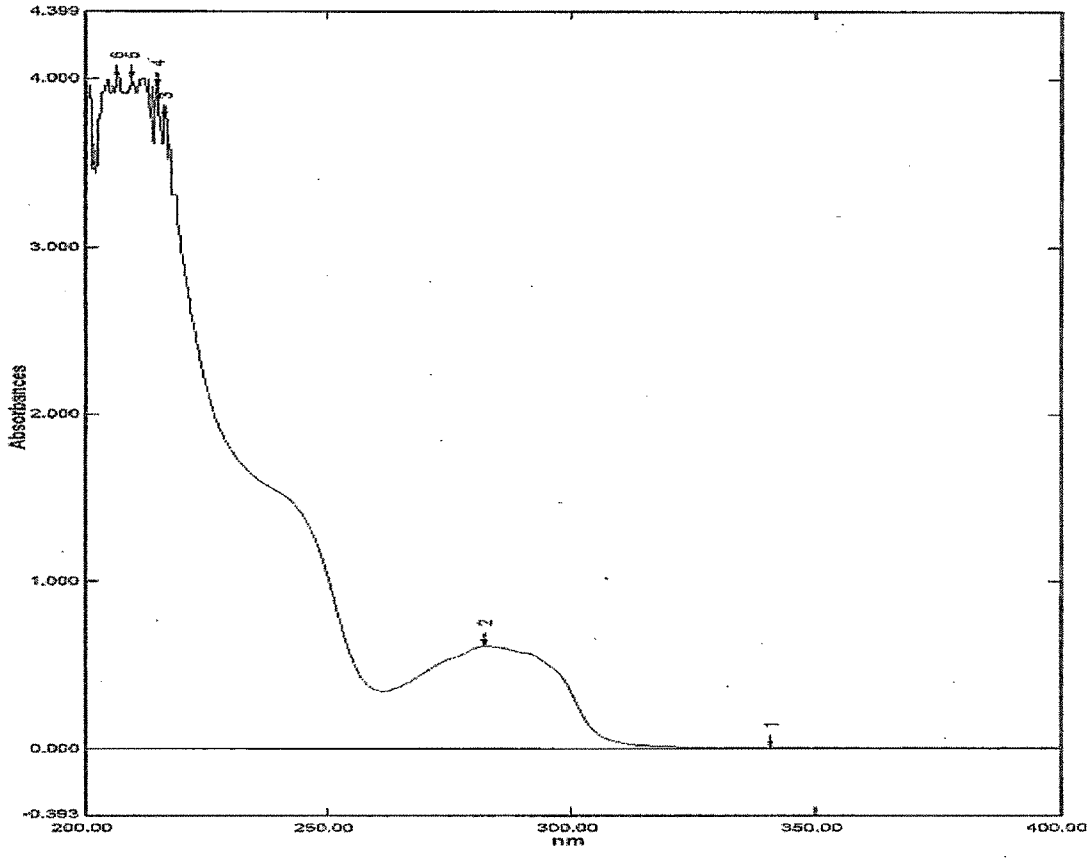

Figure 3.1 Wavelength scan of ETO in ACN at concentration of 20 µg/ml

Concentration (µg/ml)	Mean Absorbance
10	0.072 ± 0.008
20	0.143 ± 0.010
30	0.223 ± 0.007
40	0.293 ± 0.011
50	0.366 ± 0.012
60	0.439 ± 0.009
70	0.507 ± 0.016
80	0.572 ± 0.014

Results are mean ± S.D. and n= 6

Table 3.1 Regressed calibration curve of ETO in ACN at 284 nm

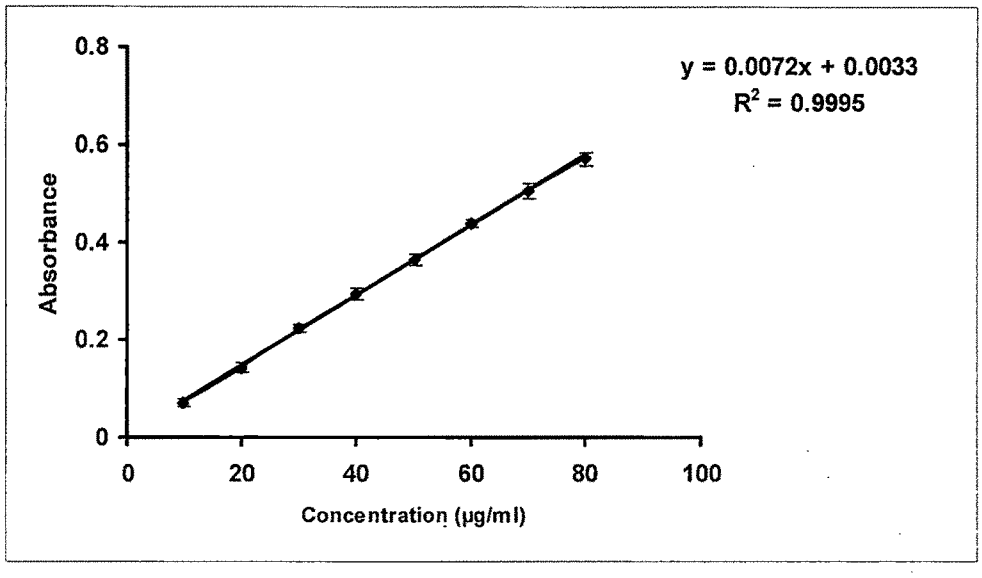

Figure 3.2 Regressed calibration curve of ETO in ACN

Concentration (µg/ml)	Mean Absorbance
10	0.080 ± 0.010
20	0.140 ± 0.012
30	0.216 ± 0.009
40	0.284 ± 0.008
50	0.356 ± 0.013
60	0.416 ± 0.011
70	0.480 ± 0.009
80	0.551 ± 0.015

Results are mean ± S.D. and n= 6

Table 3.2 Regressed calibration curve of ETO in PBS pH 7.4 at 284 nm

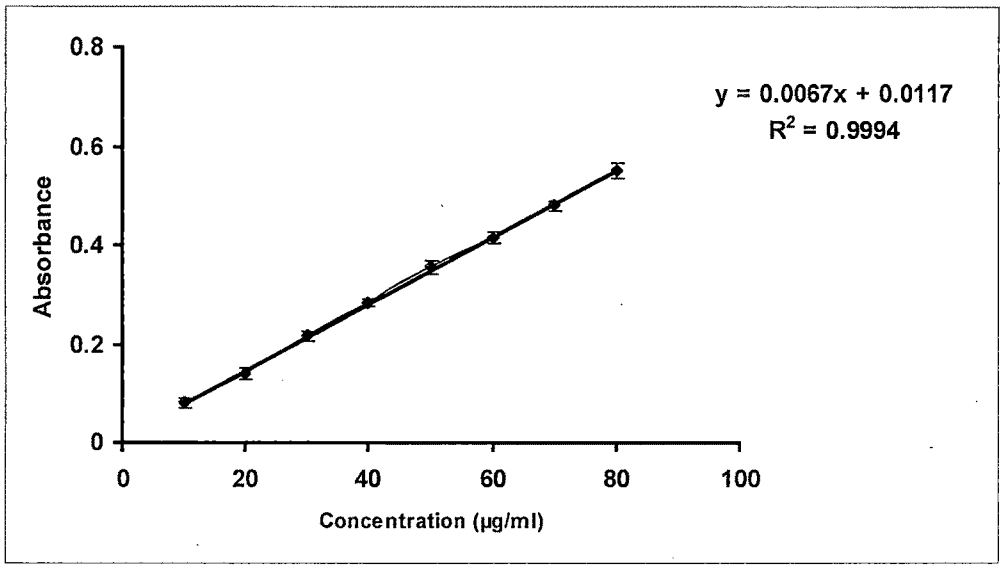

Figure 3.3 Regressed calibration curve of ETO in PBS pH 7.4

Drug concentration (µg/ml)		Precision[a]	Accuracy[b]
Actual	Obtained		
5	5.04 ± 0.10	2.38	100.80 ± 2.00
10	10.20 ± 0.24	2.35	100.45 ± 2.40
15	15.08 ± 0.20	1.32	98.32 ± 1.33
20	20.18 ± 0.31	1.53	101.43 ± 1.55

[a] Expressed as % relative standard deviation (% RSD)

[b] % Recovery ± S.D.

Table 3.3 Intraday precision and accuracy of ETO in ACN (n= 6)

Drug concentration (µg/ml)		Precision[a]	Accuracy[b]
Actual	Obtained		
5	5.05 ± 0.11	2.17	101.00 ± 2.20
10	9.98 ± 0.15	1.50	99.80 ± 1.50
15	14.91± 0.28	2.01	99.40 ± 1.86
20	20.12 ± 0.31	1.14	115.00 ± 1.55

[a] Expressed as % relative standard deviation (% RSD)

[b] % Recovery ± S.D.

Table 3.4 Interday precision and accuracy for ETO in ACN (n= 6)

Drug concentration (µg/ml)		Precision[a]	Accuracy[b]
Actual	Obtained		
5	4.87 ± 0.23	4.60	97.40 ± 4.6
10	10.08 ± 0.27	2.67	100.80 ± 2.70
15	15.32 ± 0.31	2.02	102.13 ± 2.06
20	19.93 ± 0.28	1.40	99.65 ± 1.40

[a] Expressed as % relative standard deviation (% RSD)

[b] % Recovery ± S.D.

Table 3.5 Intraday precision and accuracy of ETO in PBS pH 7.4 (n= 6)

Drug concentration (µg/ml)		Precision[a]	Accuracy[b]
Actual	Obtained		
5	4.94 ± 0. 15	3.03	98.80 ± 3.00
10	10.15 ± 0.26	2.56	101.50 ± 2.60
15	15.36 ± 0.22	1.43	102.40 ± 1.46
20	20.35 ± 0.33	1.62	101.75 ± 1.65

[a] Expressed as % relative standard deviation (% RSD)

[b] % Recovery ± SD

Table 3.6 Interday precision and accuracy for ETO in PBS pH 7.4 (n= 6)

Table 3.3 to 3.6 shows intraday and interday precision and accuracy for ETO in ACN and PBS pH 7.4 by UV spectroscopy. All R.S.D. values observed were lower than the acceptance limit of 15%, illustrating a very good precision of the proposed method. It can be seen from the results, that the proposed method was accurate, since the different tolerance limits of the bias were below the acceptance limits (± 15 %) for all the concentration levels tested including the lowest one (Boulanger et al., 2003; Guidance for industry, 2001).

3.2.2 Analytical method development of ETO in cell lysate by HPLC method

3.2.2.1 HPLC conditions

The drug content in cell lysate was determined by reverse phase HPLC method as described earlier (Kang et al., 2005; Lee & Lim, 2006). The HPLC system was

composed of UV-visible spectrophotometer detector (SPD-20A, Prominence), sampling injector (Model 231 XL, Gilson) and syringe pump (Model 402, Gilson). The separation was performed on reversed phase C18 HPLC column (Phenomenex Luan 5μ C18 (2) 250 X 4.6 mm i.d., 5 μm). The mobile phase, ACN: water: glacial acetic acid (35:61:1, v/v/v) was run at a flow rate of 1 ml/min and the column effluent was monitored by UV detector set at 254 nm. Diazepam was used as an internal standard and samples were extracted using a liquid–liquid extraction process.

B16F10 cells were grown in Iscove's Minimum Dulbecco's Medium (IMDM) supplemented with 10 % Fetal Bovine Serum (FBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 in air and the media was replenished every third day. The cells were harvested using trypsin-EDTA in their exponential growth phase. B16F10 cells were washed with PBS twice and lysed using probe sonicator (Branson Sonifier S-450, USA), which was used as blank cell lysis solution.

3.2.2.2 Stock solution and working standard solutions

Accurate quantity of weighed ETO was transferred to volumetric flask and dissolved in ACN to make final concentration of 50 μg/ml. Diazepam was also dissolved in ACN to make final concentration of 1 mg/ml. Stock solution of ETO was diluted to 4, 8, 10, 15, 20, 25 μg/ml with ACN, which were used for spiking cell lysis solution. These solutions were kept at 4 °C with protection from light when not in use.

3.2.2.3 Sample preparation

Fifty-microlitre of 10 μg /ml diazepam was added to 2.0 ml aliquots of cell lysis solution as an internal standard followed by addition of 1 ml of 6 M hydrochloric acid for precipitation of proteins. The mixture was extracted with 3.0 ml of dichloromethane, vortexed for 2 min and centrifuged for 15 min at 2500 rpm. The upper organic layer was collected and evaporated to dryness with N_2. The residue was reconstituted in 80 μl mobile phase before analysis and injected to HPLC system.

3.2.2.4 Calibration curve

To prepare calibration curves, standard samples of ETO were added to blank cell lysis solution to give final concentrations of 0.4, 0.8, 1.0, 1.5, 2.0, 2.5 μg/ml. Extraction of

ETO from cell lysate was performed in similar method as mentioned above in sample preparation. Peak area ratios of ETO to internal standard were used for construction of calibration curve by linear regression analysis method.

3.2.2.5 Precision and accuracy

The intraday precision of the assays performed in replicate (n= 5) were tested by using three concentrations of the ETO namely, 1.0, 2.0 and 3.0 μg/ml. The interday precision of the assays was estimated from the results of three replicate assays on 5 different days within 1 week. The precision was evaluated by the relative standard deviation (R.S.D.).

3.2.2.6 Extraction efficiency

Extraction efficiency of ETO from cell lysis solution was evaluated (n= 5) at 1.0, 2.0 and 3.0 μg/ml by comparing the peak areas of an extracted sample containing a known amount of ETO with the peak areas obtained from direct injections of the solution containing the same concentration of ETO in pure solvent.

3.2.2.7 Results and discussion

3.2.2.7.1 Chromatographic specificity

Separation of ETO from the internal standard in cell lysis solution was achieved using the C18 column. There were no interfering peaks co-eluting with the compounds of interest was seen (Figure 3.4). The retention time for ETO and diazepam were approximately 5.25 and 4.74 min.

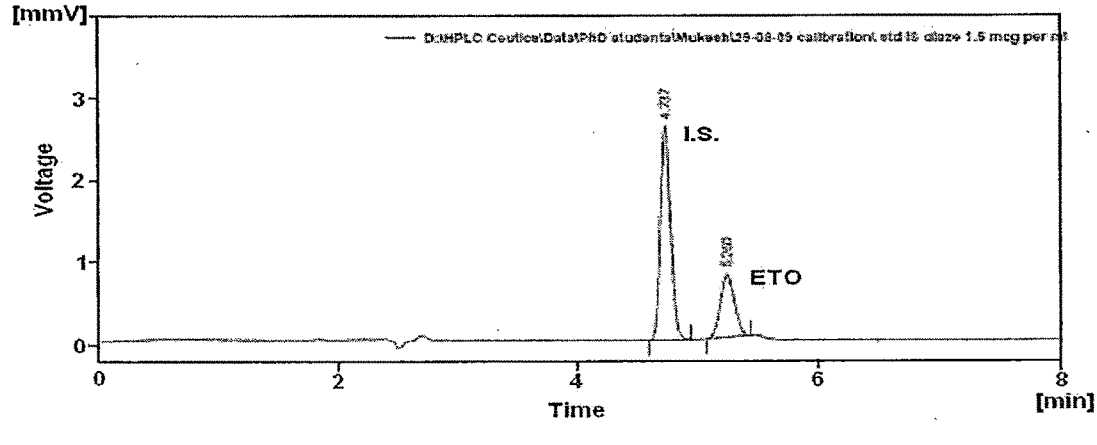

Figure 3.4 Typical chromatogram of cell lysis solution spiked with ETO along with internal standard (diazepam)

3.2.2.7.2 Calibration curve

A calibration curve for ETO was constructed by analyzing a series of blank cell lysis solution, spiked with ETO at the concentration range from 0.4 to 2.5 μg/ml. Peak area ratios (y) of the ETO to the internal standard diazepam were measured (Table 3.7) and plotted against the concentration (x) of ETO. The calibration curves for ETO was linear in the concentration range measured (Figure 3.5). The regression equations of the calibration curve of ETO obtained was $y = 0.2849x + 0.0146$ ($R^2 = 0.9975$).

Concentration of ETO (μg/ml)	Ratio of peak area of ETO to I.S.
0.4	0.109 ± 0.011
0.8	0.245 ± 0.015
1.0	0.304 ± 0.019
1.5	0.448 ± 0.019
2.0	0.600 ± 0.036
2.5	0.725 ± 0.039
3.0	0.888 ± 0.033
3.5	0.986 ± 0.041

Results are mean ± S.D. and n= 5

Table 3.7 Regressed calibration curve of ETO in cell lysate

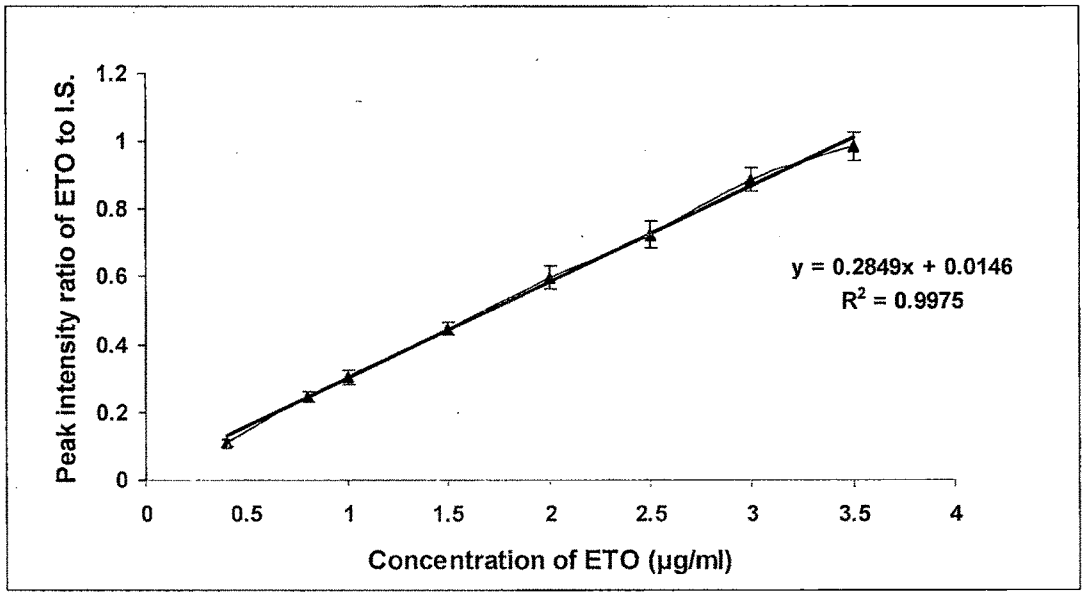

Figure 3.5 Regressed calibration curve of ETO in cell lysate

3.2.2.7.3 Precision and accuracy

The precision and accuracy of the method were assessed by intraday and interday assay validations (n= 5) at concentrations of 1.0, 2.0 and 3.0 µg/ ml. The results showed that the intra and interday percent relative standard deviations were less than 5 % (R.S.D.) (Table 3.8). The percent recovery of the assay was between 98.0 and 102.0 %.

Drug concentration (µg/ml)	Precision[a]		Accuracy[b]	
	Intraday	Interday	Intraday	Interday
1.0	4.08	6.18	98.00 ± 4.00	97.00 ± 6.00
2.0	3.46	4.52	101.00 ± 3.50	99.50 ± 4.50
3.0	3.02	2.62	99.33 ± 3.01	101.66 ± 2.66

[a] Expressed as % relative standard deviation (% RSD)

[b] % Recovery ± S.D.

Table 3.8 Intraday and interday precision and accuracy of ETO from cell lysis solution (n= 5)

3.2.2.7.4 Extraction efficiency

A series of blank cell lysis solutions, spiked with various amounts of ETO, was processed according to determine extraction efficiency. The mean extraction recovery (%) of ETO from cell lysis solution obtained is represented in Table 3.9.

Added concentration (µg/ml)	Extraction recovery (%)
1.0	69.77 ± 4.83
2.0	72.50 ± 4.06
3.0	77.24 ± 2.10

Table 3.9 Extraction recovery of ETO from cell lysate (mean ± S. D., n= 5)

3.3 ANALYTICAL METHOD DEVELOPMENT OF POLY(ETHYLENE GLYCOL)

3.3.1 Calibration curve of polyethylene glycol

Calibration curve of monomethoxy poly(ethylene) glycol [PEG] was constructed as reported earlier with slight modification (Bin et al., 2005; Peracchia et al., 1997). A stock solution of CH_3O-PEG-OH of molecular weight 2000/ 5000 was prepared by dissolving 10 mg of CH_3O-PEG-OH in 100 ml of distilled water. Serial dilutions of stock solution were made and to 5 ml of diluted solution, 2 ml of 2 N NaOH added and kept for 5 days at 50 °C. Samples were neutralized to pH 7.0 with 1 N HCl followed by addition of 250 µl of I_2/KI solution (I_2 10g/l and KI 20 g/l) and final volume was made to 10 ml with distilled water. Final concentration of CH_3O-PEG-OH in solution was kept in the range of 2 to 12 µg/ml. Samples were mixed well and absorbance was measured at 525 nm using UV visible spectrophotometer (UV 1700, PharmaSpec, Shimadzu, Japan). Blank samples were prepared in same manner without use of PEG and used for measuring absorbance of samples. Calibration plot was constructed plotting absorbance vs. concentration.

3.3.2 Results and discussion

A regressed calibration curve of CH_3O-PEG-OH of molecular weight 2000 and 5000 was plotted by concentration vs. absorbance as shown in Figure 3.6 and 3.7. In both the cases, a linear relationship was observed with the tested concentration and the regression coefficient was found 0.99. Table 3.10 and 3.11 shows the absorbance recorded with respective concentration at λ of 525 nm.

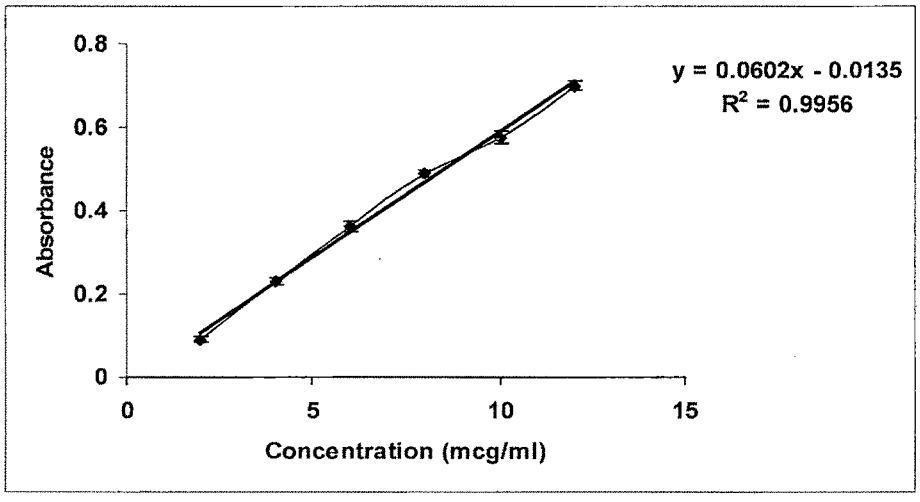

Figure 3.6 Regressed calibration curve of CH_3O-PEG-OH (M.W. 2000) at λ 525 nm

Conc. of CH_3O-PEG-OH (μg/ml)	Absorbance
2	0.091 ± 0.006
4	0.229 ± 0.009
6	0.362 ± 0.011
8	0.489 ± 0.010
10	0.576 ± 0.015
12	0.700 ± 0.010

Results are mean ± S.D. of three experiments

Table 3.10 Regressed calibration curve of CH_3O-PEG-OH (M.W. 2000) at λ 525 nm

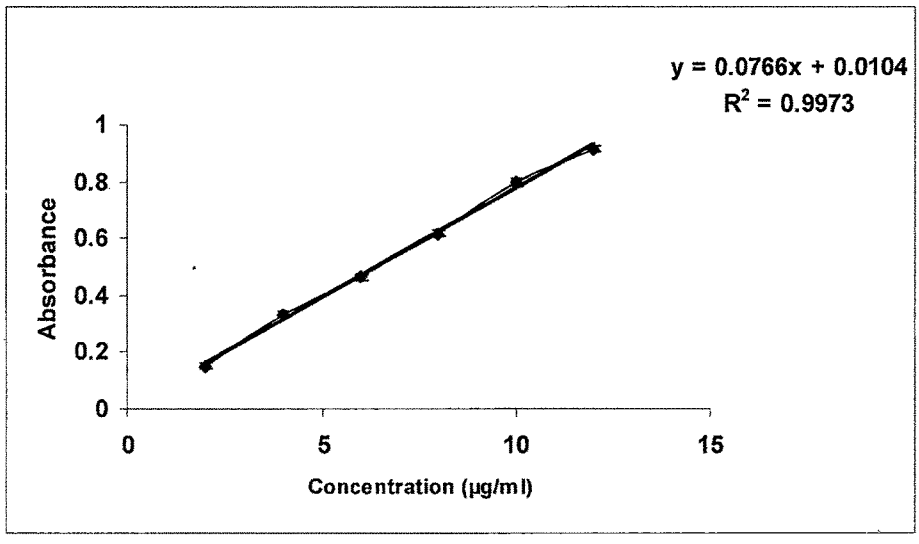

Figure 3.7 Regressed calibration curve of CH_3O-PEG-OH (M.W. 5000) at λ 525 nm

Conc. of CH_3O-PEG-OH (µg/ml)	Absorbance
2	0.152 ± 0.008
4	0.332 ± 0.010
6	0.465 ± 0.009
8	0.618 ± 0.012
10	0.798 ± 0.014
12	0.914 ± 0.011

Results are mean ± S.D. of three experiments

Table 3.11 Regressed calibration curve of CH_3O-PEG-OH (M.W 5000) at λ 525 nm

3.4 ANALYTICAL METHOD DEVELOPMENT OF YIGSR-NH_2 & EILDV-NH_2

3.4.1 Calibration curve of peptides (YIGSR-NH_2 & EILDV-NH_2)

TNBS assay is a rapid and sensitive assay for determination of free amino groups. Primary amines, upon reaction with 2,4,6-trinitrobenzene sulfonic acid (TNBSA or TNBS) form a highly chromogenic derivative, which can be measured by UV visible

spectrophotometer at 345 nm. A calibration curve for peptides (YIGSR-NH_2/ EILDV-NH_2) were developed as reported previously for proteins with slight modification (Habeeb et al., 1966; Snyder & Sobocinski, 1975).

Stock solution of YIGSR-NH_2/EILDV-NH_2 (200 mcg/ml) was prepared by dissolving 2 mg of peptide in 10 ml of distilled water. To various concentrations of YIGSR-NH_2/ EILDV-NH_2 (1ml), 2 ml of 4 % $NaHCO_3$ and 1 ml of 0.02 % TNBS were added. The resulting solution was incubated for 2 h at room temperature followed by addition of 1 ml of 0.5 N HCl to stop reaction. The absorbance of colored solution was measured at 345 nm by UV Visible spectrophotometer (UV 1700, PharmaSpec, Shimadzu, Japan) against blank. Blank sample was prepared by same method without use of peptide.

3.4.2 Results and discussion

Absorbance measured after reaction with TNBS of both the peptide is represented in Table 3.12 and 3.13. A regressed calibration curve was plotted of concentration vs. absorbance and shown in Figure 3.8 and 3.9. A linear relationship between absorbance and concentration of the peptide was observed with regression coefficient near to one.

Concentration of YIGSR-NH2 (µg/ml)	Absorbance
4	0.028 ± 0.005
8	0.062 ± 0.004
12	0.092 ± 0.006
16	0.124 ± 0.008
20	0.150 ± 0.007
24	0.182 ± 0.007

Results are mean ± S.D. of three experiments

Table 3.12 Regressed calibration curve of YIGSR-NH_2 at λ 345 nm

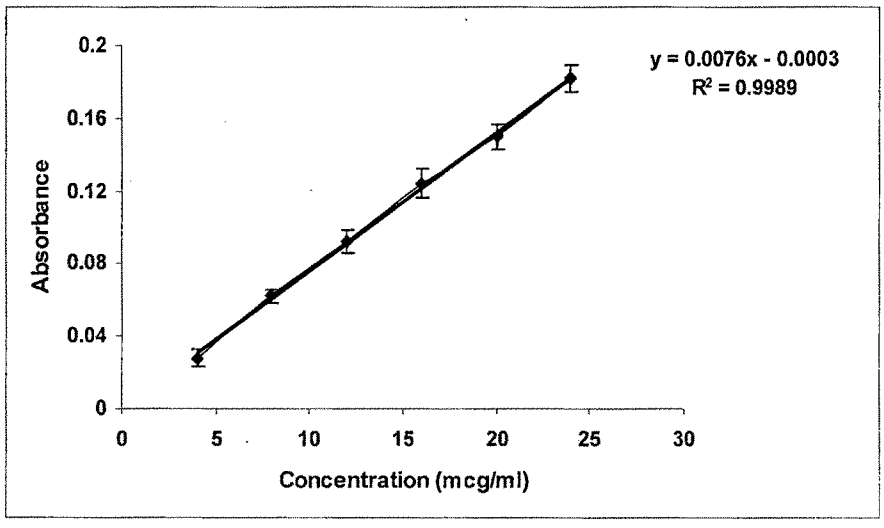

Figure 3.8 Regressed calibration curve of YIGSR-NH_2 at λ 345 nm

Concentration of EILDV-NH2 (µg/ml)	Absorbance
4	0.049 ± 0.004
8	0.095 ± 0.005
12	0.134 ± 0.007
16	0.175 ± 0.004
20	0.217 ± 0.006
24	0.268 ± 0.007

Results are mean ± S.D. of three experiments

Table 3.13 Regressed calibration curve of EILDV-NH_2 at λ 345 nm

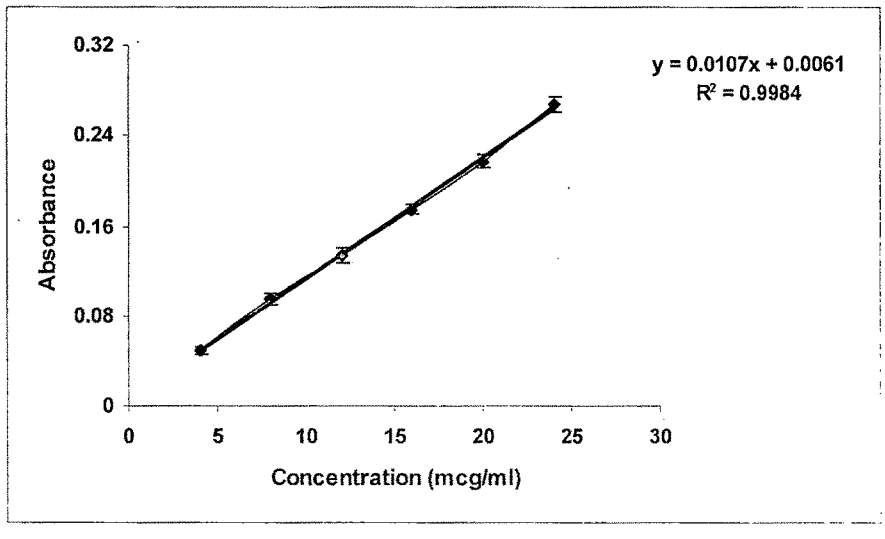

Figure 3.9 Regressed calibration curve of EILDV-NH_2 at λ 345 nm

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