CHAPTER 4: ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

4.1. Introduction:

Analytical method development is a crucial and fundamental part in the formulation development and estimation. The quantification of selected drugs is essential at every step of *in vitro* release and *in vivo* pharmacokinetic study. This could become possible only after successful development and validation of the analytical methods. The RLX and BXR samples obtained from the in vitro release were estimated by optimized and validated UV spectrometric method. Furthermore, the HPLC methods for RLX and BXR were also developed in order to carry out the analysis of in vivo pharmacokinetics samples.

4.2. Materials and methods:

RLX and BXR were obtained as a gratis sample from Zydus research center, Gujarat, India and Apicore Pharmaceutical Ltd, Vadodara, Gujarat, India respectively. Analytical grade (AR) and HPLC grade reagents viz, methanol (MeOH) and acetonitrile (ACN) were procured from Fisher Scientific (Vadodara, Gujarat) to carry out UV spectrophotometric and chromatographic analysis respectively. Double distilled water was used throughout the experiment and was filtered through 0.22µ filters to carry out chromatographic analysis.

4.2.1. UV spectrophotometric method development

4.2.1.1. UV spectrophotometric method for RLX:

- **Preparation of calibration curve:** 10 mg of RLX was dissolved in 2 mL MeOH, sonicated and the volume was made to 10 mL MeOH to have 1000 µg/mL concentration. Then after, 1 mL of the stock solution was transferred to 10 mL volumetric flask and made upto the mark with MeOH. From this second stock solution, 0.1, 0.3, 0.5, 0.7, 0.9, 1.1 and 1.3 mL was withdrawn and transferred to 10 mL volumetric flask and this was diluted upto the mark with water in order to have 1, 3, 5, 7, 9, 11, and 13 µg/mL RLX concentration. Now, the wavelength maxima was decided by scanning the drug solution from 200 to 400 nm on a UV spectrophotometer (Shimadzu 1800, Japan). All the measurements were made in triplicate.
- **Analytical method validation:** The developed analytical method was validated as per the ICH Q2(R1) guidelines for linearity, precision and accuracy, limit of detection and limit of quantitation.

- **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. The linearity of the method was obtained for 1-13 µg/mL RLX solution analyzed at 287 nm.
- **Accuracy:** Accuracy refers to the closeness of an individual observation or the mean of the observations to true value. Accuracy was performed by the standard addition method at three levels: 80%, 100% and 120% of 5 µg/mL (three replicates for each concentration) into a standard solution and by calculating the percent recovery of active ingredient from the solution. The percent recovery was calculated for nine determinations.
- **Precision:** It refers to the degree 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 could be determined by performing inter-day and intra-day precision and %RSD is calculated. Herein, three concentration viz., LQC (low quality control), MQC (mid quality control), and HQC (high quality control) i.e. 4 µg/mL, 8 µg/mL and 12 µg/mL were selected experiment was repeated 3 times in a day for intra-day and on 3 different days for inter-day precision.
- **Limit of detection (LOD) and limit of quantitation (LOQ):** It gives a clear vision upto which level the developed method can detect the drug and quantify it respectively. This was calculated following standard deviation of the response and the slope method.
- **Assay:** For the assay, the marketed formation (equivalent to 60mg RLX) was crushed and dissolved in 10 mL of MeOH in order to extract RLX (6000 µg/mL). Then, 0.1 mL of this solution was transferred to 10 mL volumetric flask and made the volume upto 10 mL with MeOH to have 60 µg/mL RLX concentration. Finally, from this, 1 mL of the solution was transferred to 10 mL volumetric flask and diluted upto 10mL with water (6 µg/mL) and analyzed by UV at λ_{max} and %RSD was calculated.

4.2.1.2. UV spectrophotometric method development for BXR:

- **Preparation of calibration curve:** 10 mg of BXR was dissolved in 2 mL MeOH, sonicated and the volume was made to 10 mL MeOH to have 1000 µg/mL concentration. Then after, 1 mL of the stock solution was transferred to 10 mL volumetric flask and made upto the mark with MeOH (100 µg/mL). From this second stock solution, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3

and 1.5 mL was withdrawn and transferred to 10 mL volumetric flask and this was diluted upto the mark with water in order to have 3, 5, 7, 9, 11, 13 and 15 µg/mL BXR concentration. Now, the wavelength maxima was decided by scanning the drug solution from 200 to 400 nm on the UV spectrophotometer (Shimadzu 1800, Japan). All the measurements were made in triplicate.

- **Analytical method validation:** The developed analytical method was validated as per the ICH Q2(R1) guidelines for linearity, precision and accuracy, limit of detection and limit of quantitation (1). 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. The linearity of the method was obtained for 3-15 µg/mL BXR solution analyzed at 262 nm. The other validation parameters, *i.e.* accuracy, precision, LOD and LOQ were calculated following a similar procedure as followed for RLX.

4.2.2. RP-HPLC method development:

4.2.2.1. RP-HPLC method development RLX

- **Preparation of Stock solution:** The primary and secondary stock solution of 1000 µg/mL and 100 µg/mL RLX respectively were prepared as mentioned in section 4.2.1.1.
- **Chromatographic condition:** The chromatographic analysis was performed on the WATERS® HPLC instrument consisting of binary pump system and equipped with PDA detector. Ammonium formate buffer (20mM, pH:2.9) and ACN mixture in the ratio of 30:70 (v/v) were employed to carry out HPLC investigation using Kromasil® C18 column (250 × 4.6 mm, 5.0 µm) and withholding 1mL/min flow rate. The aqueous phase was duly filtered by the 0.22 µm membrane filter (Milipore, Billerica, USA) and the mobile phase was degassed by sonicating it for 10min prior to use. All the analysis were performed at 287 nm. Data acquisition and quantification were performed using Empower™ software version 3.
- **Validation of developed RP-HPLC method:**
  - **Preparation of calibration curve:** 10 mg of RLX was dissolved in 2 mL MeOH, sonicated and the volume was made to 10 mL MeOH to have 1000 µg/mL concentration. Thena fter, 1 mL of the stock solution was transferred to 10 mL volumetric flask and made upto the mark with MeOH (100 µg/mL). From this second stock solution, 0.01, 0.05, 0.2, 0.5, 1 and 20 mL were withdrawn and transferred to 10 mL volumetric flask and this was diluted upto the mark with water in order to have 0.1,0.5,2,5,10 and 20 µg/mL RLX concentration. 20 µL of each concentrations were

injected and chromatographed under the optimized condition as mentioned above. Calibration curve was developed by plotting peak area versus concentration of RLX (µg/mL) and regression equation was calculated.

- **Accuracy:** Accuracy was performed by the standard addition method at three levels: 80%, 100% and 120% of 5 µg/mL (three replicates for each concentration) into a standard solution and by calculating the percent recovery of active ingredient from the solution. The percent recovery was calculated for nine determinations.
- **Precision:** Precision provides an indication of random errors and is generally subdivided into two cases: repeatability and reproducibility which could be determined by performing inter-day and intra-day precision and %RSD is calculated. Herein, three concentration viz., LQC (low quality control), MQC (mid quality control), and HQC (high quality control) i.e. 1 µg/mL, 9 µg/mL and 18 µg/mL were selected experiment was repeated three times in a day for intra-day and on three different days for inter-day precision.
- **Limit of detection (LOD) and limit of quantitation (LOQ):** It gives a clear vision upto which level the developed method can detect the drug and quantify it respectively. This was calculated following standard deviation of the response and the slope method.
- **Assay of the selected marketed formulation:** For the assay, the marketed formation (equivalent to 60mg RLX) was crushed and dissolved in 10 mL of MeOH in order to extract RLX (6000 µg/mL). Then, 0.1 mL of this solution was transferred to 10 mL volumetric flask and made the volume upto 10 mL with MeOH to have 60 µg/mL RLX concentration. Finally, from this, 1 mL of the solution was transferred to 10 mL volumetric flask and diluted upto 10mL with water (6 µg/mL) and analyzed by UV at λmax and %RSD was calculated.
- **System suitability:** This was performed by injecting six replicates of 10 µg/mL RLX solution and deviation in retention time and area was reported.

➢ The developed HPLC method was validated for various validation parameters as per the ICH Q2(R1) guideline.

4.2.2.2. RP-HPLC method development BXR

➢ **Preparation of Stock solution:** The primary and secondary stock solution of 1000 µg/mL and 100 µg/mL BXR respectively was prepared as per earlier.

- **Chromatographic condition:** The chromatographic analysis was performed on the WATERS® HPLC instrument consisting of binary pump system and equipped with PDA detector. Ammonium formate buffer (20mM, pH:2.9) and ACN mixture in the ratio of 10:90 (v/v) were employed to carry out the HPLC investigation using Kromasil® C18 column (250 × 4.6 mm, 5.0 µm) and withholding 1mL/min flow rate. The aqueous phase was duly filtered with the 0.22 µm membrane filter (Milipore, Billerica, USA) and the mobile phase was degassed by sonicating it for 10min prior to use. All the analysis was performed at 262 nm. Data acquisition and quantification were performed on Empower™ software version 3.
- **Preparation of calibration curve:** 10 mg of BXR was dissolved in 2 mL MeOH, sonicated and the volume was made to 10 mL MeOH to have 1000 µg/mL concentration. Then after, 1 mL of the stock solution was transferred to 10 mL volumetric flask and made upto the mark with MeOH (100 µg/mL). From this second stock solution, 0.01, 0.05, 0.2, 0.5, 1 and 20 mL were withdrawn and transferred to 10 mL volumetric flask and this was diluted upto the mark with water in order to have 0.1,0.5,2,5,10 and 20 µg/mL BXR concentration. 20 µL was injected for each concentrations and chromatographed under the optimized condition as mentioned above. Calibration curve was developed by plotting peak area versus concentration of BXR (µg/mL) and regression equation was calculated.
- The developed HPLC method was validated for various validation parameters as per the ICH Q2(R1) guideline as mention in 4.2.2.1.

4.2.3. Bioanalytical method development:

4.2.3.1. Bioanalytical method development for RLX

- **Procedure for Stock solution preparation:** The primary and secondary stock solution of 1000 µg/mL and 100 µg/mL RLX respectively was prepared as per mention in section 4.2.1.1. Furthermore, the 1000 µg/mL edaravone (EDA, internal standard) stock solution was prepared in the identical way.
- **Procedure for un-extracted sample preparation:** 100 µL of 100 µg/mL RLX solution was mixed with equal volume of 20 µg/mL EDA in the pre-labelled eppendorf. Finally, 800 µL of the mobile phase was added to the above mixture to have 10 µg/mL RLX solution. Similarly, 100 µL of 1, 5, 20, 50 and 200 µg/mL RLX solution is added and proceeded in the same way to have 0.1, 0.5, 2, 5 and 20 µg/mL un-extracted RLX solution
- **Procedure for plasma sample preparation:** The protein precipitation method was optimized to recover the sample from the bioanalytical matrix as it is having high protein

binding affinity. Briefly, to the 150 µL human plasma, 100 µL 100 µg/mL RLX solution was added and vortexed for 2 min followed by the addition of 100 µL of 20 µg/mL EDA and vortexed again for 2 min. Subsequently, 800 µL ACN was added, vortexed and centrifuged at 5000 rpm at 4 ºC for 10 min and the supernatant was collected and analyzed by HPLC. Similarly, 100 µL of 1, 5, 20, 50 and 200 µg/mL RLX solution is added and proceeded in the same way to have 0.1, 0.5, 2, 5 and 20 µg/mL un-extracted RLX solution.

- **Chromatographic condition:** The chromatographic procedure was kept same as described in 4.2.2.1.
- **Method Validation:** Developed bioanalytical method was validated as per the ICH Q2(R1) guideline (1).
    - System suitability: This was performed by injecting MQC concentration of RLX 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 lower limit of quantitation (LLOQ) concentration of RLX solution for 6 times and %RSD was calculated.
    - Linearity: The linearity of the RLX was taken in the range of 0.1 to 12 µg/mL and regression equation and correlation coefficient (r^2) was calculated.
    - Accuracy: Accuracy was performed taking 80, 100 and 120% concentration of RLX from the fixed concentration and %accuracy was founded.
    - Precision: Intra-day and inter-day precision was performed taking LLOQ, LQC, MQC and HQC RLX 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 slope method.
    - %Recovery: %recovery of drug from the biological matrix was calculated by extracting LQC, MQC and HQC level RLX sample and calculated by following equation:

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

    - ***Bench Top stability:*** Bench top stability of the spiked quality control samples was determined for a period of 6 h. 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 RP-HPLC method for RLX 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 tissues 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.

4.2.3.2. Bioanalytical method development for BXR

- **Procedure for Stock solution preparation:** The primary and secondary stock solutions of 1000 µg/mL and 100 µg/mL BXR respectively were prepared as per described previously in section 4.2.1.2.
- **Procedure for un-extracted sample preparation:** 100 µL of 100 µg/mL BXR solution was mixed with equal volume of 20 µg/mL clopidogrel (CLOPI, internal standard) in the pre-labelled eppendorf. Finally, 800 µL of the mobile phase was added to the above mixture to have 10 µg/mL BXR solution. Similarly, 100 µL of 1, 5, 20, 50 and 200 µg/mL BXR solution is added and proceeded in the same way to have 0.1, 0.5, 2, 5 and 20 µg/mL un-extracted BXR solution.
- **Procedure for plasma sample preparation:** The rotein precipitation method was optimized to recover the sample from the bioanalytical matrix as it is having high protein binding affinity. Briefly, to the 150 µL human plasma, 100 µL 100 µg/mL BXR solution was added and vortexed for 2 min followed by the addition of 100 µL of 20 µg/mL CLOPI (internal standard) and vortexed again for 2 min. Subsequently, 800 µL ACN was added, vortexed and centrifuged at 5000 rpm at 4 ºC for 10 min and the supernatant was collected and analyzed by HPLC. Similarly, 100 µL of 1, 5, 20, 50 and 200 µg/mL BXR solution is added and proceeded in the same way to have 0.1, 0.5, 2, 5 and 20 µg/mL un-extracted BXR solution.

- **Chromatographic condition:** The chromatographic analysis was kept same as described in 4.2.2.2.
- **Method Validation:** It was performed in identical manner as mentioned in section 4.2.3.1.
- **Application of developed HPLC method for BXR estimation in different tissue homogenate:** It was performed in the same way as mentioned in section 4.2.3.1.

4.3. Result and discussion

4.3.1. UV method development:

4.3.1.1. UV spectrophotometric method development for RLX:

After scanning the RLX solution from 200 to 400 nm, the λmax obtained was 287 nm (figure 4.1) which was used as an analytical wavelength. The developed UV spectrophotometric method was validated for various parameters and the results were in accordance with the ICH Q2(R1) criteria and listed in the table 4.1.

Table 4.1. UV method validation parameters for RLX

PARAMETERS	RESULTS	
Wavelength (nm)	287	
Concentration range (µg/mL)	1-13	
Regression equation	y= 0.0634x-0.0059	
Correlation coefficient (r^2)	0.9993	
Intra-day precision (%RSD)	LQC (4 µg/mL)	0.88%
	MQC (8 µg/mL)	0.69%
	HQC (12 µg/mL)	0.92%
Inter-day precision (%RSD)	LQC (4 µg/mL)	0.51%
	MQC (8 µg/mL)	0.48%
	HQC (12 µg/mL)	0.43%
Accuracy (%Recovery)	80%	98.53%
	100%	98.14%
	120%	99.25%
%Assay	99.81%	
LOD (µg/mL)	0.17	
LOQ (µg/mL)	0.52	

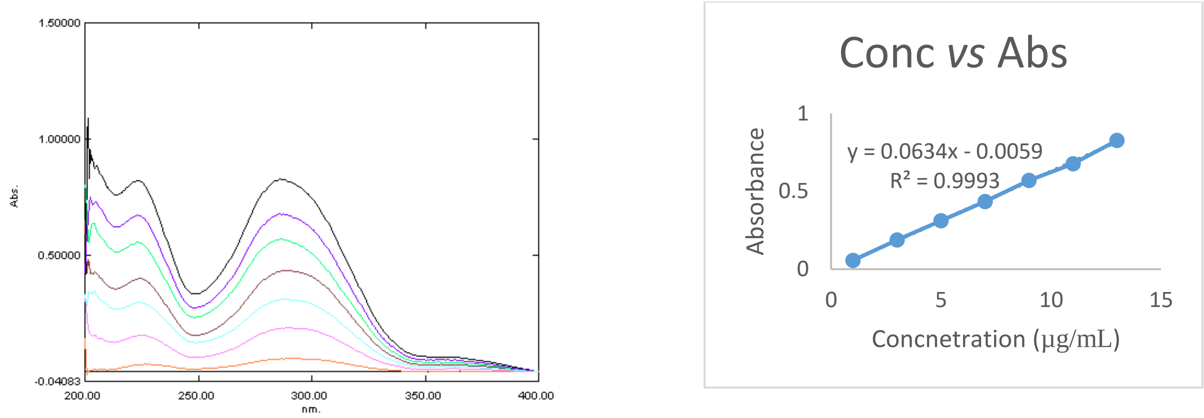


Figure 4.1: Calibration curve for RLX at 283nm for 1 to 13 µg/mL

4.3.1.2. UV spectrophotometric method development for BXR:

After scanning the RLX solution from 200 to 400 nm, the λmax obtained was 262 nm (figure 4.2) which was used as an analytical 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 4.2.

Table 4.2. UV method validation parameters for BXR

PARAMETERS	RESULTS	
Wavelength (nm)	262	
Concentration range (µg/mL)	3-15	
Regression equation	y= 0.0519x-0.0099	
Correlation coefficient (r^2)	0.9985	
Intra-day precision (%RSD)	LQC (4 µg/mL)	0.99%
	MQC (8 µg/mL)	0.84%
	HQC (12 µg/mL)	0.83%
Inter-day precision (%RSD)	LQC (4 µg/mL)	0.62%
	MQC (8 µg/mL)	0.47%
	HQC (12 µg/mL)	0.63%
Accuracy (%Recovery)	80%	99.12%
	100%	99.56%
	120%	98.93%
LOD (µg/mL)	0.21	
LOQ (µg/mL)	0.64	

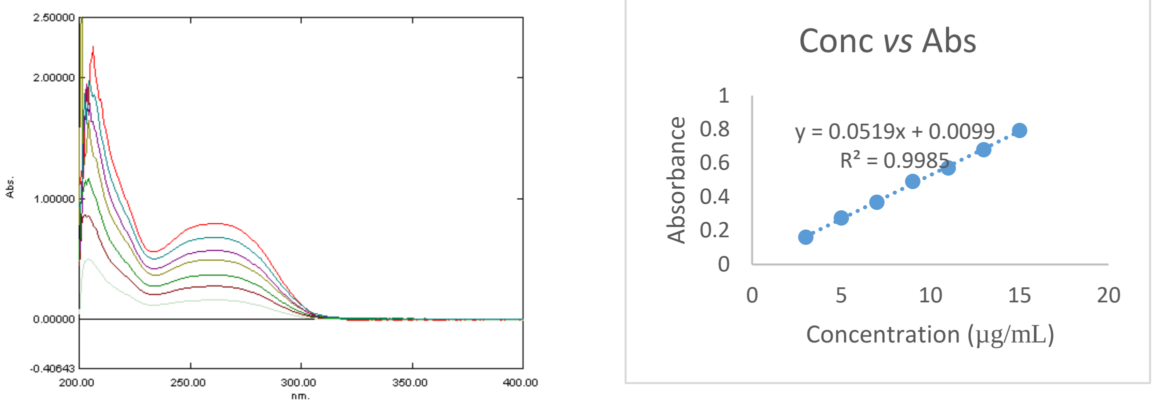


Figure 4.2: Calibration curve for BXR at 262 nm for 3 to 15 µg/mL

4.3.2. RP-HPLC method development:

4.3.2.1. RP-HPLC method development for RLX:

The RP-HPLC method showed RLX retention time at 10.8±0.85 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.

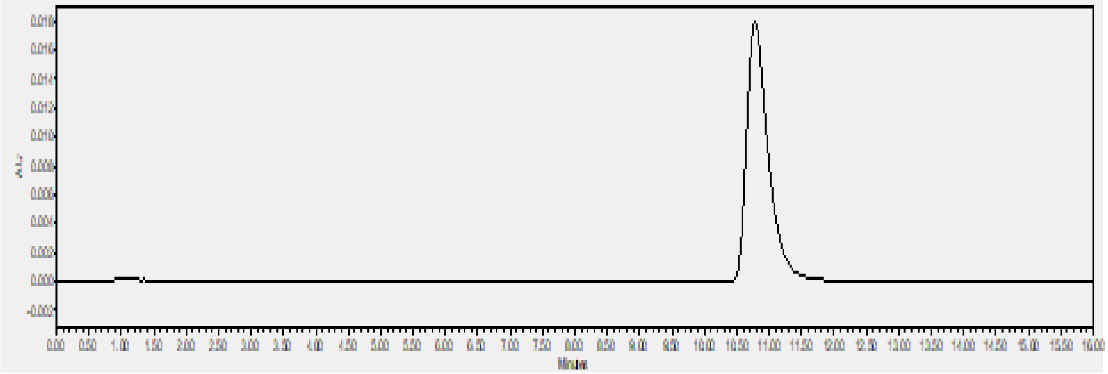
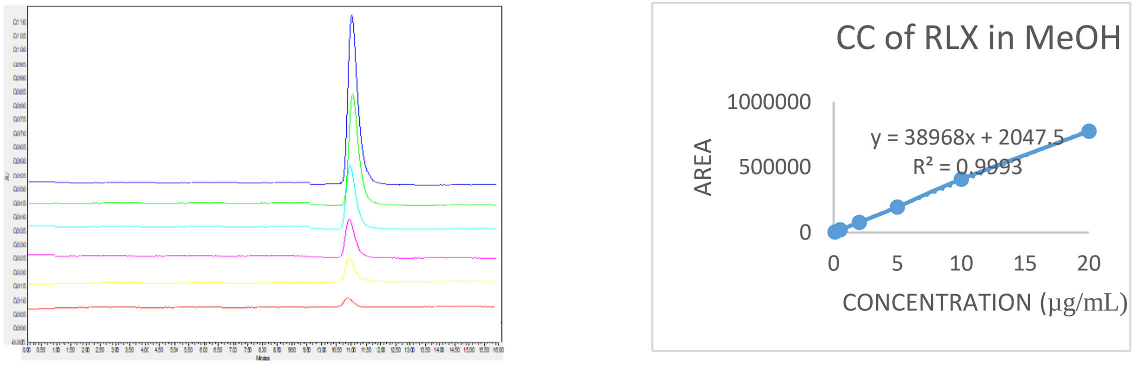


Figure 4.3. RP-HPLC chromatograph for RLX


(A)

(B)

Figure 4.4. (A) Overlain RP-HPLC chromatogram and (B) calibration curve for RLX (0.1-20 µg/mL)

Table 4.3. RP-HPLC method validation parameters for RLX

PARAMETERS	RESULTS	
Wavelength (nm)	287	
Concentration range (µg/mL)	0.1-20	
Regression equation	y= 90369x-27754	
Correlation coefficient (r^2)	0.9992	
Intra-day precision (%RSD)	LQC (1 µg/mL)	0.66%
	MQC (9 µg/mL)	0.56%
	HQC (18 µg/mL)	0.62%
Inter-day precision (%RSD)	LQC (1 µg/mL)	0.33%
	MQC (9 µg/mL)	0.38%
	HQC (18 µg/mL)	0.47%
Accuracy (%Recovery)	80%	99.17%
	100%	99.62%
	120%	99.18%
LOD (µg/mL)	0.01	
LOQ (µg/mL)	0.03	
System suitability	Retention time	10.8±1.16 min
	Area	415795±0.52

4.3.2.2. RP-HPLC method development for BXR

The RP-HPLC method showed BXR retention time at 8.8±1.13 min at 1mL/min flow rate (figure 4.5). 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 along with the linearity chromatographs as depicted in figure 4.6.

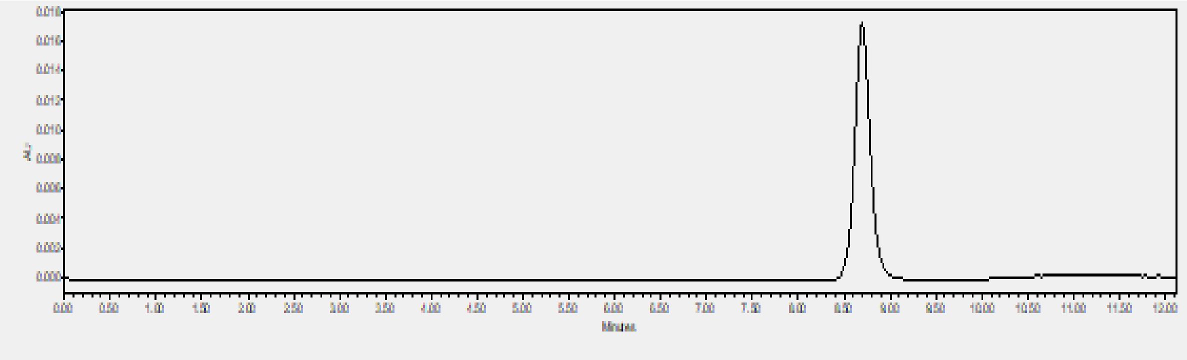


Figure 4.5. RP-HPLC chromatograph for BXR

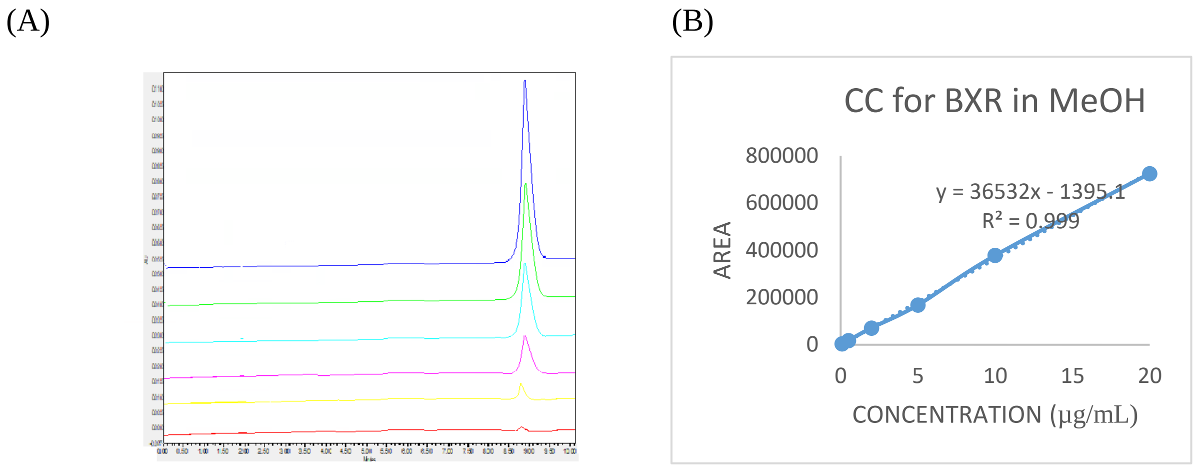


Figure 4.6. (A) Overlain RP-HPLC chromatogram and (B) calibration curve for BXR (0.1-20 µg/mL)

Table 4.4. RP-HPLC method validation parameters for BXR

PARAMETERS	RESULTS	
Wavelength (nm)	262	
Concentration range (µg/mL)	0.1-20	
Regression equation	y= 79852x+27846	
Correlation coefficient (r^2)	0.9992	
Intra-day precision (%RSD)	LQC (1 µg/mL)	0.57%
	MQC (9 µg/mL)	0.43%
	HQC (18 µg/mL)	0.55%
Inter-day precision (%RSD)	LQC (1 µg/mL)	0.23%
	MQC (9 µg/mL)	0.20%
	HQC (18 µg/mL)	0.31%
Accuracy (%Recovery)	80%	99.88%
	100%	99.16%
	120%	98.37%
LOD (µg/mL)	0.012	
LOQ (µg/mL)	0.037	
System suitability	Retention time	8.8±1.21 min
	Area	385895±0.78

4.3.3. Bioanalytical method development

4.3.3.1. Bioanalytical method development for RLX

- The RP-HPLC method for un-extracted and plasma extracted RLX and EDA showed RLX retention time at 10.9±1.06 min and EDA at 6.7±0.98 min at 1mL/min flow rate (figure 4.7. and figure 4.8.). The linearity data and calibration curve for both un-extracted samples and plasma extracted samples is depicted in figure 4.9A and 4.9B 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 4.5.
- Additionally, in order to calculate the amount of the drug goes to different organs, the linearity of RLX in different the tissue matrixes were taken and calibration curve was generated as depicted in figure 4.10.

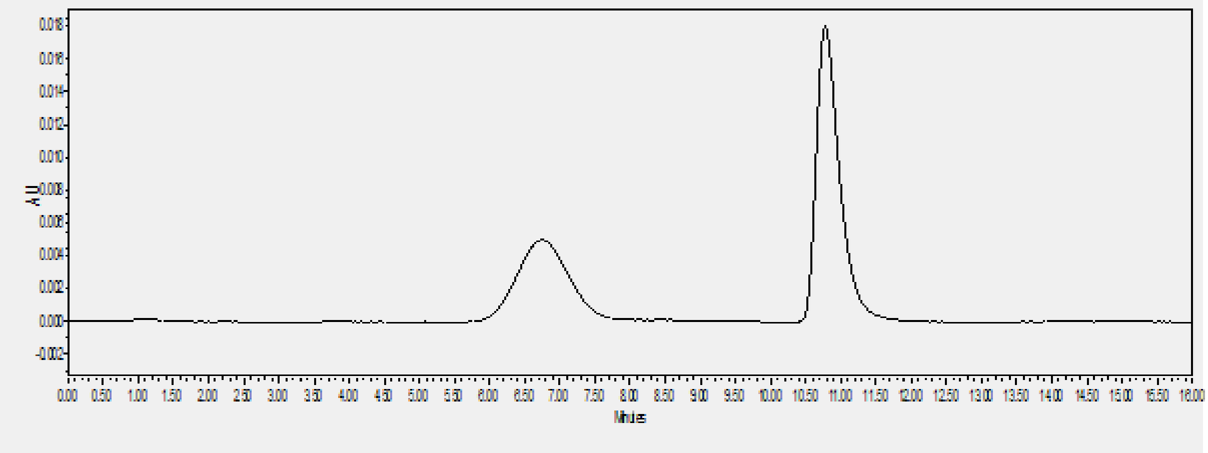


Figure 4.7: RP-HPLC Chromatogram for un-extracted RLX and EDA

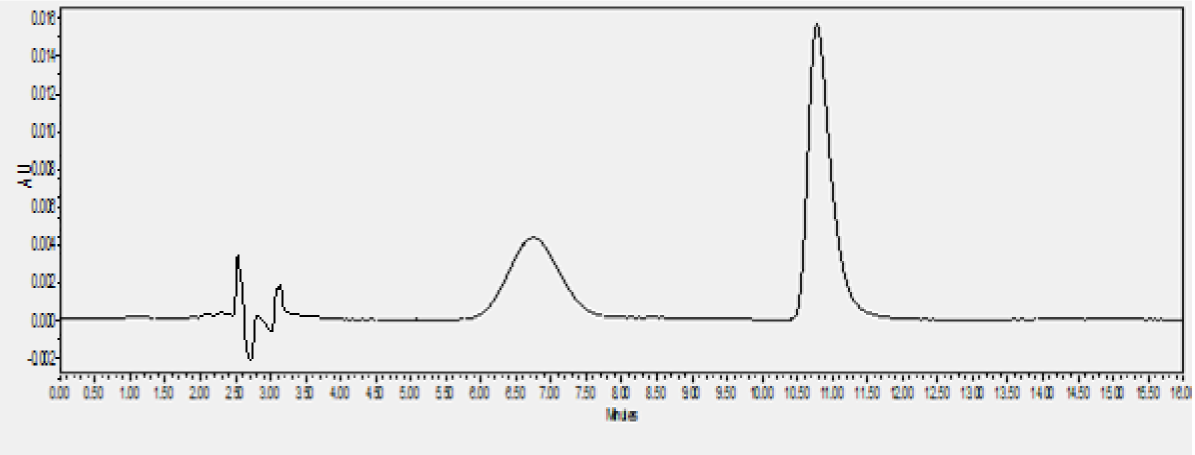


Figure 4.8: RP-HPLC Chromatogram for RLX and EDA extracted from plasma

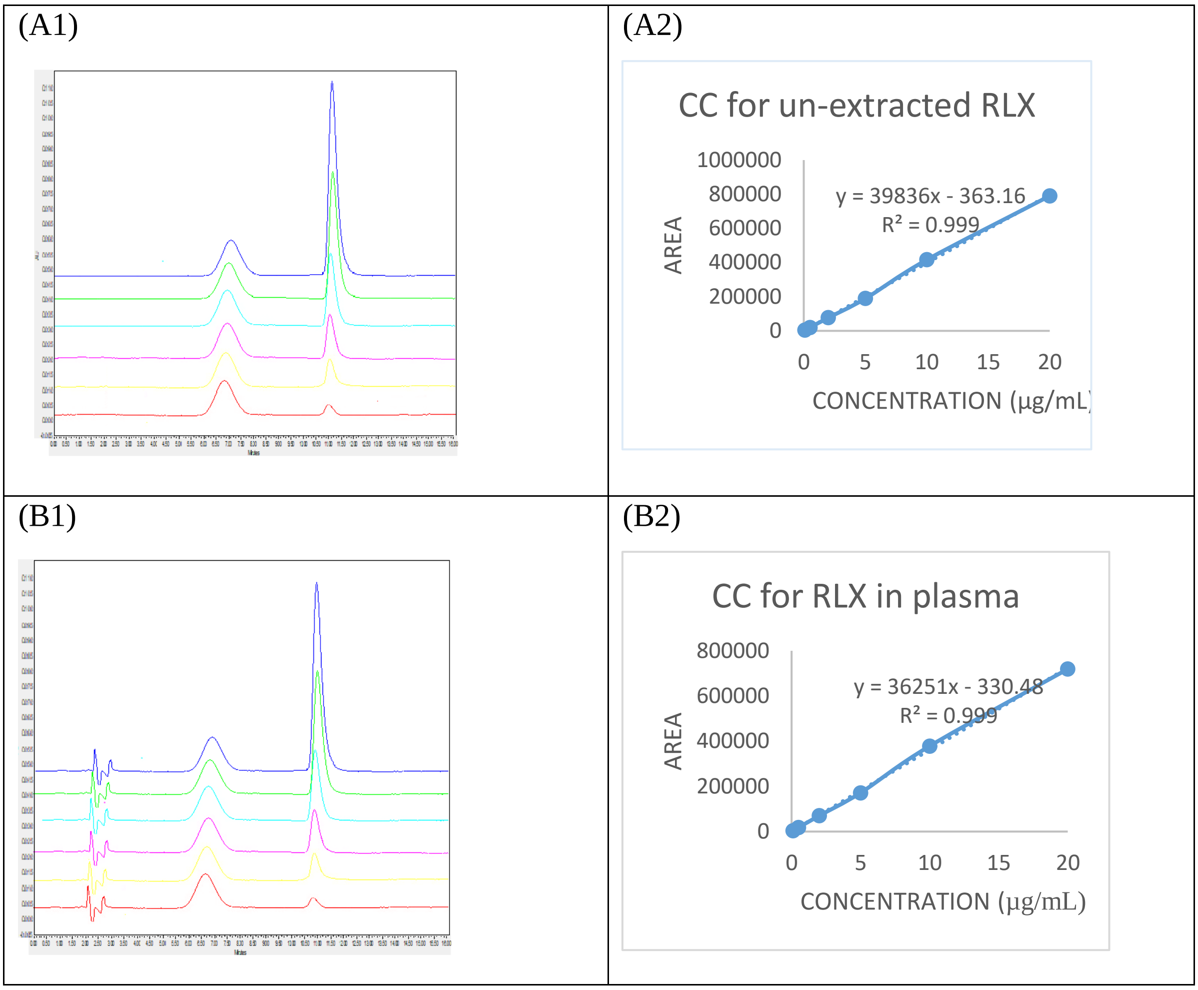


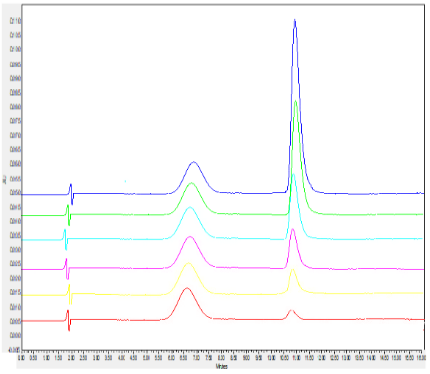
Figure 4.9. Overlain RP-HPLC chromatogram for (A1) un-extracted RLX and (B1) extracted RLX, calibration curve for (A2) un-extracted RLX and (B2) extracted RLX (0.1-20 µg/mL)

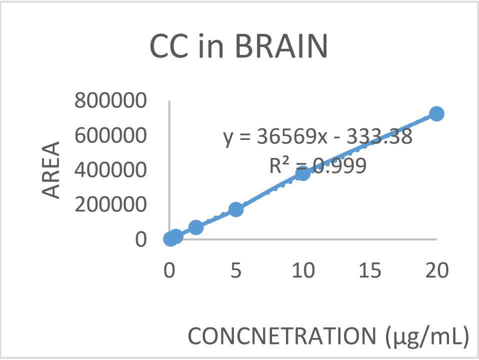
Table 4.5. RP-HPLC method validation parameters for RLX

PARAMETERS	RESULTS OBTAINED	
Wavelength (nm)	287	
Concentration range (µg/mL)	0.1-20	
Regression equation	y= 91478x+29451	
Correlation coefficient (r^2)	0.999	
Intra-day precision (%RSD)	LLOQ (0.1 µg/mL)	1.12%
	LQC (1 µg/mL)	0.85%
	MQC (9 µg/mL)	0.96%
	HQC (18 µg/mL)	0.92%
Inter-day precision (%RSD)	LLOQ (0.1 µg/mL)	0.98%
	LQC (1 µg/mL)	0.39%

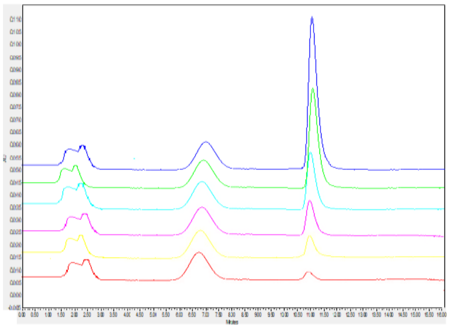
	MQC (9 µg/mL)	0.41%
	HQC (18 µg/mL)	0.33%
Accuracy (%Recovery)	80%	98.11%
	100%	101.22%
	120%	98.96%
LOD (µg/mL)	0.05	
LOQ (µg/mL)	0.15	
%Recovery	LQC (1 µg/mL)	89.31%
	MQC (9 µg/mL)	85.95%
	HQC (18 µg/mL)	88.56%
Bench-top Stability	LQC (1 µg/mL)	102.55%
	HQC (18 µg/mL)	101.89%
Freeze-thaw stability	LQC (1 µg/mL)	99.84%
	HQC (18 µg/mL)	100.26%
Long-term stability	LQC (1 µg/mL)	101.23%
	HQC (18 µg/mL)	101.07%
System suitability	Retention time	10.9±1.06 min
	Area	386782±0.87

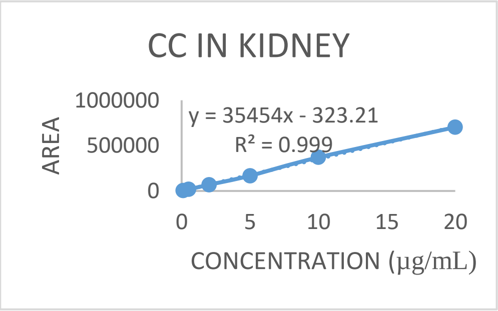
(A)





(B)





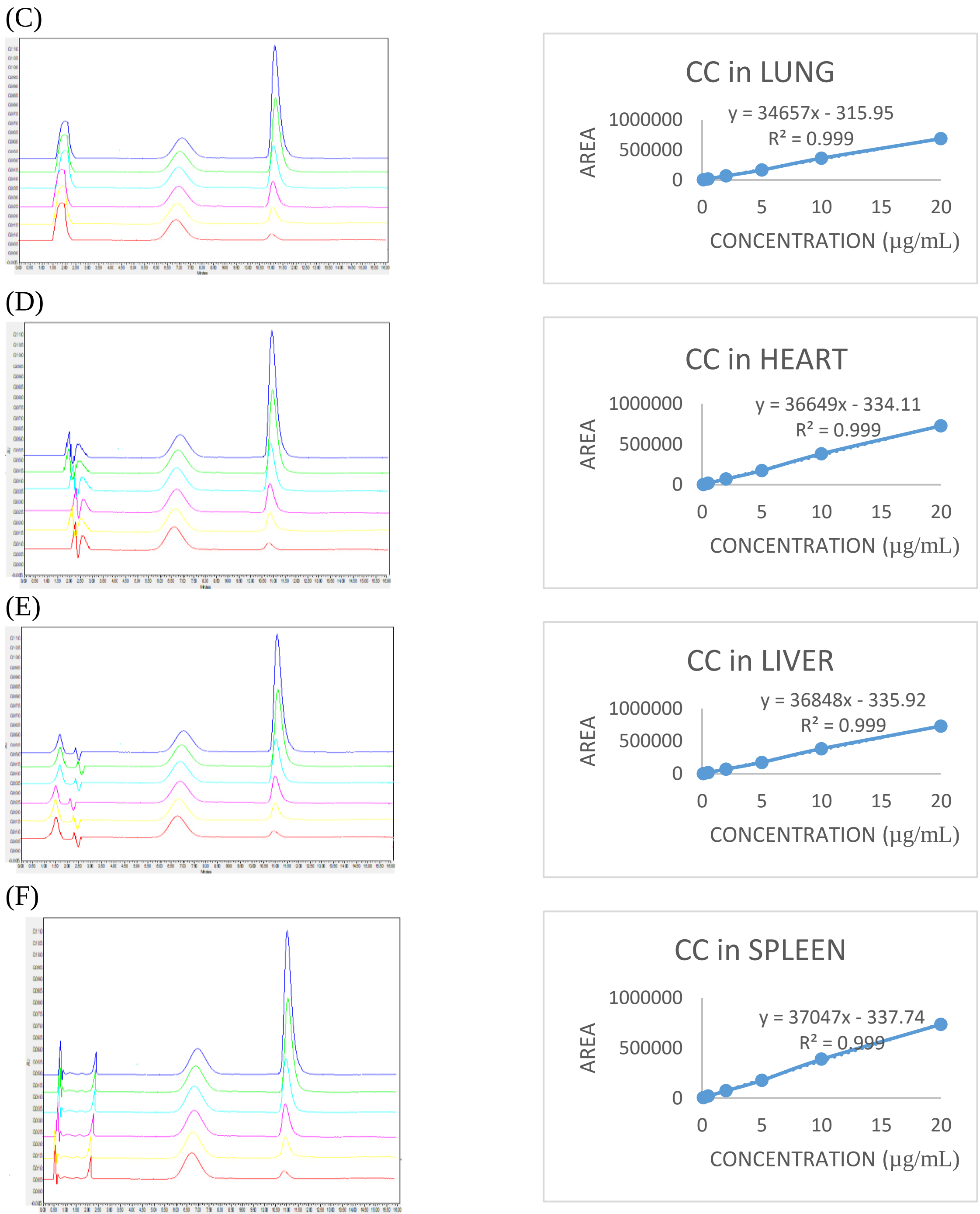


Figure 4.10. Overlain RP-HPLC images and calibration curve of RLX in (A) Brain, (B) Kidney, (C) Lung, (D) Heart, (E) Liver and (F) Spleen matrix

4.3.3.2. Bioanalytical method development for BXR

- The RP-HPLC method for un-extracted and plasma extracted BXR and CLOPI showed BXR retention time at 8.8±0.21 min and CLOPI at 5.5±0.26 min at 1mL/min flow rate (figure 4.11. and figure 4.12.). The linearity data and calibration curve for both un-extracted samples and plasma extracted samples is depicted in figure 4.13A and 4.13B 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 4.6.

- Additionally, in order to calculate the amount of drug goes to different organs, the linearity of BXR in different tissue matrix were taken and calibration curve was generated as depicted in figure 4.14.

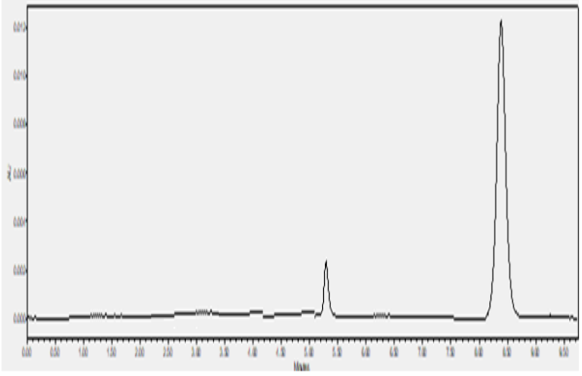


Figure 4.11: RP-HPLC Chromatogram for un-extracted BXR and CLOPI

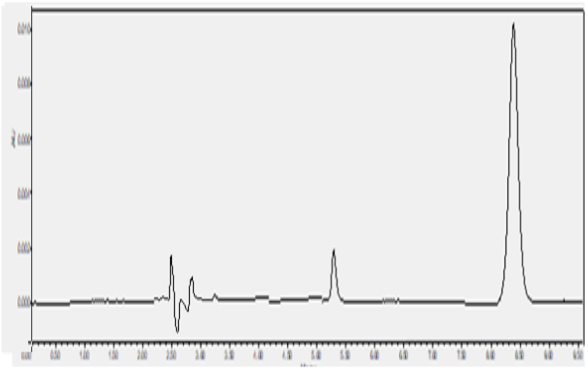
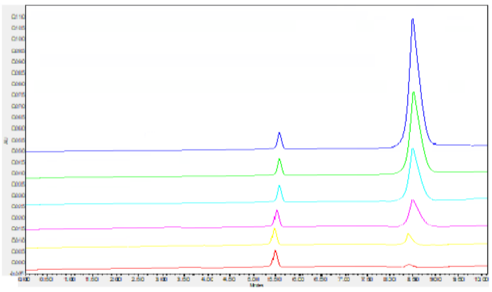
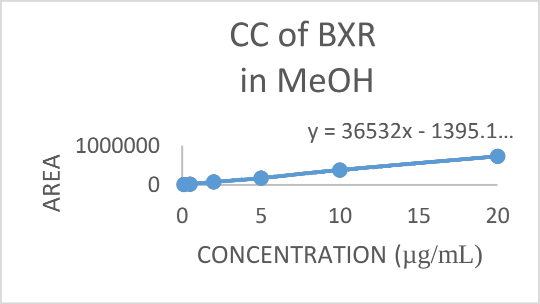


Figure 4.12: RP-HPLC Chromatogram for BXR and CLOPI extracted from plasma

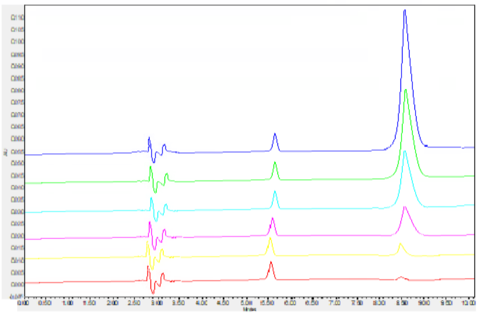
(A)



(B)



(B)



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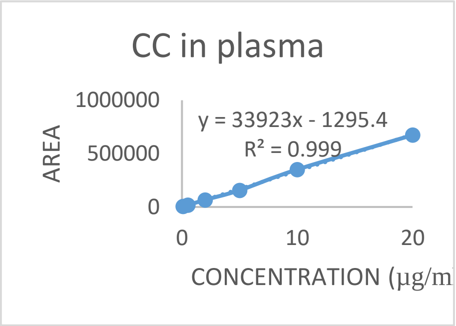
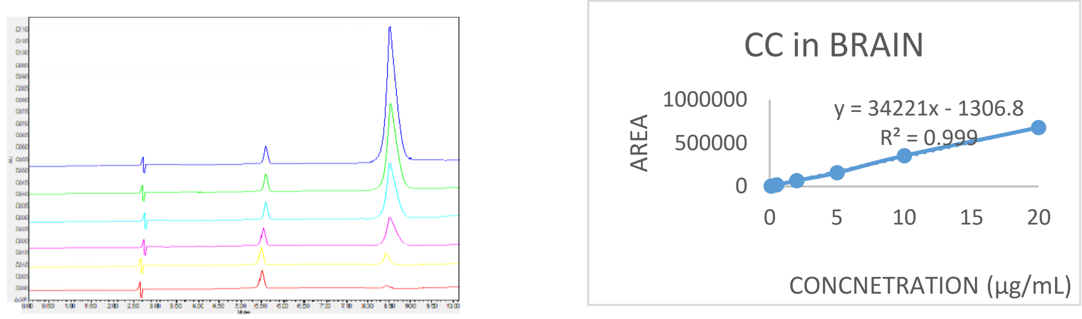


Figure 4.13. Overlain RP-HPLC chromatogram for (A1) un-extracted BXR and (B1) extracted BXR, calibration curve for (A2) un-extracted BXR and (B2) extracted BXR (0.1-20 µg/mL)

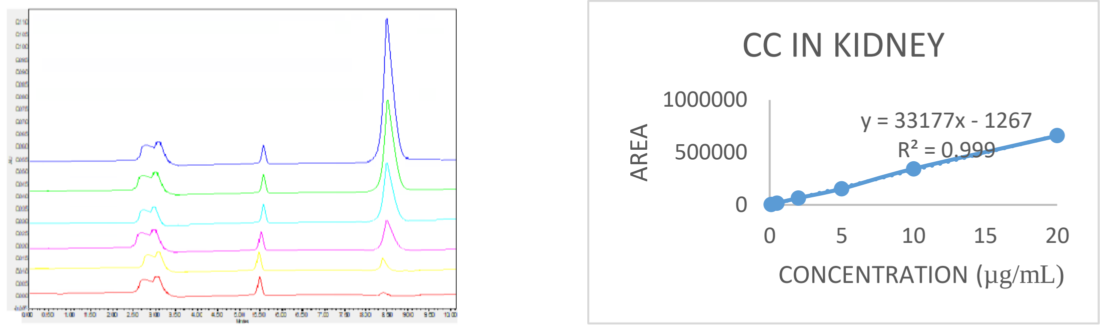
Table 4.6. RP-HPLC method validation parameters for BXR

PARAMETERS	RESULTS	
Wavelength (nm)	267	
Concentration range (µg/mL)	0.1-20	
Regression equation	y= 81456x+31578	
Correlation coefficient (r^2)	0.999	
Intra-day precision (%RSD)	LLOQ (0.1 µg/mL)	1.56%
	LQC (1 µg/mL)	0.55%
	MQC (9 µg/mL)	0.61%
	HQC (18 µg/mL)	0.72%
Inter-day precision (%RSD)	LLOQ (0.1 µg/mL)	0.83%
	LQC (1 µg/mL)	0.41%
	MQC (9 µg/mL)	0.38%
	HQC (18 µg/mL)	0.32%
Accuracy (%Recovery)	80%	100.22%
	100%	102.14%
	120%	101.96%
LOD (µg/mL)	0.07	
LOQ (µg/mL)	0.22	
%Recovery	LQC (1 µg/mL)	88.66%
	MQC (9 µg/mL)	86.12%
	HQC (18 µg/mL)	91.22%
Bench-top Stability	LQC (1 µg/mL)	101.67%
	HQC (18 µg/mL)	102.49%
Freeze-thaw stability	LQC (1 µg/mL)	102.55%
	HQC (18 µg/mL)	101.38%
Long-term stability	LQC (1 µg/mL)	99.61%
	HQC (18 µg/mL)	101.33%
System suitability	Retention time	8.8±0.21 min
	Area	355023±0.87

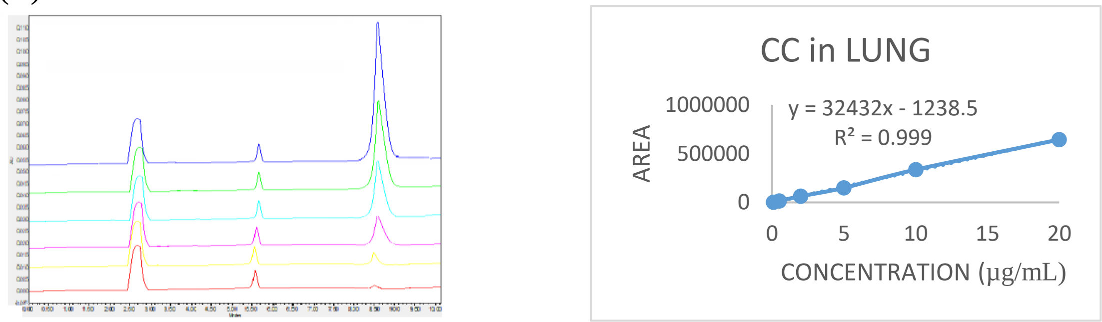
(A)


CC in BRAIN
AREA
1000000
500000
0
y = 34221x - 1306.8
R² = 0.999
0
5
10
15
20
CONCNETRATION (µg/mL)

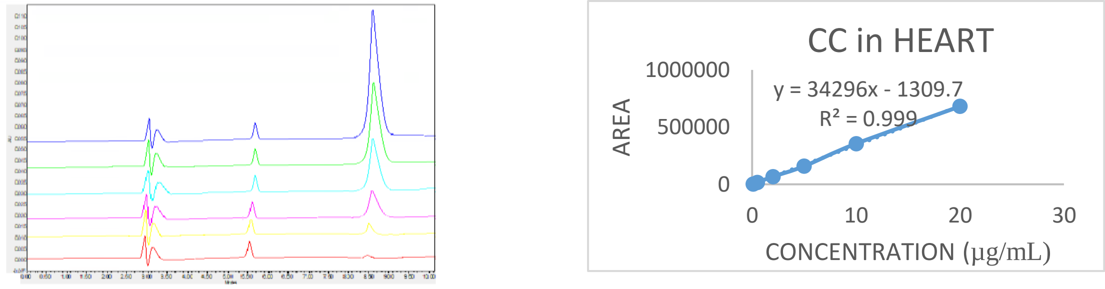
(B)


CC IN KIDNEY
AREA
1000000
500000
0
y = 33177x - 1267
R² = 0.999
0
5
10
15
20
CONCENTRATION (µg/mL)

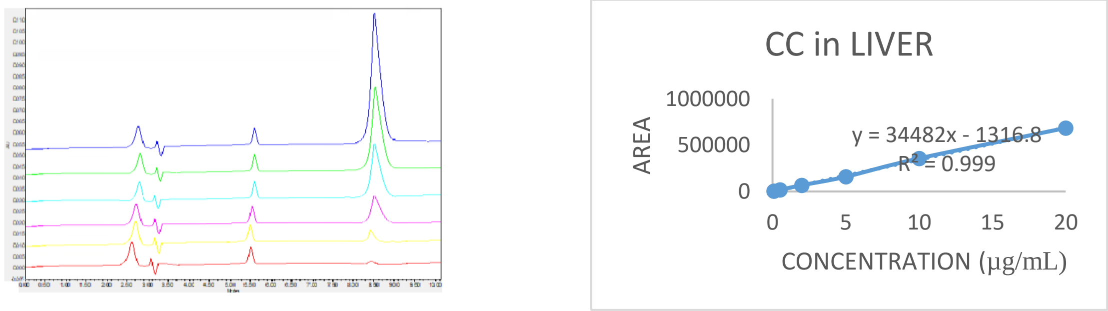
(C)


CC in LUNG
AREA
1000000
500000
0
y = 32432x - 1238.5
R² = 0.999
0
5
10
15
20
CONCENTRATION (µg/mL)

(D)


CC in HEART
AREA
1000000
500000
0
y = 34296x - 1309.7
R² = 0.999
0
10
20
30
CONCENTRATION (µg/mL)

(E)


CC in LIVER
AREA
1000000
500000
0
y = 34482x - 1316.8
R² = 0.999
0
5
10
15
20
CONCENTRATION (µg/mL)

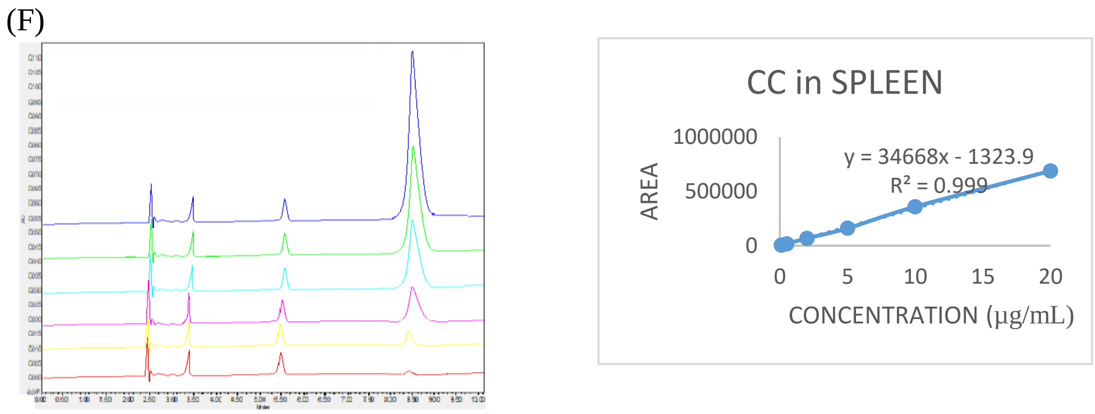


Figure 4.14. RP-HPLC images and calibration curve of BXR in (A) Brain, (B) Kidney, (C) Lung, (D) Heart, (E) Liver and (F) Spleen matrix

4.4. Summary:

The UV and RP-HPLC methods were developed and validated successfully as per the ICH Q2(R1) guideline for RLX and BXR and were used for estimation of *in vitro* and *in vivo* sample analysis.

4.5. References:

1. Guideline IHT, editor Validation of analytical procedures: text and methodology Q2 (R1). International Conference on Harmonization, Geneva, Switzerland; 2005.