CHAPTER 4: ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

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4.1 Introduction

Analytical method development and validation form a crucial part of formulation development and analysis. The assay and quantification of active pharmaceutical ingredients (API) in the developed formation is extremely important and a highly sensitive and accurate analytical method ensures that each and every step of formulation evaluation like in-vitro drug release and in vivo pharmacokinetic study is carried out meticulously. For estimation of ETO and BIC loading and dissolution samples estimation UV spectrophotometric method was developed and validated. For determining the drug release during diffusion study spectrofluorimetric method was developed and successfully validated for both the drugs. For analyzing samples of Caco-2 permeability study a highly sensitive HPLC -Fluorescence (HPLC-Fl) method was developed for ETO and BIC. Finally a successful bioanalytical HPLC-Fl method was developed and validated for both the drugs in combination with their respective internal standards. The matrix were plasma and different organ matrix. Tapentadol and Tadalafil were chosen as internal standards for ETO and BIC respectively.

4.2 Materials and Methods

Pure ETO and BIC active pharmaceutical ingredients were kindly gifted by Intas Pharmaceuticals limited, Ahmedabad, Gujarat. Tapentadol (TAP) and Tadalafil (TAD) were obtained as sampel gratis from Ami Lifesciences Pvt Ltd, Vadodara, Gujarat, India. Marketed formulations for ETO 50 mg Lastet® capsules and BIC 50 mg Cassotide 50® tablets manufactured by Khandelwal laboratories and Cipla respectively were purchased from local pharmacy stores. Analytical and HPLC grade reagents Acetonitrile (ACN) and Methanol (MeOH) were purchased from Fischer scientific (Mumbai, Maharashtra, India). Double distilled water was used throughout the study and filtered through 0.22µ filters prior to

chromatographic analysis. Ammonium Formate (AR grade) was procured from S.D. Fine chem, Mumbai. Formic acid was obtained from Merck laboratories.

4.3 Etoposide

Fresh UV Spectrophotometric, Fluorometric and HPLC-Fl method was developed for ETO. Although several methods in biological fluids are also reported(1-4), but none could be used in laboratory.

4.3.1 UV spectrophotometric method development for ETO

- **Preparation of calibration curve:** 10 mg of ETO was dissolved in 2mL MeOH and sonicated. Later, the volume was made up to 10 mL with MeOH to obtain a stock solution of concentration 1000µg/mL. Further, 5 mL was withdrawn from stock solution and transferred to a 50mL volumetric flask, with dilution up to the mark using MeOH to get 100 µg/mL. From this second stock solution, dilution was made withdrawing 1,2,3,4,5 and 6 mL with water in 10mL volumetric flask to get final solutions of concentrations 10,20,30,40,50 and 60 µg/mL respectively. The scanning range for obtaining the wavelength maxima was 200-400 nm on UV Spectrophotometer (Shimadzu 1800, Kyoto, Japan). All the measurements were done in triplicates.
- **Analytical method validation:** The developed analytical method was successfully validated as per the ICH Q2R1 guidelines. The parameters included in validation were Linearity, precision, accuracy, limit of detection and limit of quantitation.
  - **Linearity:** The linearity for an analytical method can be defined as its ability to elicit test results which are directly proportional to the concentration of the analyte in samples within a selected range. Linearity range for ETO solution analysed at 285 nm was 10-60µg/mL
  - **Accuracy:** Accuracy is defined as the closeness of individual values to the true values. Accuracy was executed at three levels: 80%, 100% and 120% of 10µg/mL

concentration by standard addition methods using three replicates for each concentration. These into a standard solution and calculation of %recovery of API from the solution. The percentage recovery was estimated for 9 samples in total.

- **Precision:** It is defined the closeness of the values to each other. It is generally divided into repeatability and reproducibility, determined by performing inter-day and intra-day precision and calculating %RSD. Here, three level concentration, Lower quality control (LQC=15), mid quality control (MQC=35) and high quality control (HQC=55) were selected and experiment was repeated thrice for inter day and intraday in triplicates.
- **Limit of detection (LOD) and limit of quantitation (LOQ):** It was calculated based on the standard deviation of response and slope method. LOD gives the minimum concentration to which the method is capable of detecting the drug and LOQ denotes the minimum level or concentration for quantification.
- **Assay of the marketed formulation:** For this purpose the marketed formulation of ETO equivalent to 50mg of ETO was crushed and dissolved in 10 mL MeOH and concentration of 5000 µg/mL was obtained. From this 1 mL of solution was withdrawn and transferred to a volumetric flask of 10 mL. The volume was made upto the mark with MeOH to get concentration of 500 µg/mL. Again same dilution pattern was repeated to get the concentration of 50 µg/mL. This was analysed by UV spectrophotometer at 285 nm and %RSD was calculated.

4.3.2 Spectrofluorometric method development for ETO

- Preparation of calibration standards: 10 mg ETO was weighed accurately and transferred to a 10 mL volumetric flask. The volume was made up to 10 mL with MeOH (1000µg/mL). Further, 1 mL solution was withdrawn and volume was made up to the mark in 10mL volumetric flask with MeOH (100µg/mL). Finally, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6 and 0.8 mL was withdrawn and made upto the mark in 10mL volumetric flask with

water in order to have 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6 and 8µg/mL. The readings were taken at 323 nm and 247 nm emission and excitation wavelength respectively on SHIMADZU Spectrofluorophotometer RF 5301 PC (Kyoto, Japan).

- **Analytical method validation:** The developed spectrofluorometric method was validated as per the ICH Q2R1 guidelines. for various parameters viz, linearity, precision, accuracy, LOD and LOQ. Linearity of the method was tested in range 0.1-8 µg/mL.
  - **Accuracy:** Accuracy was performed by standard addition method. The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found (5). It is also termed as trueness. The accuracy was established across the selected range of the analytical procedure. The API was kept constant at 3 µg/mL. The formulation was added at 80%, 100% and 120% levels.
  - **Precision:** The precision of a method is the degree of closeness of agreement between series of measurements obtained from multiple sampling under prescribed conditions. Both intraday and inter-day precision were determined and calculated in % RSD. The three LQC, MQC and HQC levels taken were 0.3, 3 and 7 µg/mL respectively.
  - **LOD and LOQ:** The detection limit of an analytical procedure is the lowest amount of sample which can be detected with ease but not necessary quantitated. LOQ is defined as the lowest amount of analyte in the sample which can be accurately and precisely determined.
  - **Assay of marketed formulation:** For this purpose the marketed formulation of ETO equivalent to 50mg of ETO was crushed and dissolved in 10 mL MeOH and concentration of 5000 µg/mL was obtained. From this 1 mL of solution was withdrawn and transferred to a volumetric flask of 10 mL. The volume was made upto the mark with MeOH to get concentration of 500 µg/mL. Again from this, 0.1mL 500 µg/mL

solution was withdrawn and made upto the mark in 10mL volumetric flask to get the concentration of 5 µg/mL. This was analysed by spectrofluorometer with excitation wavelength 247 nm and emission wavelength at 323 nm and %RSD was calculated.

4.3.3 RP-HPLC-FL method development for ETO

- **Preparation of stock solution:** The primary and secondary stock solution of 1000 µg/mL and 100 µg/mL ETO respectively were prepared as mentioned in section 4.3.1.
- **Chromatographic condition:** The chromatographic analysis was performed on a SHIMADZU® HPLC LC-20AD equipped with RF-20A prominence fluorescence detector and binary pump. Samples were injected through Rheodyne 7725 injector valve with fixed loop at 20µL. The mobile phase consisted of Ammonium formate buffer (20mM strength) of pH 3.9 and MeOH in the ratio 49:51. The separation was carried out on a waters symmetry 300 column (250mm × 4.6 mm i.d., 5µ particle size). The mobile phase was vacuum filtered through 0.45µ nylon membrane filter followed by degassing in an ultrasonic bath before use. The data was analysed at excitation wavelength 247nm and emission wavelength at 323 nm with 1mL/min flow rate. Data acquisition and integration was performed on LC solutions™ software.
- **Validation of the developed RP-HPLC-FL method for ETO**

- **Preparation of calibration solution:** 10 mg ETO was weighed accurately and transferred to a 10 mL volumetric flask. The volume was made up to 10 mL with MeOH (1000 µg/mL). Further, 1 mL solution was withdrawn and transferred to a 10 mL volumetric flask and volume was made up to the mark with MeOH (100 µg/mL). The further dilutions were done in water by withdrawing 0.01, 0.05, 0.2, 0.4, 0.6, 0.8, 1, 2 and 5 mL of 100 µg/mL solution and the volume was made upto the mark in 10mL volumetric flask with water in order to have 0.1, 0.5, 2, 4, 6, 8, 10, 20 and 50 µg/mL. The readings were taken at emission of

323nm and excitation 247 nm on SHIMADZU HPLC equipped with the fluorescence detector (Kyoto, Japan).

- **Accuracy:** The standard API solution concentration was fixed at 10µg/mL. And spiked with formulation at 80%, 100% and 120% of the fixed API concentration. The % RSD was calculated. Each analysis was done in triplicate.
- **Precision:** The precision was determined at both intra-day and inter-day level. The three concentrations of LQC, MQC and HQC taken were 0.3, 15 and 40 µg/mL. The experiment was repeated thrice for both intra-day and inter-day precision. The % RSD was determined.
- **LOD and LOQ:** This was calculated based on the standard deviation of slope and response method. The developed fluorescence method was highly sensitive and accurate.
- **Assay of the marketed formulation:** For this purpose the marketed formulation of ETO equivalent to 50mg of ETO was dissolved in 10 mL MeOH and filtered, concentration of 5000 µg/mL was obtained. From this 1 mL of solution was withdrawn and transferred to a volumetric flask of 10 mL. The volume was made upto the mark with MeOH to get concentration of 500 µg/mL. Again same dilution pattern was repeated to get the concentration of 50 µg/mL. This was analysed by spectrofluorometer with excitation wavelength 247 nm and emission wavelength at 323 nm and %RSD was calculated.
- **System suitability:** This was performed by injecting six replicates of 10 µg/mL ETO solution and deviation in retention time and area was reported

4.3.4 RP-HPLC-FL bioanalytical method development for ETO

Bioanalytical method relates to determination of drug or metabolite or both in biological matrices like plasma, serum, urine etc. It is major stay in clinical and pre-clinical studies for quantitative estimation and efficacy of the formulation developed(6).

- **Preparation of stock solution:** The primary and secondary stock solution of 1000 µg/mL and 100 µg/mL ETO respectively were prepared as mentioned in section 4.2.3. Further, internal standard Tapentadol (TAP) stock solution of 1000µg/mL and 100 µg/mL was prepared in a similar manner. Further, 0.5mL of 100 µg/mL TAP solution was withdrawn and diluted to 10mL with mobile phase to get 5 µg/mL TAP solution.
- **Chromatographic condition:** The chromatographic procedure was kept same as described in 4.3.3.
- **Validation of developed RP-HPLC method:**

- **Procedure for unextracted sample preparation:** 100 µL of 100 µg/mL ETO solution was mixed with equal volume of 5 µg/mL TAP in the pre-labelled Eppendorf. Finally, 800 µL of mobile phase was added to above mixture to have solution containing 10 µg/mL ETO and 5 µg/mL TAP solution. Similarly, 100 µL of 1, 5, 20, 40, 60, 80, 100, 200 and 500 µg/mL ETO solution is added and proceeded in the same way to have 0.1, 0.5, 2, 4, 6, 8, 10, 20 and 50 µg/mL un-extracted ETO solution.
- **Procedure for plasma sample preparation:** Protein precipitation method was optimized to recover the sample from the bioanalytical matrix as the drug is having high protein binding affinity. Briefly, to the 150 µL human plasma, 100 µL 100 µg/mL ETO solution was added and vortexed for 2 min followed by addition of 100 µL of 5 µg/mL TAP (internal standard) and vortexed again for 2 min. Subsequently, 800 µL MeOH was added, vortexed and centrifuged at 5000 rpm at 4 ºC for 10 min and supernatant was collected and analysed by HPLC. Similarly, 100 µL of 1, 5, 20, 40, 60, 80, 100, 200 and 500 µg/mL ETO solution

is added and proceeded in the same way to have 0.1, 0.5, 2, 4, 6, 8, 10, 20 and 50 µg/mL plasma extracted ETO solution.

- **Method Validation:** Developed bioanalytical method was validated as per the ICH Q2(R1) guideline.
- System suitability: This was performed by injecting MQC concentration of ETO solution for 6 times and deviation system suitability parameter viz., deviation in retention time, peak area, asymmetry was determined
- Sensitivity: This was performed by injecting 6 replicates of ETO solution at LLOQ concentration and %RSD was calculated
- Linearity: The linearity of the ETO was taken on the range of 0.1 to 50 µg/mL and regression equation and correlation coefficient (r^2) was calculated.
- Accuracy: Accuracy was performed taking 80, 100 and 120% concentration of ETO from the fixed concentration and %accuracy was founded.
- Precision: Intra-day and inter-day precision was performed taking LLOQ, LQC, MQC and HQC ETO concentration and analysis was done on the same day and within the day respectively for 3 times and %RSD was calculated.
- LOD and LOQ: This was calculated following standard deviation of the response and the slop method.
- %Recovery: %recovery of drug from biological matrix was calculated by extracting LQC, MQC and HQC level ETO sample and calculated by following equation:

$$\%Recovery = \frac{Area\ of\ extracted\ sample}{Area\ of\ unextraxted\ sample} * 100 \ldots (4.1)$$

- ***Bench Top stability:*** Bench top stability of the spiked quality control samples was determined for a period of 6 hr. stored at room temperature. Stability was assessed by comparing them against the freshly spiked calibration standards.

- ***Freeze thaw stability:*** Freeze thaw stability of the spiked quality control samples was determined after three freeze thaw cycles stored at -80 °C. Stability was assessed by comparing them against the freshly spiked calibration standards.
- ***Long term stability:*** Long term stability of the spiked quality control samples was determined after stored at -80 °C for 14 days. Stability was assessed by comparing them against the freshly spiked calibration standards.

➢ **Application of developed HPLC method for ETO estimation in different tissue homogenate:** To make this possible, different tissue homogenates were prepared by collecting tissue of interest viz., brain, lung, liver, spleen, kidney and heart immediately after sacrificing the animals. The tissues were rinsed with normal saline and dried on the tissue paper followed by freeze drying at -20 ºC until analysis. Then the tissue were thawed to room temperature, weighed and homogenized. Then, the tissue homogenates were treated same as the plasma and the calibration curve was generated in the identical concentration range that was considered for plasma matrix.

4.4 Result and discussion for Etoposide

4.4.1 UV spectrophotometric method development

After scanning in the range of 200-400 nm; the λ_{max} obtained was 285 nm (Fig 4.1). This was selected as analytical wavelength and further analysis was carried out at this wavelength. The developed UV spectrophotometric method was validated for various parameters and the results were in accordance to the ICH Q2(R1) criteria and listed in the table 4.1.

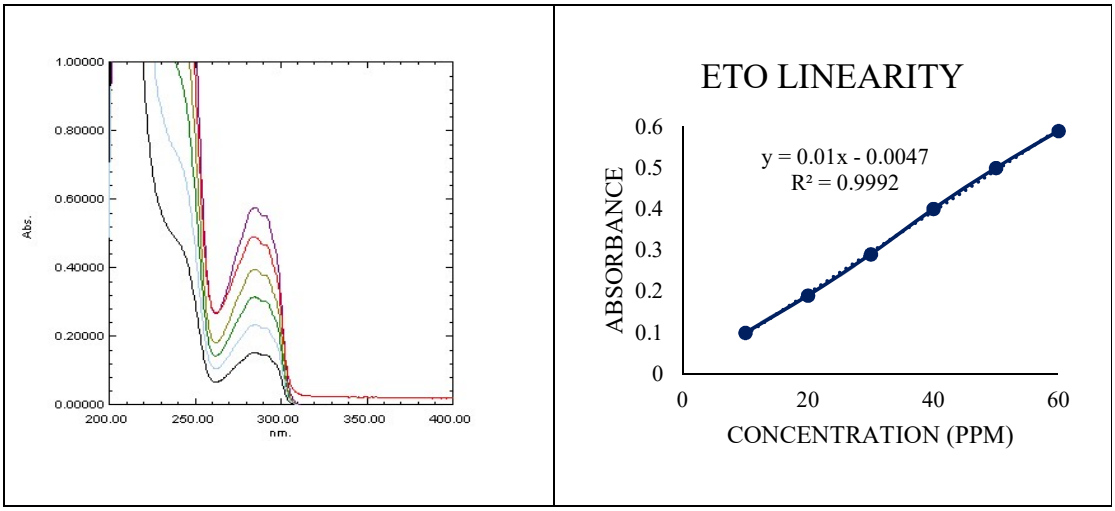


Figure 4.1. Calibration curve for ETO at 285 nm for 10-60 µg/mL.

PARAMETERS	**RESULTS OBTAINED**	
Wavelength (nm)	285	
Concentration range (µg/mL)	10-60	
Regression equation	y= 0.01x-0.0047	
Correlation coefficient (r^2)	0.9992	
Intra-day precision (%RSD)	LQC (15 µg/mL)	0.38
	MQC (35 µg/mL)	0.72
	HQC (55 µg/mL)	0.79
Inter-day precision (%RSD)	LQC (15 µg/mL)	0.33
	MQC (35 µg/mL)	0.70
	HQC (55 µg/mL)	0.75
Accuracy (%Recovery)	80%	98.87±0.59
	100%	99.01±0.97
	120%	99.22±0.52
%Assay	99.45±1.45%	
LOD (µg/mL)	2	
LOQ (µg/mL)	6	

Table 4.1. UV method validation parameters for ETO

4.4.2 Spectrofluorimetric method development

After scanning the excitation and emission wavelength for ETO were determined as 247 and 323 nm respectively (Fig 4.2). This was selected as analytical wavelength and further analysis was carried out at this wavelength. The developed spectrofluorophotometric method was validated for various parameters and the results were in accordance to the ICH Q2(R1) criteria and listed in the table 4.2.

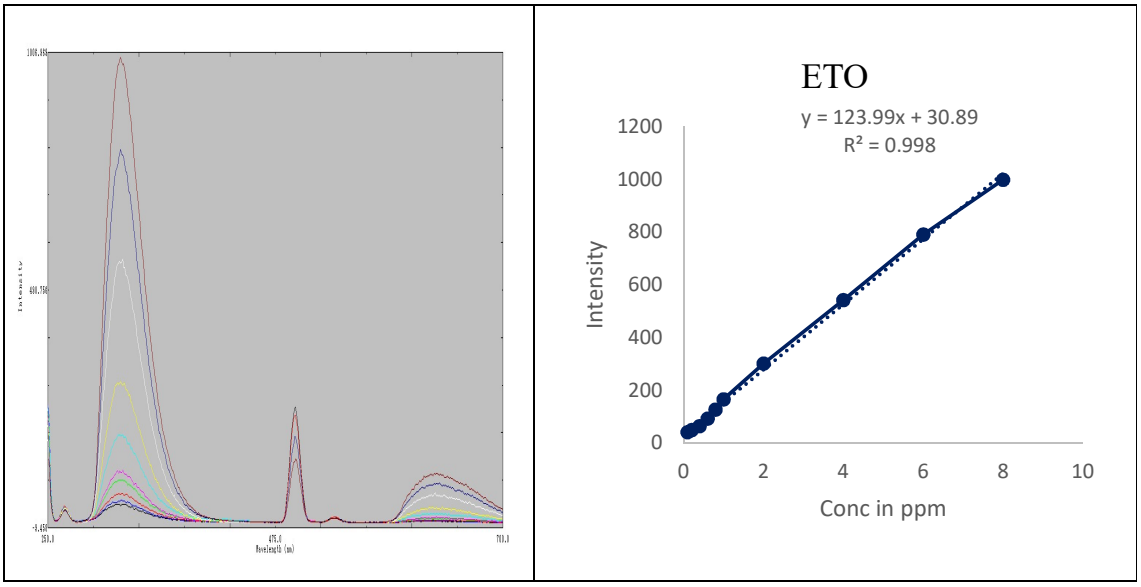


Figure 4.2. Calibration curve for ETO Excitation at 247 nm and emission at 323nm for 0.1-8 µg/mL.

PARAMETERS	**RESULTS OBTAINED**	
Wavelength (nm)	247 nm excitation and 323 nm emission	
Concentration range (µg/mL)	0.1-8	
Regression equation	y= 123.99x+30.89	
Correlation coefficient (r^2)	0.998	
Intra-day precision (%RSD)	LQC (0.3 µg/mL)	0.29
	MQC (3 µg/mL)	0.67
	HQC (7 µg/mL)	0.86
Inter-day precision (%RSD)	LQC (0.3 µg/mL)	0.28
	MQC (3 µg/mL)	0.62
	HQC (7 µg/mL)	0.82
Accuracy (%Recovery)	80%	99.71±0.51
	100%	99.82±0.28
	120%	99.02±0.41
%Assay	99.92±0.62%	
LOD (µg/mL)	0.05	
LOQ (µg/mL)	0.15	

Table 4.2 Spectrofluorimetric method validation parameters for ETO

4.4.3 RP-HPLC-FL method development for ETO

The RP-HPLC-FL method showed ETO retention time at 5.9 ± 0.1 min at 1mL/min flow rate (figure 4.3). The method was validated as per the ICH Q2(R1) guideline for various parameters

and the outcomes of that are reported in the following table 4.3 along with the linearity chromatographs as depicted in figure 4.4.

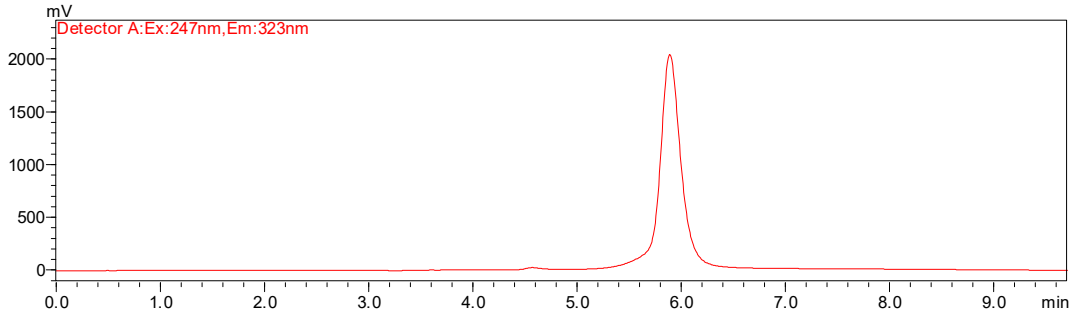


Figure 4.3. RP-HPLC-Fl chromatogram for ETO

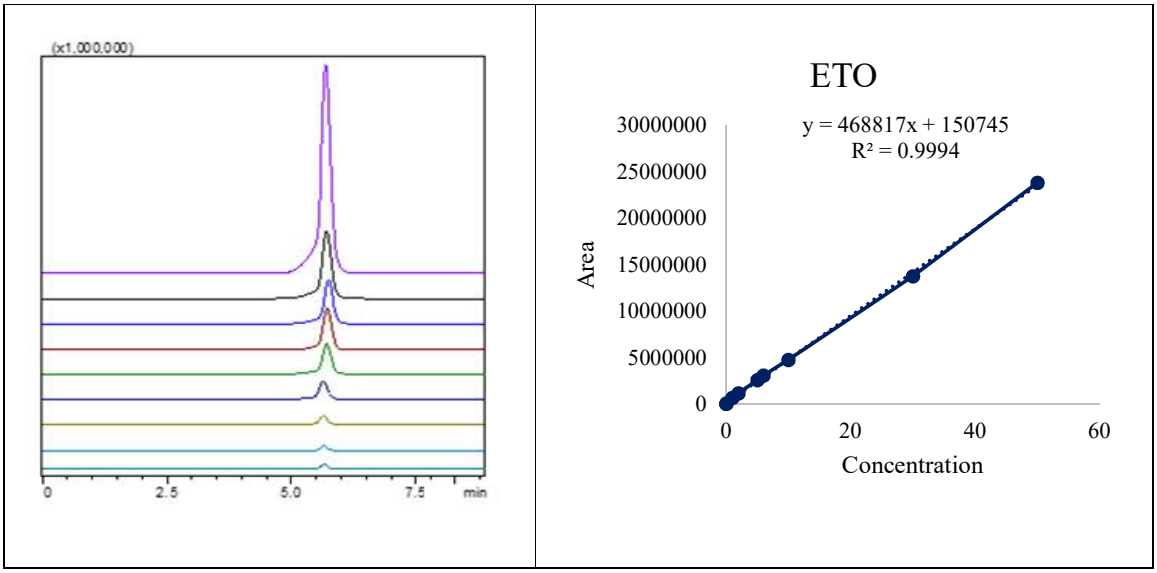


Figure 4.4. RP-HPLC-Fl chromatogram image and calibration curve for ETO

PARAMETERS	**RESULTS OBTAINED**	
Wavelength (nm)	247 nm excitation and 323 nm emission	
Concentration range (µg/mL)	0.1-50	
Regression equation	468817x + 150745	
Correlation coefficient (r^2)	0.9994	
Intra-day precision (%RSD)	LQC (0.3 µg/mL)	0.58%
	MQC (15 µg/mL)	0.47%
	HQC (40 µg/mL)	0.51%
Inter-day precision (%RSD)	LQC (0.3 µg/mL)	0.33%
	MQC (15 µg/mL)	0.41%

	HQC (40 µg/mL)	0.39%
Accuracy (%Recovery)	80%	99.55±0.64%
	100%	98.87±0.42%
	120%	99.64±0.32%
% Assay	99.87±0.54%	
LOD (µg/mL)	0.01	
LOQ (µg/mL)	0.03	

Table 4.3. RP-HPLC-Fl method validation parameters for ETO

4.4.4. RP-HPLC-FL bioanalytical method development for ETO

- The RP-HPLC method for un-extracted and plasma extracted ETO and TAP showed ETO retention time at 5.9±0.1 min and TAP at 4.5±0.1 min at 1mL/min flow rate (figure 4.5. and figure 4.6.). The linearity data and calibration curve for both un-extracted samples and plasma extracted samples is depicted in figure 4.7A and 4.7B respectively. The method was validated as per the ICH Q2(R1) guideline for various parameters and the outcomes of that are reported in the following table 4.4 .
- Additionally, in order to calculate the amount of drug goes to different organs, the linearity of ETO in different tissue matrix were taken and calibration curve was generated as depicted in figure 4.8.

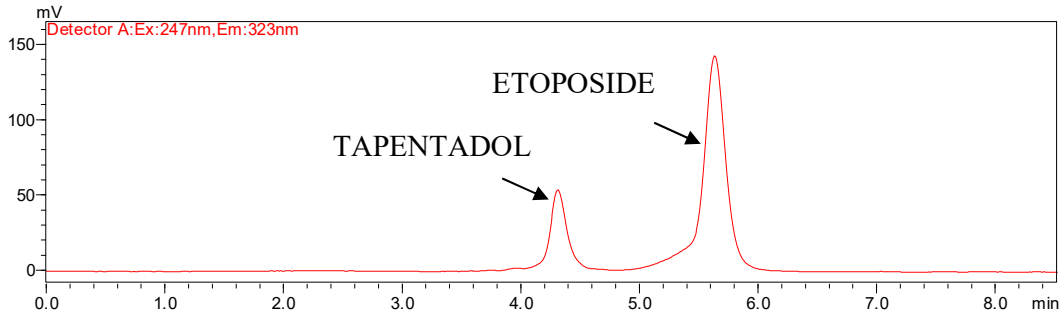


Figure 4.5. RP-HPLC Chromatogram for un-extracted TAP and ETO

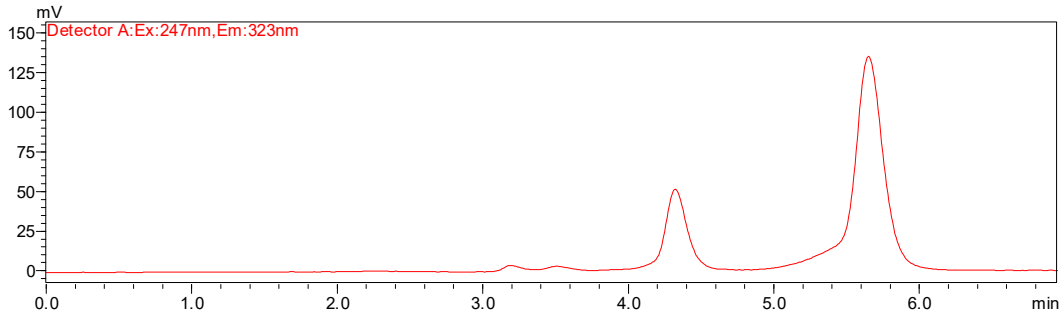


Figure 4.6. RP-HPLC Chromatogram for TAP and ETO extracted from plasma

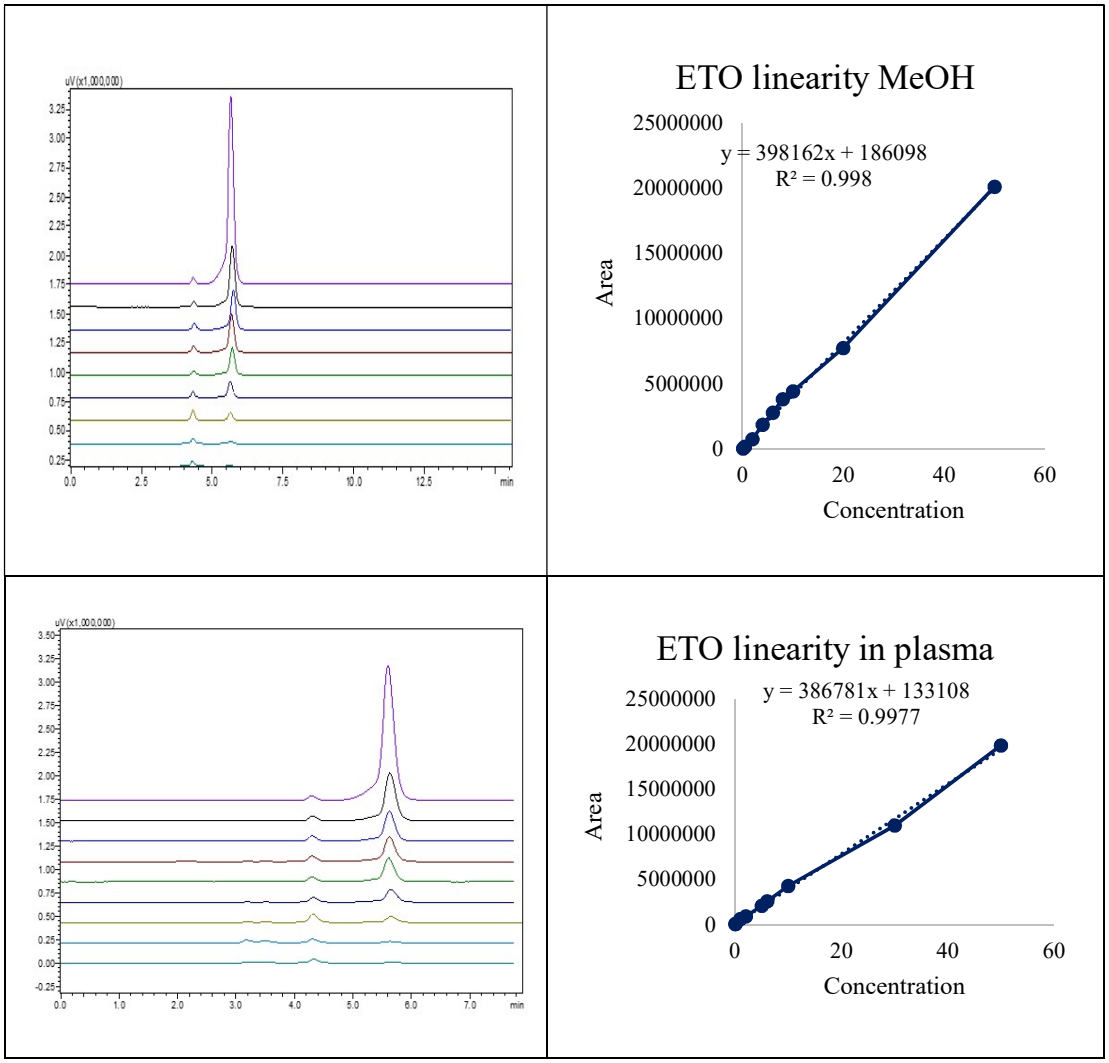
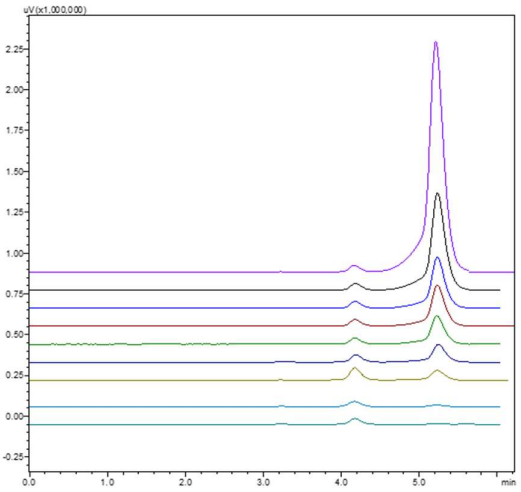
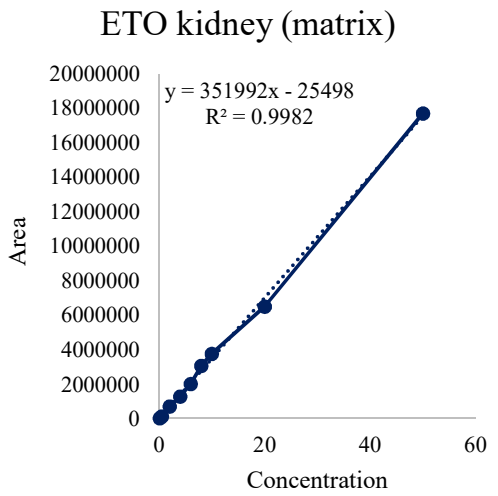


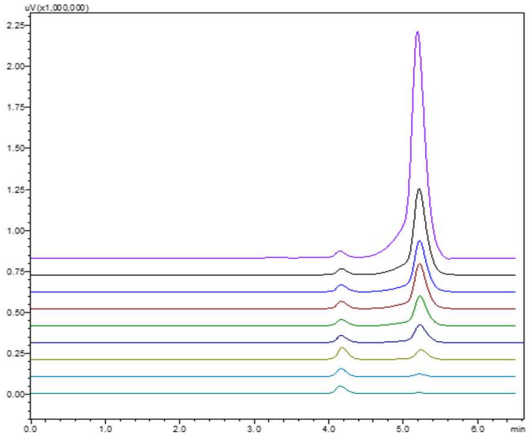
Figure 4.7. RP-HPLC images and calibration curve for (A) un-extracted ETO and (B) ETO extracted from plasma

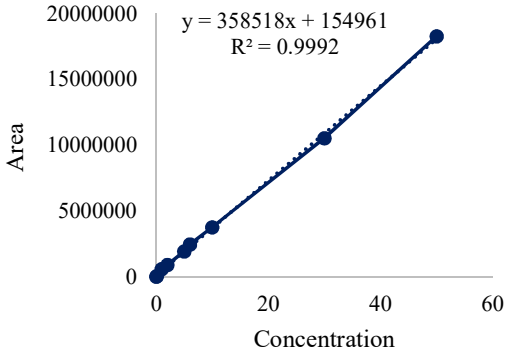
PARAMETERS	RESULTS OBTAINED	
Wavelength (nm)	247 nm excitation and 323 nm emission	
Concentration range (µg/mL)	0.1-50	
Regression equation	y = 386781x + 133108	
Correlation coefficient (r^2)	0.9977	
Retention time	5.9±0.2 min (ETO) and 4.5±0.1 min (TAP)	
Intra-day precision (%RSD)	LLOQ (0.1 µg/mL)	1.15%
	LQC (0.3 µg/mL)	0.85%
	MQC (15 µg/mL)	0.77%
	HQC (40 µg/mL)	0.56%
Inter-day precision (%RSD)	LLOQ (0.1 µg/mL)	0.99%
	LQC (0.3 µg/mL)	0.71%
	MQC (15 µg/mL)	0.57%
	HQC (40 µg/mL)	0.66%
Accuracy (%Recovery)	80%	98.66±0.70%
	100%	101.47±0.41%
	120%	99.56%±0.98%
LOD (µg/mL)	0.03	
LOQ (µg/mL)	0.09	
%Recovery	LQC (0.3µg/mL)	86.66±0.14%
	MQC (15µg/mL)	85.47%1.05%
	HQC (40µg/mL)	89.57%0.84%
Bench-top Stability	LQC (0.3µg/mL)	99.22%
	HQC (15µg/mL)	98.89%
Freeze-thaw stability	LQC (40µg/mL)	101.55%
	HQC (0.3µg/mL)	100.85%
Long-term stability	LQC (15µg/mL)	99.28%
	HQC (40µg/mL)	101.45%
System suitability	Theoretical plates	6945
	Area	3929648

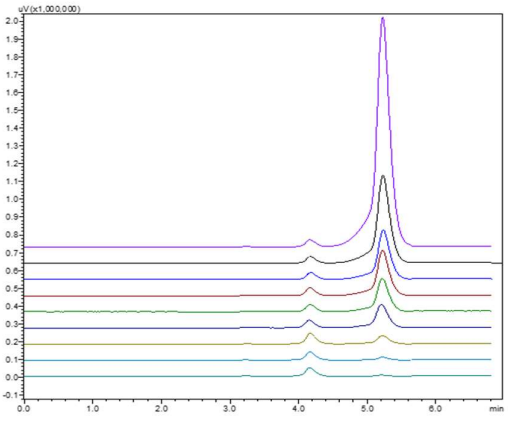
Table 4.4. RP-HPLC-Fl bioanalytical method validation parameters for ETO

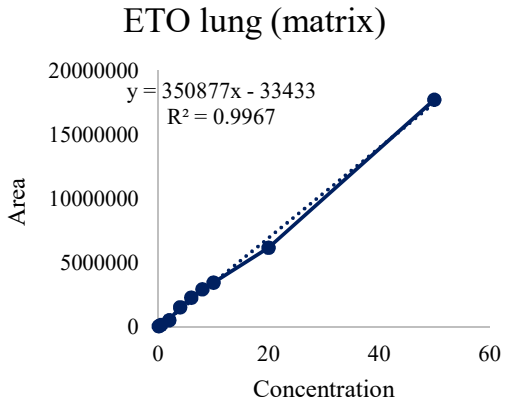


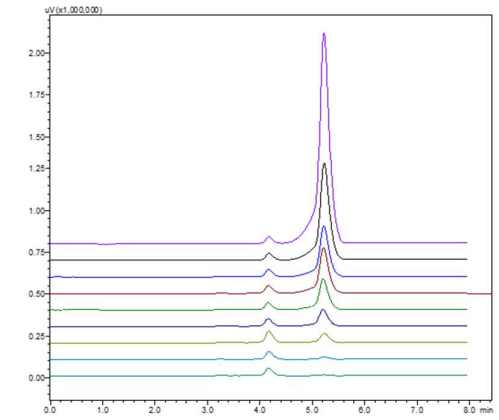

ETO kidney (matrix)
y = 351992x - 25498
R² = 0.9982
Area
Concentration

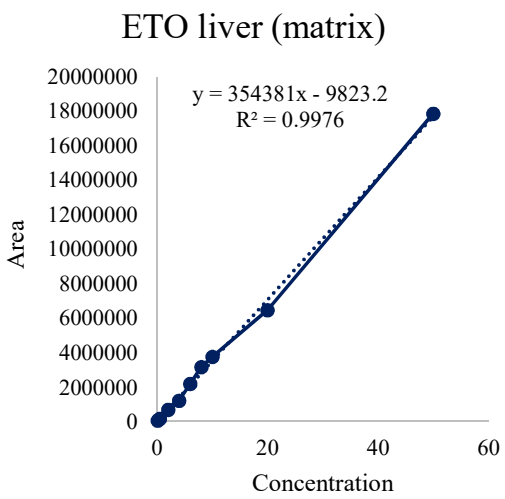


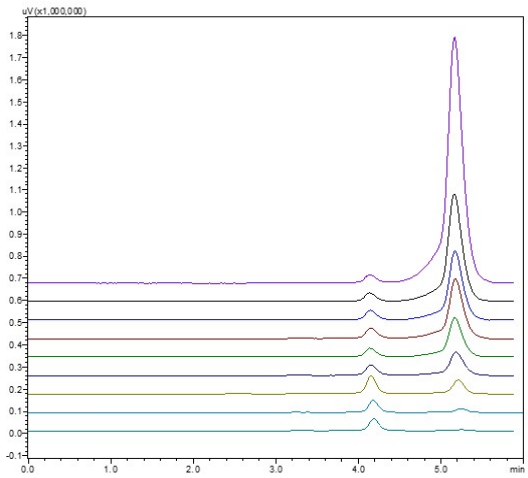

ETO Brain (matrix)
y = 358518x + 154961
R² = 0.9992
Area
Concentration

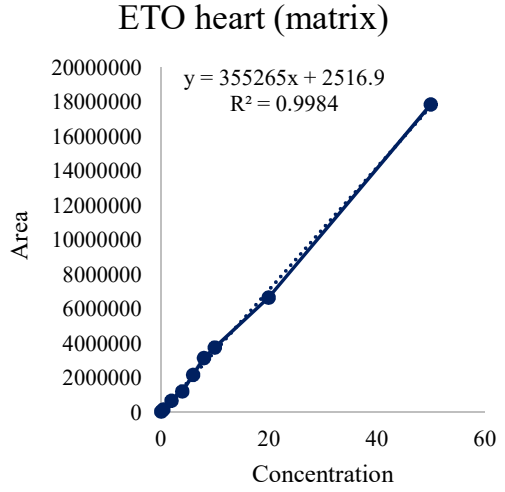


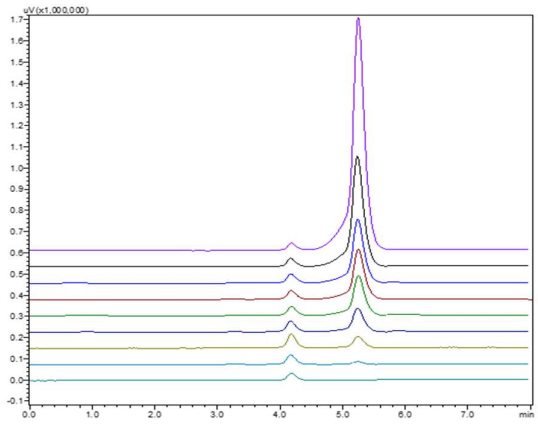

ETO lung (matrix)
y = 350877x - 33433
R² = 0.9967
Area
Concentration




ETO liver (matrix)
y = 354381x - 9823.2
R² = 0.9976
Area
Concentration




ETO heart (matrix)
y = 355265x + 2516.9
R² = 0.9984
Area
Concentration



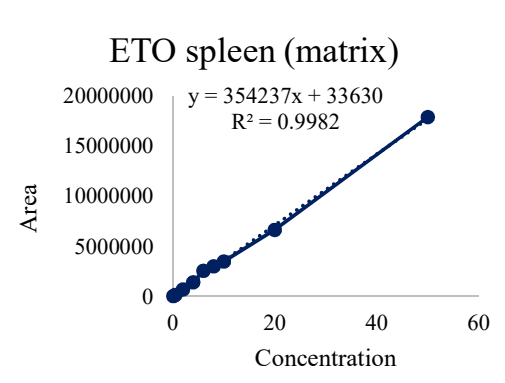

ETO spleen (matrix)
y = 354237x + 33630
R² = 0.9982
Area
Concentration

Figure 4.8. RP-HPLC images and calibration curve of ETO in (a) kidney (b) brain (c) lung (d) liver (e) heart (f) spleen matrix

4.5 Bicalutamide

UV Spectrophotometric, Fluorometric and HPLC-Fl methods were developed for BIC. Several literature reported methods were available for estimation of BIC in biological fluids(7-10) but none could be adopted in the laboratory.

4.5.1 UV spectrophotometric method development for BIC

- **Preparation of calibration curve:** 10 mg of BIC was dissolved in 2mL MeOH, sonicated well and volume was made up to 10 mL with MeOH (1000µg/mL concentration). Thereafter 1 mL solution was transferred to a 10 mL volumetric flask and MeOH was added up to 10mL mark labelled as second stock solution of 100 µg/mL. From this solution further dilutions of 2,4,6,8,10 and 12 µg/mL were made by withdrawing 0.2,0.4.0.6.0.8.1 and 1.2 mL respectively and adding water up to the final mark. The wavelength at which maximum absorbance occurred was obtained by scanning drug solution from 200-400 nm on UV Spectrophotometer (Shimadzu 1800, Kyoto, Japan). All the measurements were taken in triplicates.
- **Analytical method validation:** The developed UV spectrophotometric method was validated as per the ICH Q2 R1 guidelines for various parameters viz, linearity, precision, accuracy, LOD and LOQ. Linearity of the method was tested in range 2-12 µg/mL. BIC solution was analysed at 272 nm. A similar approach was used for validation as followed for ETO, except for the step of crushing the tablets prior to dissolving in MeOH followed by filtration.

4.5.2 Spectrofluorimetric method development for BIC

- **Preparation of calibration standards:** 10 mg BIC was weighed accurately and transferred to a 10 mL volumetric flask. The volume was made up to 10 mL with MeOH.

Further, 1 mL solution was withdrawn and transferred to a 10 mL volumetric flask. Again volume was made up to the mark with MeOH. The further dilutions were done in water. For calibration curve final concentrations taken were 0.1, 0.2, 0.5, 1, 2, 4, 6, 8 and 10µg/mL. The readings were taken at emission of 323nm and excitation 247 nm on SHIMADZU Spectrofluorophotometer RF 5301 PC (Kyoto, Japan).

- **Analytical method validation:** The developed spectrofluorometric method was validated as per the ICH Q2R1 guidelines. for various parameters viz, linearity, precision, accuracy, LOD and LOQ. Linearity of the method was tested in range 0.1-10 µg/mL. The spectrofluorometric method was validated in a similar way as mentioned earlier for ETO.

4.5.3 RP-HPLC-FL method development for BIC

- **Preparation of calibration standards:** 10 mg BIC was weighed accurately and transferred to a 10 mL volumetric flask. The volume was made up to 10 mL with MeOH (1000 µg/mL). Further, 1 mL solution was withdrawn and transferred to a 10 mL volumetric flask and volume was made up to the mark with MeOH (100 µg/mL). The further dilutions were done in water by withdrawing 0.01, 0.05, 0.2, 0.4, 0.6, 0.8, 1, 2 and 5 mL of 100 µg/mL solution and the volume was made upto the mark in 10mL volumetric flask with water in order to have 0.1, 0.5, 2, 4, 6, 8, 10, 20 and 50 µg/mL. The readings were taken at emission of 323nm and excitation 247 nm on SHIMADZU HPLC equipped with the fluorescence detector (Kyoto, Japan).

- **Analytical method validation:** The developed spectrofluorometric method was validated as per the ICH Q2R1 guidelines. for various parameters viz, linearity, precision, accuracy, LOD and LOQ. Linearity of the method was tested in range 0.1-50 µg/mL. The spectrofluorometric method was validated in a similar way as mentioned earlier for ETO except for the step of crushing the tablets prior to dissolving in MeOH followed by filtration.

4.5.4 RP-HPLC-FL bioanalytical method development for BIC

- **Preparation of stock solution:** The primary and secondary stock solution of 1000 µg/mL and 100 µg/mL BIC respectively were prepared as mentioned in section 4.5.3.Further, internal standard Tadalafil (TAD) stock solution of 1000µg/mL and 100µg/mL was prepared in a similar manner. Lastely, 0.5mL of 100 µg/mL TAD solution was withdrawn and diluted to 10mL with mobile phase to get 5 µg/mL TAD solution.

- **Procedure for unextracted sample preparation:** 100 µL of 100 µg/mL BIC solution was mixed with equal volume of 5 µg/mL TAP in the pre-labelled Eppendorf. Finally, 800 µL of mobile phase was added to above mixture to have solution containing10 µg/mL and 5 µg/mL BIC and TAD. Similarly, 100 µL of 1, 5, 20, 40, 60, 80, 100, 200 and 500 µg/mL BIC solution is added and proceeded in the same way to have 0.1, 0.5, 2, 4, 6, 8, 10, 20 and 50 µg/mL un-extracted BIC solution.

- **Procedure for plasma sample preparation:** Protein precipitation method was optimized to recover the sample from the bioanalytical matrix as the drug is having high protein binding affinity. Briefly, to the 150 µL human plasma, 100 µL 100 µg/mL BIC solution was added and vortexed for 2 min followed by addition of 100 µL of 5 µg/mL TAD (internal standard) and vortexed again for 2 min. Subsequently, 800 µL MeOH was added, vortexed and centrifuged at 5000 rpm at 4 ºC for 10 min and supernatant was collected and analysed by HPLC. Similarly, 100 µL of 1, 5, 20, 40, 60, 80, 100, 200 and 500 µg/mL BIC solution is added and proceeded in the same way to have 0.1, 0.5, 2, 4, 6, 8, 10, 20 and 50 µg/mL plasma extracted BIC solution.

- **Chromatographic condition:** The chromatographic procedure was kept same as described in 4.5.3.

- **Method Validation:** Developed bioanalytical method was validated as per the ICH Q2(R1) guideline for system suitability, sensitivity, linearity (0.1 to 50 µg/mL), accuracy and precision.

Freshly prepared solutions were utilized in accuracy and precision studies.

- LOD and LOQ: This was calculated following standard deviation of the response and the slop method.
- %Recovery: %recovery of drug from biological matrix was calculated by extracting LQC, MQC and HQC level BIC sample and calculated by following equation:

$$\%Recovery = \frac{Area\ of\ extracted\ sample}{Area\ of\ unextraxted\ sample} * 10 \text{ .....(4.2)}$$

- The stability of BIC in biological matrix plasma was established at QC concentrations with three replicates.
- Bench top, freeze thaw and long term stability were determined as described earlier.

- **Application of developed HPLC method for BIC estimation in different tissue homogenate:** To make this possible, different tissue homogenates were prepared by collecting tissue of interest viz., brain, lung, liver, spleen, kidney and heart immediately after sacrificing the animals. The tissues were rinsed with normal saline and dried on the tissue paper followed by freeze drying at -20 ºC until analysis. Then the tissue were thawed to room temperature, weighed and homogenized. Then, the tissue homogenates were treated same as the plasma and the calibration curve was generated in the identical

4.6 Result and discussion for Bicalutamide

4.6.1 UV spectrophotometric method development for BIC

After scanning in the range of 200-400 nm; the λ_{max} obtained was 272 nm (Fig 4.9). This was selected as analytical wavelength and further analysis was carried out at this wavelength. The

developed UV spectrophotometric method was validated for various parameters and the results were in accordance to the ICH Q2(R1) criteria and listed in the table 4.5.

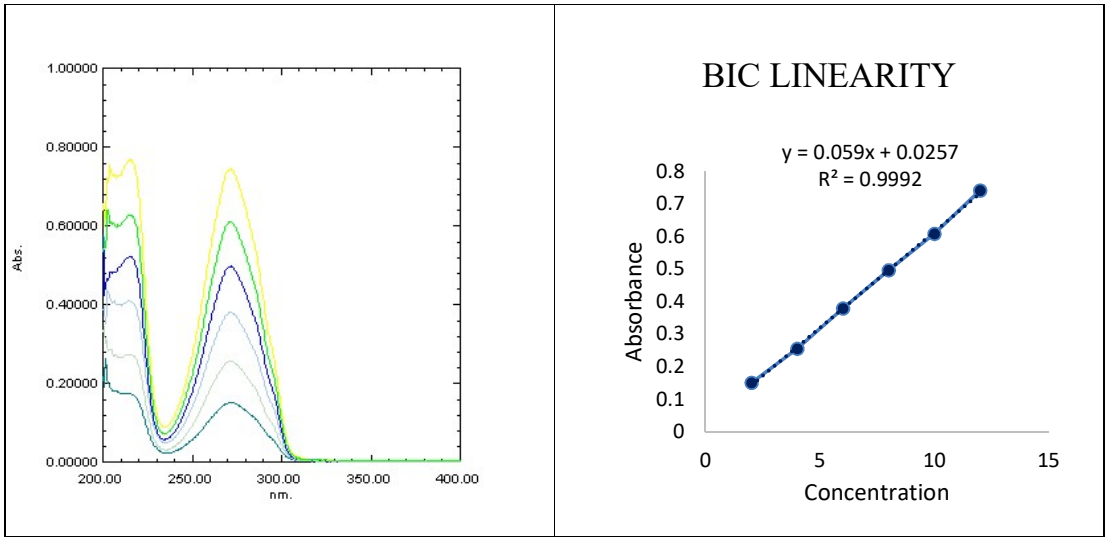


Figure 4.9. Calibration curve for BIC at 272 nm for 2-12 µg/mL.

PARAMETERS	RESULTS OBTAINED	
Wavelength (nm)	272	
Concentration range (µg/mL)	2-12	
Regression equation	y= 0.059x+0.0257	
Correlation coefficient (r^2)	0.9992	
Intra-day precision (%RSD)	LQC (3 µg/mL)	0.78
	MQC (5 µg/mL)	0.86
	HQC (9 µg/mL)	0.82
Inter-day precision (%RSD)	LQC (3 µg/mL)	0.72
	MQC (5 µg/mL)	0.84
	HQC (9 µg/mL)	0.81
Accuracy (%Recovery)	80%	99.14±0.65
	100%	98.47±0.36
	120%	99.77±0.51
%Assay	99.02%±0.55%	
LOD (µg/mL)	0.1	
LOQ (µg/mL)	0.3	

Table 4.5. UV method validation parameters for BIC

4.6.2 Spectrofluorometric method development for BIC

After scanning the excitation and emission wavelength for BIC were determined as 260 and 323 nm respectively (Fig 4.10). This was selected as analytical wavelength and further analysis was carried out at this wavelength. The developed spectrofluorophotometric method was validated for various parameters and the results were in accordance to the ICH Q2(R1) criteria and listed in the table 4.6.

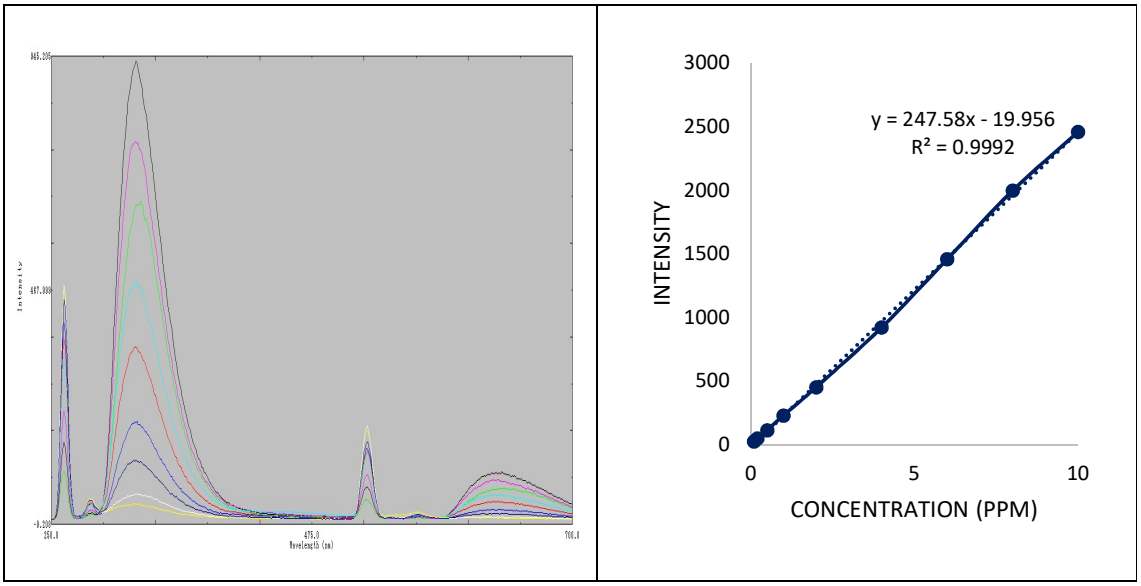


Figure 4.10. Calibration curve for BIC Excitation at 260 nm and emission at 323nm for 0.1-10 µg/mL

PARAMETERS	RESULTS OBTAINED	
Wavelength (nm)	260 nm excitation and 323 nm emission	
Concentration range (µg/mL)	0.1-10	
Regression equation	y= 247.58x+19.956	
Correlation coefficient (r^2)	0.999	
Intra-day precision (%RSD)	LQC (0.3 µg/mL)	0.28
	MQC (3 µg/mL)	0.62
	HQC (9 µg/mL)	0.78
Inter-day precision (%RSD)	LQC (0.3 µg/mL)	0.21
	MQC (3 µg/mL)	0.60
	HQC (9 µg/mL)	0.88
Accuracy (%Recovery)	80%	98.82±0.72
	100%	98.89±0.12
	120%	99.24±0.89
%Assay	99.23%±1.77	

LOD (µg/mL)	0.05
LOQ (µg/mL)	0.15

Table 4.6. Spectrofluorimetric method validation parameters for BIC

4.6.3 RP-HPLC-FL method development for BIC

The RP-HPLC-FL method showed BIC retention time at 5.7±0.1 min at 1mL/min flow rate (figure 4.11). The method was validated as per the ICH Q2(R1) guideline for various parameters and the outcomes of that are reported in the following table 4.7 along with the linearity chromatographs as depicted in figure 4.12.

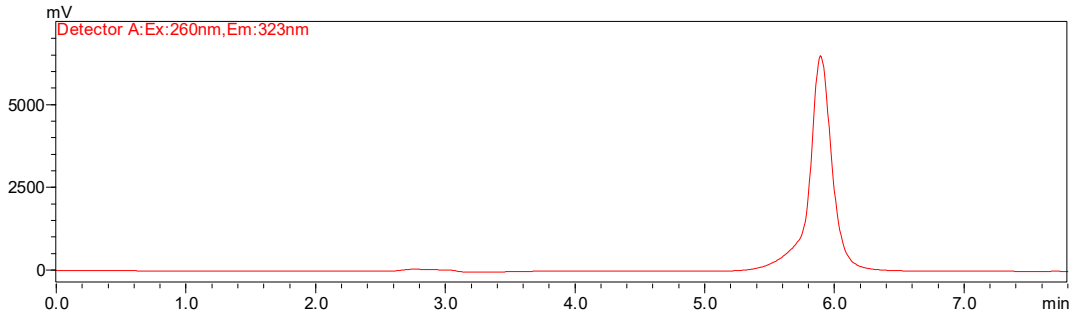


Figure 4.11. RP-HPLC-FL chromatogram for BIC

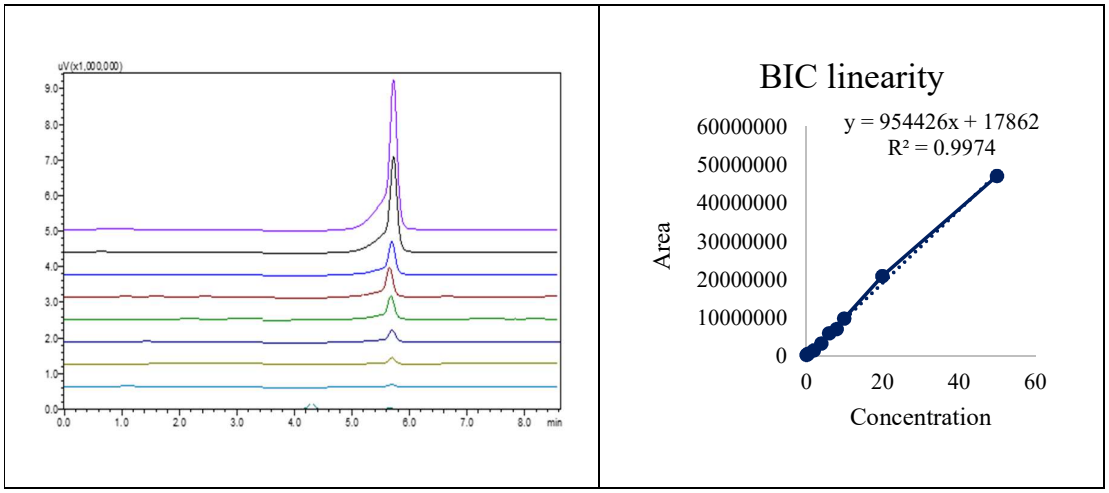


Figure 4.12. RP-HPLC-FL chromatogram image and calibration curve for BIC

PARAMETERS	RESULTS OBTAINED
Wavelength (nm)	260 nm excitation and 323 nm emission
Concentration range (µg/mL)	0.1-50
Regression equation	y = 954426x + 17862

Correlation coefficient (r^2)	0.9974	
Intra-day precision (%RSD)	LQC (0.3 µg/mL)	0.49%
	MQC (15 µg/mL)	0.55%
	HQC (40 µg/mL)	0.51%
Inter-day precision (%RSD)	LQC (0.3 µg/mL)	0.34%
	MQC (15 µg/mL)	0.47%
	HQC (40 µg/mL)	0.31%
Accuracy (%Recovery)	80%	99.74±0.84%
	100%	99.31±0.22%
	120%	98.93±1.01%
% Assay	100.89±0.67%	
LOD (µg/mL)	0.005	
LOQ (µg/mL)	0.015	

Table 4.7. RP-HPLC-FL method validation parameters for BIC

4.6.4 RP-HPLC-FL Bioanalytical method development

- The RP-HPLC method for un-extracted and plasma extracted BIC and TAD showed BIC retention time at 5.7±0.1 min and TAP at 4.3±0.1 min at 1mL/min flow rate (figure 4.13. and figure 4.14.). The linearity data and calibration curve for both un-extracted samples and plasma extracted samples is depicted in figure 4.15A and 4.15B respectively. The method was validated as per the ICH Q2(R1) guideline for various parameters and the outcomes of that are reported in the following table 4.8.
- Additionally, in order to calculate the amount of drug goes to different organs, the linearity of BIC in different tissue matrix were taken and calibration curve was generated as depicted in figure 4.16.

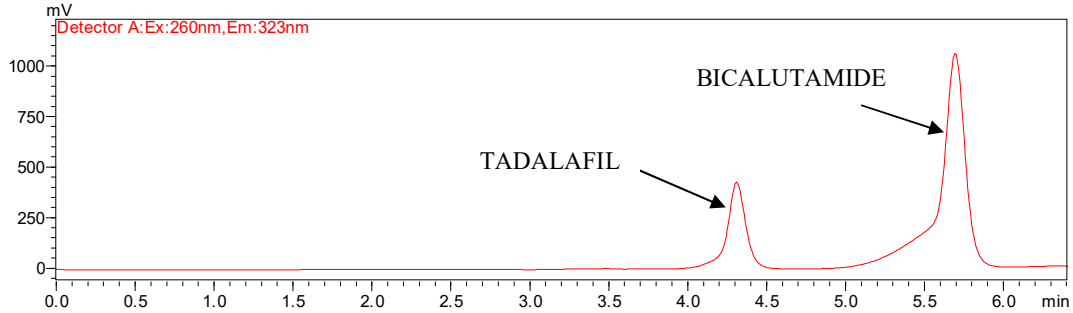


Figure 4.13. RP-HPLC Chromatogram for un-extracted TAD and BIC

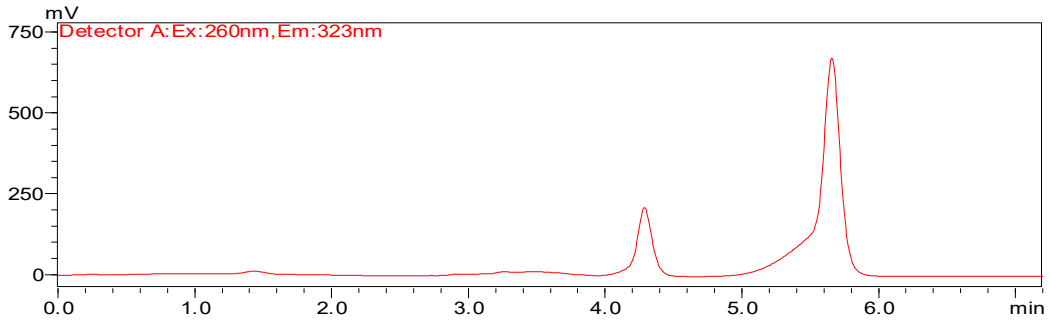


Figure 4.14. RP-HPLC Chromatogram for extracted TAD and BIC from plasma

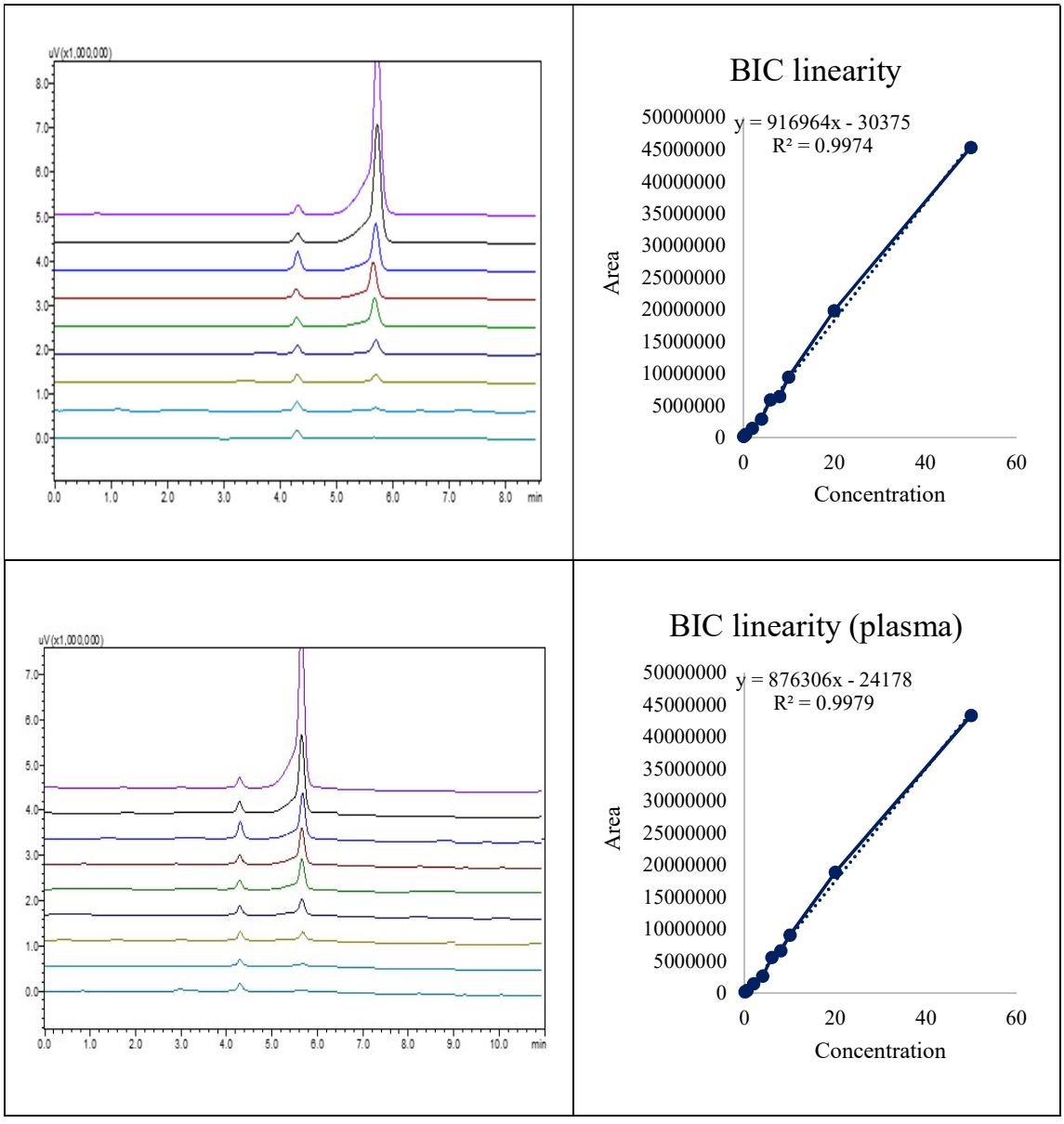
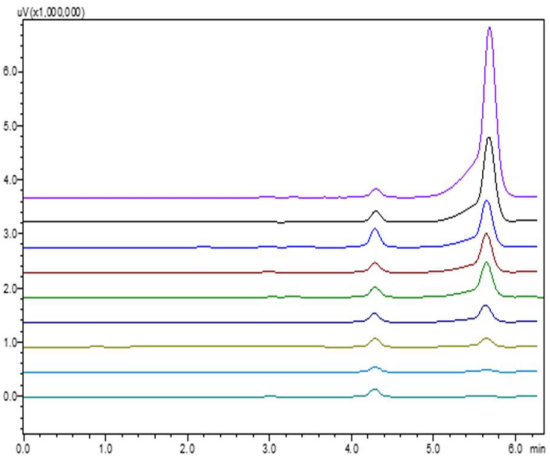
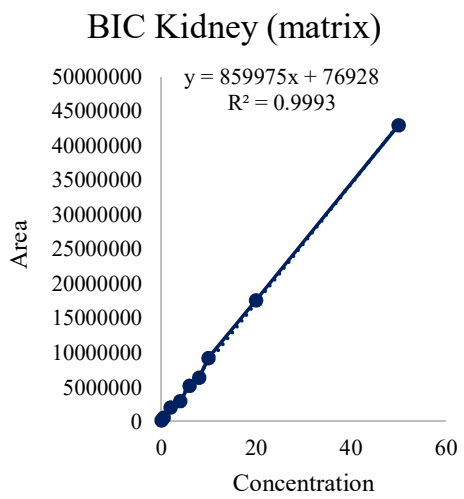


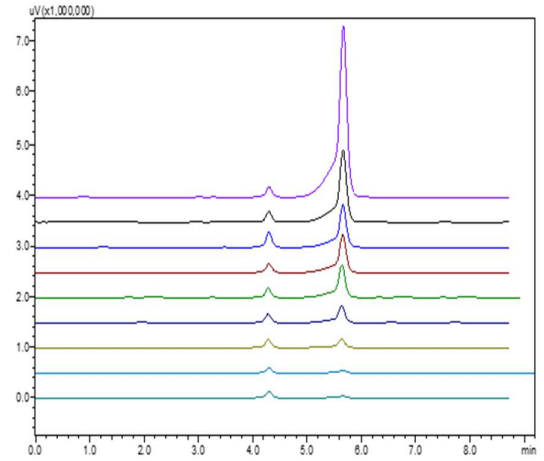
Figure 4.15. Unextracted and extracted BIC (from plasma) linearity

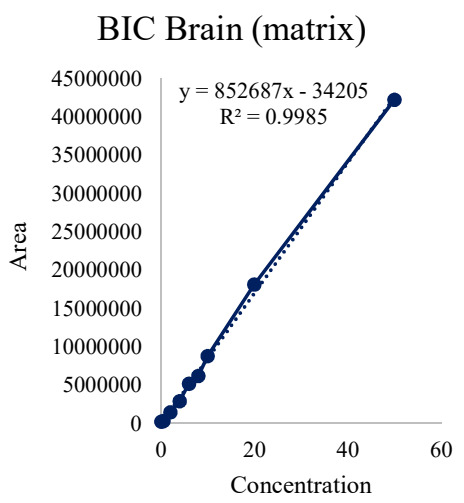
PARAMETERS	RESULTS OBTAINED	
Wavelength (nm)	260 nm excitation and 323 nm emission	
Concentration range (µg/mL)	0.1-50	
Regression equation	y = 876306x - 24178	
Retention time	4.3±0.2 min (TAD) 5.7±0.2 min (BIC)	
Correlation coefficient (r^2)	0.9979	
Intra-day precision (%RSD)	LLOQ (0.1 µg/mL)	0.58%
	LQC (0.3 µg/mL)	0.79%
	MQC (15 µg/mL)	0.81%
	HQC (40 µg/mL)	0.88%
Inter-day precision (%RSD)	LLOQ (0.1 µg/mL)	0.75%
	LQC (0.3 µg/mL)	0.77%
	MQC (15 µg/mL)	0.69%
	HQC (40 µg/mL)	0.83%
Accuracy (%Recovery)	80%	99.44±0.12%
	100%	100.77±0.42%
	120%	98.87±0.61%
LOD (µg/mL)	0.01	
LOQ (µg/mL)	0.03	
%Recovery	LQC (0.3µg/mL)	84.55%±1.12%
	MQC (15µg/mL)	88.27%±0.51%
	HQC (40µg/mL)	85.69%±0.72%
Bench-top Stability	LQC (0.3µg/mL)	99.43%
	HQC (40µg/mL)	99.17%
Freeze-thaw stability	LQC (0.3µg/mL)	100.39%
	HQC (40µg/mL)	101.08%
Long-term stability	LQC (0.3µg/mL)	99.62%
	HQC (40µg/mL)	100.33%
System suitability	Theoretical plates	7458
	Area	9025472

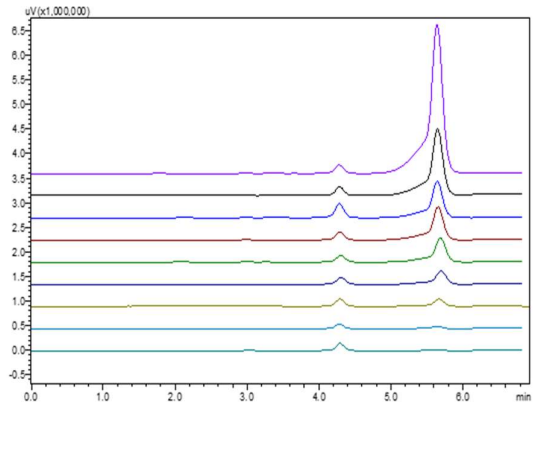
Table 4.8. RP-HPLC-Fl bioanalytical method validation parameters for BIC

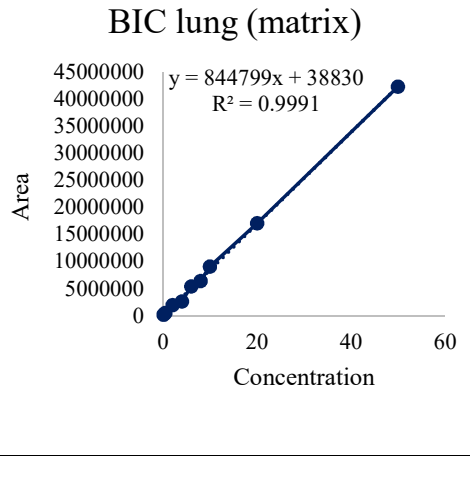

uV(x1,000,000)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.0 1.0 2.0 3.0 4.0 5.0 6.0 min

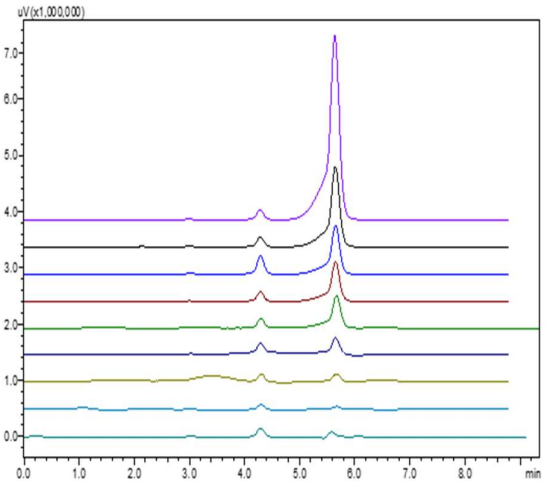

BIC Kidney (matrix)
y = 859975x + 76928
R² = 0.9993
Area
50000000
45000000
40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
0 20 40 60
Concentration

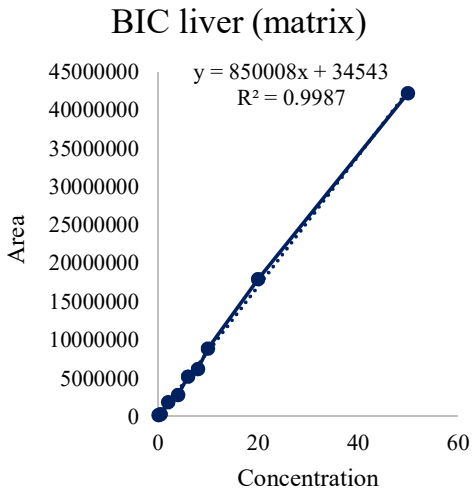

uV(x1,000,000)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 min

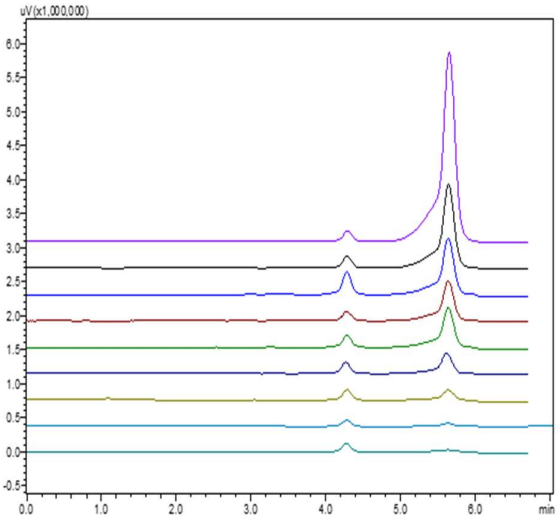

BIC Brain (matrix)
y = 852687x - 34205
R² = 0.9985
Area
45000000
40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
0 20 40 60
Concentration

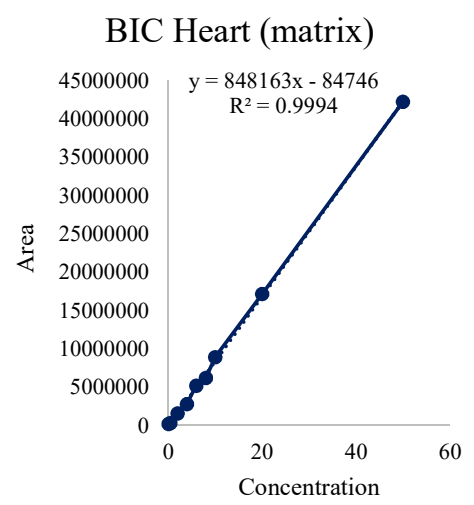

uV(x1,000,000)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0.0 1.0 2.0 3.0 4.0 5.0 6.0 min


BIC lung (matrix)
y = 844799x + 38830
R² = 0.9991
Area
45000000
40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
0 20 40 60
Concentration


uV(x1,000,000)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 min


BIC liver (matrix)
y = 850008x + 34543
R² = 0.9987
Area
45000000
40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
0 20 40 60
Concentration


uV(x1,000,000)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0.0 1.0 2.0 3.0 4.0 5.0 6.0 min


BIC Heart (matrix)
y = 848163x - 84746
R² = 0.9994
Area
45000000
40000000
35000000
30000000
25000000
20000000
15000000
10000000
5000000
0
0 20 40 60
Concentration

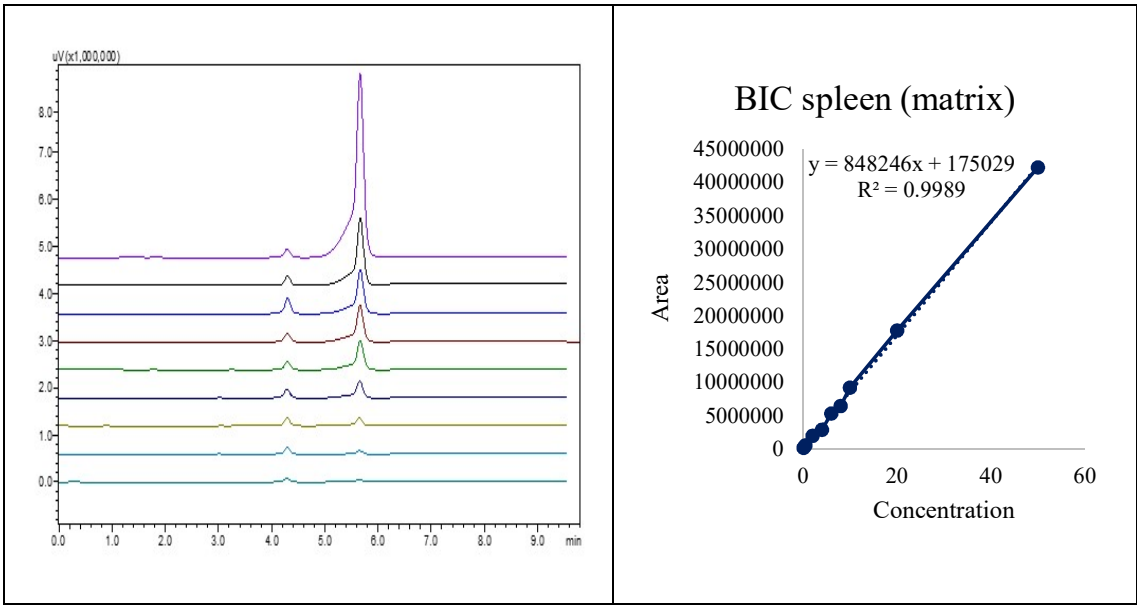


Figure 4.16. RP-HPLC images and calibration curve of BIC in (a) kidney (b) brain (c) lung (d) liver (e) heart (f) spleen

4.7 Summary

A sensitive UV, spectrofluorimetric and RP-HPLC-Fl methods were developed and validated successfully as per the ICH Q2(R1) guideline for both the drugs namely ETO and BIC and were used for estimation of *in vitro* release, permeability and *in vivo* sample analysis.

4.8 References

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