3. Analytical Method Development

3.1. Introduction

For any pharmaceutical development program, analytical method development and validation are key elements. Analytical method development is an approach which involves screening of suitable assay method for measurement of desired component(s) of a formulation at various stages of product development. The term “Validation” can be understood as an approach for establishing the acceptability of a particular analytical method which is used for the quantification of concentration of sample in labs. Validation is a very vital tool for the scientist to identify the performance of developed method and to prove its performance limits.(1, 2) Vital parameters which may be calculated during method validation are specificity, range, linearity, precision, and accuracy, LOD (limits of detection) and LOQ (limit of quantitation).(2) Additional validation may be required as and when changes are encountered during formulation development and modifications are made to existing analytical methods. For obtaining precise data, accuracy and performance of instruments is also crucial.

For the measurement of vital formulation characteristics, analytical methods are employed for the present investigation which include, percent entrapment efficiency, *in-vitro* drug release, in vivo bioavailability, ex vivo skin deposition/permeation, and drug retention during stability testing. For the quantitative analysis of FBX and TAC in formulation and in the human body, different analytical methods are reported in literatures are mentioned in table 3.1.

Table 3.1: Reported analytical methods for TAC and FBX

Analytical method	**Reference**
TAC	
Liquid chromatography-mass spectroscopy	(3)
FBX	
Ultraviolet-visible (UV) Spectroscopy	(4, 5)

From simple instrumental method like UV spectrophotometry to more sensitive and sophisticated methods like LCMS (liquid chromatography- mass spectroscopy) and HPLC (high performance liquid chromatography) were exploited depending upon the theoretical amount of drug substance present in analytical samples. Analytical methods adopted in the present investigation are briefly explained below.

3.2. Reagent Preparation

3.2.1. 6mM phosphoric acid:

For the preparation of 6mM phosphoric acid, 0.395 ml of phosphoric acid was added in 1000 ml double distilled water and then the solution was filtered employing 0.22 µm membrane filter.

3.2.2. 0.1 % formic acid:

1 ml of phosphoric acid was added in 1000 ml double distilled water and mixed thoroughly to prepare 0.1 % formic acid. Then, the solution was filtered with the help of 0.22 µm membrane filter.

3.2.3. Double Distilled Water

For the preparation of purified HPLC grade water, double distilled water was filtered with nylon filter paper pore size of pore size of 0.22 µm. The above mentioned filter paper was procured from Pall Life sciences, Mumbai, India.

3.3. Equipment

3.3.1. High Performance Liquid Chromatographic (HPLC) System

The gradient HPLC system using Agilent 1220 series, a manual Rheodyne injector (20 µl fixed loop) and Ultraviolet visible detector (Agilent, Germany) was employed together with Chem-Station as data handling software.

3.3.2. UV Visible Spectrophotometer

Shimadzu-1800 spectrophotometer was used to measure the amount of drug for the evaluation of drug entrapment and also to evaluate the concentration of drug during *in-vitro* drug release.

3.4. Analytical Methods for Tacrolimus

It is reported that TAC is greatly soluble in majority of organic solvents such as chloroform, methanol, acetonitrile, acetone and many other solvents but it

is found to be practically insoluble in water.(6, 7) TAC is official in the USP and exhibits a λ_{max} of 220 nm in UV. However, methanol and ACN exhibit a λ_{max} of 205 nm and 195 nm respectively, which are able to solubilize TAC. Due to the proximity in $\lambda_{max\ of}$ TAC and solvents, interference is observed in the estimation of the TAC by UV-Vis spectrophotometer. Hence, HPLC method was developed to quantify TAC in TAC loaded cubosomes containing microneedles and for *in-vitro* study. Due to the low dose of TAC, it is not possible to estimate its amount in plasma and organ by HPLC method. Hence, LC-MS was selected which is already reported in *in-vivo* study.(8) Materials and equipment used in analytical method development are mentioned in table 3.2.

Table 3.2: Materials & Equipment employed in analytical method development of TAC

Materials/Equiment	**Source/Manufacturer**
Acetonitrile (HPLC grade)	Rankem Fine Ltd.
Methyl Butyl tert-ether	Loba Chemi Pvt. Ltd
Ortho phosphoric acid (AR grade)	Spectrochem Labs Ltd
Filtered double distilled water	Prepared in the laboratory
Beakers, volumetric flasks, calibrated pipettes etc.	Borosil Glass wares, India
Micropipettes (10 µl, 200 µl and 1000 µl) and Microcentrifuge tubes	Tarson Pvt. Ltd., India
pH meter	Labindia Instruments Pvt. Ltd., Mumbai
HPLC	Agilent, Germany
LCMS	Sciex QTRAP® 4500, USA

3.4.1. HPLC Method for Tacrolimus

HPLC method was developed by slightly modifying the mobile phase composition given in the monograph of TAC in USP.(9)

3.4.1.1. *Instrument:* Agilent gradient HPLC (Agilent infinity 1220 system)

3.4.1.2. *Reagents/Solvents:* ACN HPLC grade, Phosphoric acid, methyl buthyl tert-ether, double distilled water

3.4.1.3. Chromatographic Conditions:

Table 3.3: Chromatographic condition for HPLC of TAC

Parameters	**Chromatographic conditions**
Mobile Phase	Solution T-A: 6 mM Phosphoric acid Solution T-B: ACN and tert-butyl methyl ether (81:19) Solution T-C: Solution A and solution B (4:1) Solution T-D: Solution A and solution B (1:4) Mobile Phase: Solution T-C:Solution T-D :: 50::50
HPLC column	C18 column, 4.6 µm, 150 mm (Thermo Scientific)
UV wavelength	220 nm
Injection volume	20 µl
Flow rate	1.5 ml/min
Run time	15 min
Temperature of column	60 °C

3.4.1.4. Mobile phase for estimation of TAC using HPLC

For the preparation of mobile phase for estimation of TAC using HPLC, 6mM phosphoric acid was prepared as described above (solution T-A). Then, solution T-B was prepared by mixing acetonitrile (ACN) and methyl butyl tert-ether in ratio of 81:19. Solution T-C and T-D were prepared by mixing solution T-A and T-B in ratio of 4:1 and 1:4 respectively. Finally, solution T-C and solution T-D in ratio of 50:50 were mixed for preparing mobile phase.(9)

3.4.1.5. Preparation of standard stock solution of Tacrolimus

10.0 mg of TAC, was taken volumetric flask having capacity of 10 ml and was dissolved in acetonitrile: water (70:30), and final volume was made up with ACN: water (70:30) (1 mg/ml).

3.4.1.6. Preparation of standard Tacrolimus solution

0.25 ml to 1.25 ml aliquots of TAC standard stock solutions were taken in calibrated volumetric flasks having capacity of 10 ml. Further they were diluted with ACN: water (70:30) up to the mark to achieve standard TAC solutions of 25, 50, 75, 100, and 125 µg/ml concentrations. These standard TAC solutions were further used to develop calibration plot for estimation of TAC in the formulation to find out entrapment and in various drug release/permeation study samples.

3.4.1.7. Preparation of calibration plot of Tacrolimus

Analytical method: Agilent infinity 1220 system (Agilent, Germany) with Thermo Scientific® C18 column, (Thermo Scientific, USA) was used for HPLC analysis of TAC as mentioned above. Resulting standard solutions of TAC were injected through Rheodyne® injectors with 20 µL sample loop. Combination of Solution T-C and Solution T-D in a ratio of 50:50 (as described in table 3.3) was employed as mobile phase at flow rate of 1.5 ml/minute at 60°C. A chromatograms of above prepared standard stock solutions were recorded at 220 nm detection wavelength for a run time of 15 minutes. The calibration curves between standard TAC concentrations (taken on X-axis) and their relevant peak area (taken on Y-axis) were generated. GraphPad Prism V6.01 (GraphPad Software) was used to obtain the equation of best fit straight line and correlation coefficient. This developed calibration plot of TAC was used to estimate TAC concentration during in-vitro release study and entrapment efficiency.

Bio-analytical method: Agilent 1220 system (Agilent, Germany) with Thermo Scientific® C18 column, (Thermo Scientific, USA) was used for HPLC analysis of TAC as mentioned above. From standard stock solution of TAC as prepared in section 3.4.1.5, different aliquots of 0.25, 0.5, 0.75, 0.1 and 1.25 ml were transferred to separate centrifuge tube (capacity 10 ml) and 4 ml of rat plasma was added in to it. Then, volume was made up to 10 ml using acetonitrile to carry out plasma protein precipitation. The final concentration ranges after adding plasma and acetonitrile was 25-125 µg/ml.

Then, these samples were vortex mixed for 5 min and after that, they were centrifuged at 5000 rpm for 15 min at 25 °C in order to separate precipitated plasma proteins from samples. Supernatant containing TAC were collected, filtered with 0.22 µ PVDF syringe filter and analyzed with HPLC using same mobile phase as described in section 3.4.1.4 at wavelength of 220 nm. All experiments were performed in triplicates. These standard TAC solutions were used to develop calibration plot for estimation of TAC in ex-vivo release study.

3.4.1.8. Validation of HPLC method of Tacrolimus

3.4.1.8.1. Linearity and range

Linearity can be defined as capability of any analytical method to get the test outcomes in accordance to the amount of analyte present in the sample, directly or after well-defined transformation. “Range” of an analytical procedure can be defined as the difference between upper and lower amount of analyte in the sample for which linearity has been shown with suitable level of precision and accuracy.(1, 2) To validate linearity and range of developed HPLC method, standard solutions of 25-125 µg/ml were prepared as described in section 3.4.1.5 and 3.4.1.6. GraphPad Prism V6.01 (GraphPad Software) was used to apply linear regression on standard calibration data and straight line equations as well as correlation coefficients (R2) were generated to validate linearity.

3.4.1.8.2. Robustness

Robustness of a system can be checked by studying the effect of minor changes in analytical methods on system suitability parameters and this can facilitate the performance the analytical method(14). The robustness was validated by observing the change in peak area of TAC standard solutions at flow rate of 1.3 and 1.7 ml/min, temperature of 58 °C and 62 °C and mobile phase composition ratio of solution T-C:solution T-D to 48:52 and 52:48.(1, 2)

3.4.1.8.3. Sensitivity

LOD and LOQ are frequently utilized for calculating even a minor concentration of an analyte that can be reliably measured by an analytical method. The LOD and LOQ of developed methods were determined from standard deviation of response and slope using Eq. 3.1 and Eq. 3.2.(1, 2)

$$LOD = 3\,X\,\frac{R}{S}$$

Equation-3.1

$$LOQ = 10\,X\,\frac{R}{S}$$

Equation-3.2

Where, S= Slope of the linearity curve

R= Standard deviation of line

3.4.1.8.4. Precision/ Repeatability

The consistency and reproducibility of an analytical method is represented by Precision and it also shows a similarity or proximity between different measurements done for the same sample. Multiple measurements for 50 µg/ml standard solutions were done on same day as well as on three consecutive days to determine intraday and interday precision, respectively. Precision can be expressed as percent RSD (relative standard deviation).(1, 2)

3.4.1.8.5. Accuracy

The closeness of agreement between the data collected by an analytical method to the accepted reference value is represented by accuracy. For the calculation of accuracy, standard addition method was employed to wherein known amounts of standard drug (80, 100 and 120 %) were added to the pre-analyzed samples and the peak area were measured. Accuracy was then assessed in terms of mean % recovery using Eq. 3.3.(1, 2)

$$\%\,Recovery = \left[\frac{(C_T - C_S)}{C_A}\right] X\,100$$

Equation-3.3

Where, C_T = total drug concentration measured after standard addition

C_S = drug concentration measured before standard addition

C_A = theoretical increase in drug concentration by standard addition

3.4.1.8.6. Specificity

Specificity can be defined as the ability to measure various components present in analyte viz., impurities, degradants, matrix, etc. which may be present.(1, 2) In the present study, the ability of the methods to accurately measure TAC in formulations was assessed via evaluation of interference by excipients (GMO, PVA, ethanol and lactose) using formulation prototype method. The formulation prototypes were prepared by spiking standard 50 µg/ml TAC solutions with other formulation components at their maximum fraction which were supposed to be present in analytical samples as given in Table 3.3.

Table 3.4: Formulation prototypes for determination of specificity of analytical methods

Microneedle patch containing TAC loaded Cubosomes	
Formulation Components	**Concentration (50 µg/ml)**
GMO in ethanol	1 ml
PVA in water	1 ml
Lactose	1 ml

The HPLC peak area of these formulation prototypes were compared with that of standard TAC solutions in respective solvents and excipients' mix which were prepared using similar portions of all the excipients and omitting the drug.

3.4.2. LC-MS Method for Tacrolimus

LC-MS for the estimation of a TAC in plasma sample was adopted from the reported method by S. Sadjadi.(3)

3.4.2.1. *Instrument:* Sciex QTRAP® 4500

3.4.2.2. *Reagents/Solvents:* Methanol HPLC grade, Formic acid, double distilled water

3.4.2.3. Chromatographic Conditions:

Table 3.5: Chromatographic condition for LC-MS of TAC

Parameters	**Chromatographic conditions**
Mobile Phase	Solution L-A: 0.1 % formic acid Solution L-B: Methanol Mobile Phase: Solution L-A:Solution L-B :: 20::80
HPLC column	C18 column, 2.1 µm, 50 mm (Thermo Scientific)
Injection volume	10 µl
Flow rate	0.3 ml/ min
Run time	1.5 min
Temperature of column	25 °C

3.4.2.4. Mobile phase for estimation of TAC using LCMS

For the preparation of mobile phase for estimation of TAC using LCMS, 0.1 % formic acid was prepared as described in section 3.2.2 (solution L-A). Then, solution L-A and methanol was mixed in ratio of 20:80 which was used as mobile phase in LCMS of TAC.(3)

3.4.2.5. Preparation of standard stock solution of Tacrolimus

10.0 mg of TAC, was taken in volumetric flask having capacity of 10 ml and methanol was added to dissolve it, and volume was made up with methanol (1 mg/ml) called solution A. 0.1 ml of solution A was withdrawn and shifted to volumetric flask (10 ml). Final volume was done using methanol to obtain 10 µg/ml (solution B). 0.1 ml of solution B was shifted in another volumetric flask having 10 ml capacity and final volume was made up to obtain 100 ng/ml standard stock solution (solution C).

3.4.2.6. Preparation of calibration plot of Tacrolimus

Sciex QTRAP® 4500 (Sciex, USA) with Thermo Scientific® C18 column, (Thermo Scientific, USA) was used for LC-MS analysis of TAC as mentioned above. Form solution C, different aliquots of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.00 ml were transferred to separate centrifuge tube with 10 ml capacity and 4 ml of rat plasma was added in to it. Then, volume was made upto 10 ml using

methanol to carry out plasma precipitation. The final concentration ranges after adding plasma and methanol was 5-30 ng/ml. Then, these samples were vortex mixed for 5 min and after that, they were centrifuged at 5000 rpm for 15 min at 25 °C in order to separate precipitated plasma proteins from samples. Supernatant containing TAC was collected, filtered with 0.22 µ syringe filter and analyzed with UPLC using same mobile phase as mentioned in Table 3.5 attached with mass detector. These standard TAC solutions were used to develop calibration plot for estimation of TAC in blood plasma.

3.4.2.7. Validation of LC-MS method of Tacrolimus

3.4.2.7.1. Linearity and range

Linearity can be defined as capability of any analytical method to get the test data in accordance to the amount of analyte present in the sample, directly or after well-defined transformation. “Range” of an analytical procedure can be defined as the difference between upper and lower amount of sample for which linearity has been shown with suitable level of precision and accuracy.(1, 2) To validate linearity and range of developed HPLC method, standard solutions of 5-30 ng/ml were prepared as described in section 3.4.2.6. GraphPad Prism V6.01 (GraphPad Software) was used to apply linear regression on standard calibration data and straight line equations as well as correlation coefficients (R2) were generated to validate linearity.

3.4.2.7.2. Robustness

Robustness of a system can be checked by studying the effect of minor changes in analytical methods on system suitability parameters and this can facilitate the performance the analytical method(14). The robustness was validated by observing the change in peak area of TAC standard solutions at flow rate of 0.2 and 0.4 ml/ min, temperature of 23 °C and 27 °C and mobile phase composition ratio of solution L-A:solution L-B to 18:82 and 22:78.(1, 2)

While other parameters like sensitivity, precision and accuracy were performed in a similar way as described in section 3.4.1.8.

3.5. Analytical Methods for Febuxostat

FBX dissolves easily in majority of organic solvents like acetonitrile, methanol, chloroform, acetone and many other solvents but it is practically insoluble in water.(10) FBX exhibits a λ_{max} of 315 nm in UV. UV visible spectrophotometric method was adopted to analyze drug concentration in formulation and during in-vitro release study.(4, 5) HPLC method was developed to estimate concentration of FBX during ex-vivo and cell permeability study. Moreover, it is also useful in animal study to find out blood plasma concentration of FBX. Materials and equipment used in analytical method development are listed in table 3.6.

Table 3.6: Materials and Equipment used in analytical method development of FBX

Materials/Equiment	**Source/Manufacturer**
Acetonitrile (HPLC grade)	Rankem Fine Ltd.
Methanol (HPLC grade)	Rankem Fine Ltd.
Formic Acid (AR grade)	Spectrochem Labs Ltd
Filtered double distilled water	Prepared in the laboratory
Beakers, volumetric flasks, calibrated pipettes etc.	Borosil Glass wares, India
Micropipettes (10 µl, 200 µl and 1000 µl) and Microcentrifuge tubes	Tarson Pvt. Ltd., India
pH meter	Labindia Instruments Pvt. Ltd., Mumbai
HPLC	Agilent, Germany
UV visible spectrophotometer (UV-1800)	Shimadzu, Japan

3.5.1. HPLC Method for Febuxostat

3.5.1.1. **Instrument:** Agilent gradient HPLC

3.5.1.2. **Reagents/Solvents:** ACN (Acetonitrile) HPLC grade, Formic acid, double distilled water

3.5.1.3. Chromatographic Conditions:

Table 3.7: Chromatographic condition for HPLC of FBX

Parameters	**Chromatographic conditions**
Mobile Phase	Solution F-A: Double distilled water pH adjusted to 2.2 using formic acid Solvent F-B: ACN Mobile Phase: Solution F-A:Solvent F-B :: 20:80
HPLC column	C18 column, 4.6 µm, 250 mm (Thermo Scientific)
UV wavelength	315 nm
Injection volume	20 µl
Flow rate	1 ml/ min
Run time	10 min
Temperature	45 °C

3.5.1.4. Mobile phase for estimation of FBX using HPLC

For the preparation of mobile phase for estimation of FBX using HPLC; two solvents were used. First; double distilled water having a pH adjusted to 2.2 using formic acid (solution F-A) and second was ACN (solvent F-B). Then, Solvent F-A and F-B were taken and mixed in a ratio of 20:80 to obtain mobile phase HPLC method.

3.5.1.5. Preparation of standard stock solution of Febuxostat

10.0 mg of FBX was taken in volumetric flask with 10 ml capacity and solubilized in acetonitrile, then further acetonitrile was added to make up the volume and a solution with 1 mg/ml concentration was obtained. This solution was further diluted by taking 0.1 ml solution in volumetric flask (10 ml capacity) and solution having 100 µg/ml amount of FBX was achieved. (Dilution was made with ACN).

3.5.1.6. Preparation of standard Febuxostat solution

FBX standard solutions were prepared by two developed methods.

Analytical Method: 0.2 to 1 ml aliquots of FBX standard stock solutions were shifted into individual calibrated volumetric flasks with 10 ml capacity and diluted using acetonitrile till the mark on the flask, for achieving standard

FBX solutions of 0.2, 0.4, 0.6, 0.8, and 1.0 µg/ml concentrations. These standard FBX solutions were used to develop calibration plot for estimation of FBX in optimized cubosomes.

Bio analytical method: Aliquots having 0.2, 0.4, 0.6, 0.8 and 1.0 ml of standard FBX solution as prepared in section 3.5.1.5 were taken into individual 10 ml centrifuge tubes and further 4 ml of plasma was poured into the tube. Then, volume of the centrifuge tube was made up to 10 ml using ACN to carry out plasma precipitation. The final concentration range after the addition of plasma and ACN was 0.2-1.0 µg/ml. Then, these samples were vortex mixed for 5 min and after that, the samples was exposed to centrifugation wherein the chosen parameters were 5000 rpm for 15 min at 4 °C to isolate precipitated plasma proteins from samples. Supernatant containing FBX was collected, filtered with 0.22µ PVDF syringe filter and analyzed using HPLC using same mobile phase as described in section 3.5.1.4 at wavelength of 315 nm. These standard FBX solutions were used to develop calibration plot for estimation of FBX in ex-vivo release study, and plasma blood concentration.

3.5.1.7. Preparation of calibration plot for Febuxostat

Agilent 1220 system (Agilent, Germany) with Thermo Scientific C18 column, (Thermo Scientific, USA) was used for HPLC analysis of FBX as mentioned above. The resulting standard solutions of FBX were injected through Rheodyne® injectors with 20 µL sample loop. A mixture of double distilled water of pH 2.2 and ACN in a ratio of 20:80 (as described in section 3.5.1.4.) was prepared. Above prepared solution was employed as a mobile phase wherein flow rate was set at 1.0 ml/minute at a temperature of 50°C. The chromatograms were recorded at 315 nm detection wavelength for a run time of 10 minutes. The calibration plot between standard FBX concentrations (taken on X-axis) and their relevant peak area (taken on Y-axis) were generated. GraphPad Prism V6.01 (GraphPad Software) was used to obtain the equation of best fit straight line and correlation coefficient.

3.5.1.8. Validation of HPLC method of Febuxostat

3.5.1.8.1. Linearity and range

Linearity can be termed as capability of any analytical method to get the test data in accordance to the amount of analyte present in the sample, directly or after well-defined transformation. “Range” of an analytical procedure can be defined as the difference between upper and lower amount of sample for which linearity has been shown with suitable level of precision and accuracy.(1, 2) To validate linearity and range of developed HPLC method, standard solutions of 0.2-1.0 µg/ml were prepared as described in section 3.5.1.6. GraphPad Prism V6.01 (GraphPad Software) was used to apply linear regression on standard calibration data and straight line equations as well as correlation coefficients (R2) were generated to validate linearity.

3.5.1.8.2. Robustness

Robustness of a system can be checked by studying the effect of minor changes in analytical methods on system suitability parameters and this can facilitate the performance the analytical method(14). The robustness was validated by observing the change in peak area of TAC standard solutions at flow rate of 0.8 and 1.2 ml/min, temperature of 43 °C and 47 °C and mobile phase composition ratio of solution F-A:solution F-B to 18:82 and 22:78.(1, 2)

While other parameters like sensitivity, precision and accuracy were performed in a similar way as described in section 3.4.1.8.

3.5.2. UV Visible Spectrophotometric Method(4, 5)

Measurement of FBX entrapped within cubosomes and microneedle patch containing FBX loaded cubosomes, UV spectrophotometric methods in ACN:methanol (ACN:MOH) in the concentration of 9:1 v/v ratio and phosphate buffer pH 7.4 were developed.

3.5.2.1. Preparation of standard stock solution of Febuxostat in ACN:Methanol

Standard stock solution comprising of 100 µg/ml FBX was made in ACN:MOH as mentioned in section 3.5.1.4. Accurately weighed 10 mg of FBX was taken into separate 10 ml calibrated volumetric flasks and solubilized in ACN:MOH in 9:1 v/v ratio. Standard solution of FBX having a concentration of 1000 µg/ ml was achieved by adding the same solvents to

above solution up to 10 ml. 1 ml from the above solution was shifted in individual calibrated volumetric flask with 10 ml capacity, further final volume was made up using ACN:MOH-9:1 to obtain standard stock solution having the concentration of 100 µg/ml.

3.5.2.2. Preparation of standard stock solution of Febuxostat in Phosphate buffer pH 7.4

Stock solution comprising of 100 µg/ ml of FBX was prepared in phosphate buffer pH 7.4 as mentioned in section 3.5.1.4. 10 mg of precisely weighed FBX was shifted in individual calibrated volumetric flask with 10 ml capacity. Then, FBX was solubilized in minimum volume of Methanol. Further, final volume was made up with phosphate buffer pH 7.4 to achieve concentration of 1000 µg/ml of standard FBX solution. Then, 1 ml of above solution was further taken in calibrated volumetric flask with 10 ml capacity and volume was made up to get standard stock solution of 100 µg/ ml.

3.5.2.3. Preparation of standard solutions of Febuxostat

In ACN:Water (9:1): Standard solution of FBX in ACN:Water was prepared using 100 µg/ml standard stock solution as prepared in section 3.5.2.1. Accurately 0.2, 0.4, 0.6, 0.8 and 1.0 ml aliquots of FBX stock solutions were taken in individual calibrated volumetric flasks (10 ml) and the volume was made up with ACN:Water to obtain standard FBX solutions having conc. in range of 2 to 10 µg/ml. These solutions were used to prepare a calibration plot of FBX in ACN:Water, which was useful to find out the entrapment efficiency of FBX in cubosomes.(4, 5)

In Phosphate buffer pH 7.4: Standard solution of FBX in Phosphate buffer pH 7.4 was prepared using 100 µg/ml standard stock solution as prepared in section 3.5.2.2. Accurately 0.2, 0.4, 0.6, 0.8 and 1.0 ml aliquots of FBX stock solutions were taken in individual calibrated volumetric flasks (10 ml) and the volume was made up with phosphate buffer pH 7.4 to obtain standard FBX solutions having concentration in range of 2 to 10 µg/ml. These solutions were used to prepare a calibration plot of FBX in phosphate buffer pH for quantification of FBX during in-vitro release..(4, 5)

3.5.2.4. Determination of analytical wavelengths

The spectrum of 10 µg/ml standard FBX solutions in both solvents were obtained by scanning the solutions over 200-400 nm wavelength (λ) using UV Visible spectrophotometer against respective solvents as blank. A wavelengths with maximum absorbance (λmax) were selected as analytical wavelengths for photometric measurements in respective solvents during preparation of calibration plots as well as during drug quantification in analytical samples.(4)

3.5.2.5. Preparation of calibration plots

UV visible spectrophotometer was used to record absorbance of standard FBX solutions (as prepared section 3.5.2.3.) at wavelength of 315 nm. Calibration plots between standard FBX concentrations (taken on X-axis) and their corresponding absorbance (taken on Y-axis) were generated.

3.5.2.6. Validation of UV spectrophotometric methods for Febuxostat

3.5.2.6.1. Linearity and range

Linearity can be termed as the capability of any analytical method to get the test data in accordance to the amount of analyte present in the sample, directly or after well-defined transformation. “Range” of an analytical procedure can be defined as difference between upper and lower amount of sample for which linearity has been shown with suitable level of precision and accuracy.(1, 2) To validate linearity and range of developed HPLC method, standard solutions of 0.2-1.0 µg/ml were prepared as described in section 3.5.2.3. GraphPad Prism V6.01 (GraphPad Software) was used to apply linear regression on standard calibration data and straight line equations as well as correlation coefficients (R2) were generated to validate linearity.

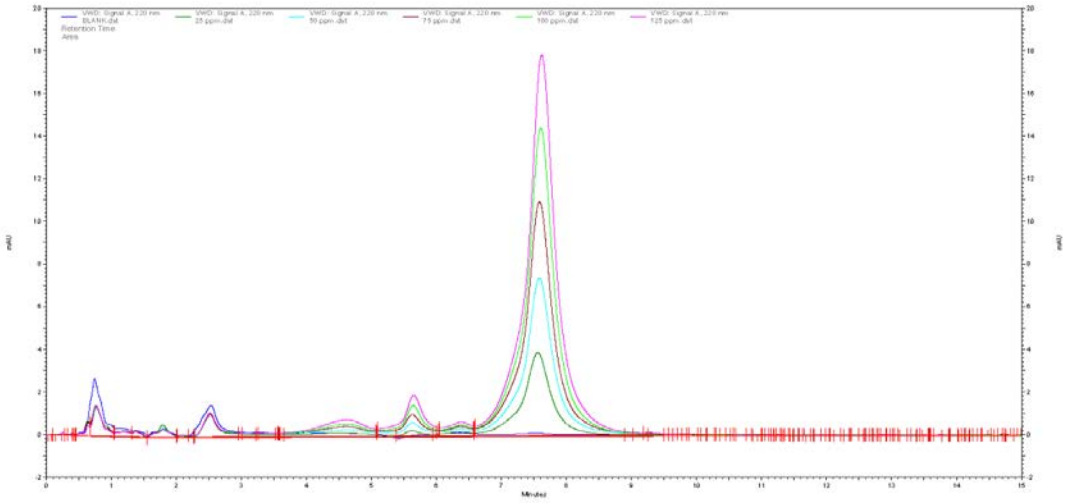
While other parameters like sensitivity, precision and accuracy were performed in a similar way as described in section 3.4.1.8.

3.6. RESULTS AND DISCUSSION

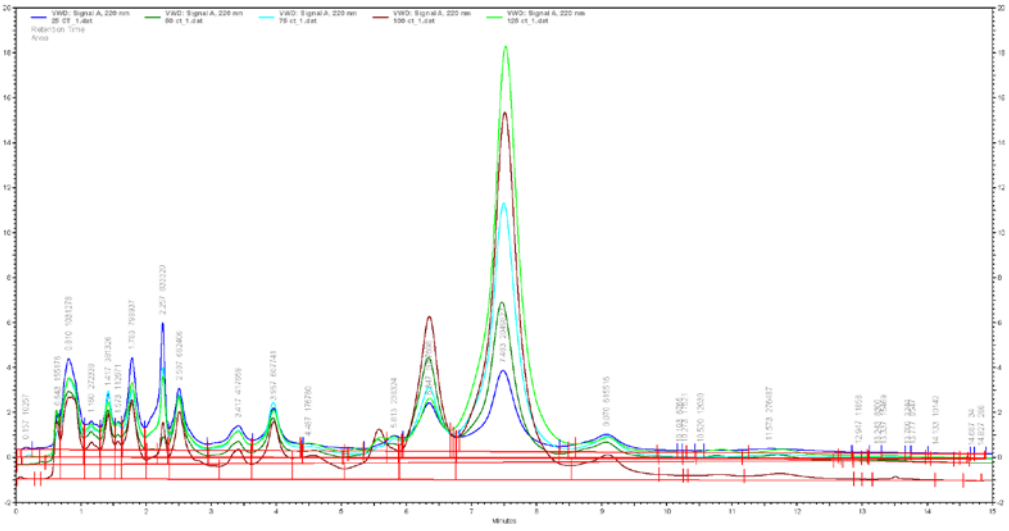
Obtained results and observations are given below.

3.6.1. Estimation of Tacrolimus using HPLC

Typical overly chromatograms obtained from RP-HPLC analysis using C18 column is shown in Fig 3.1. Sharp, symmetric peaks were observed with average retention time of 7.58 min at 220 nm detection wavelength and 1.5 ml/min flow rate.



(A)



(B)

Figure 3.1: Typical HPLC overlay plot of TAC (25-125 µg/ml) (A) For analytical method and (B) For bioanalytical method

The peak area values corresponding to selected concentration range of TAC for both the methods are given in Table 3.8 and calibration plots for the same are illustrated in Fig. 3.2 & 3.3.

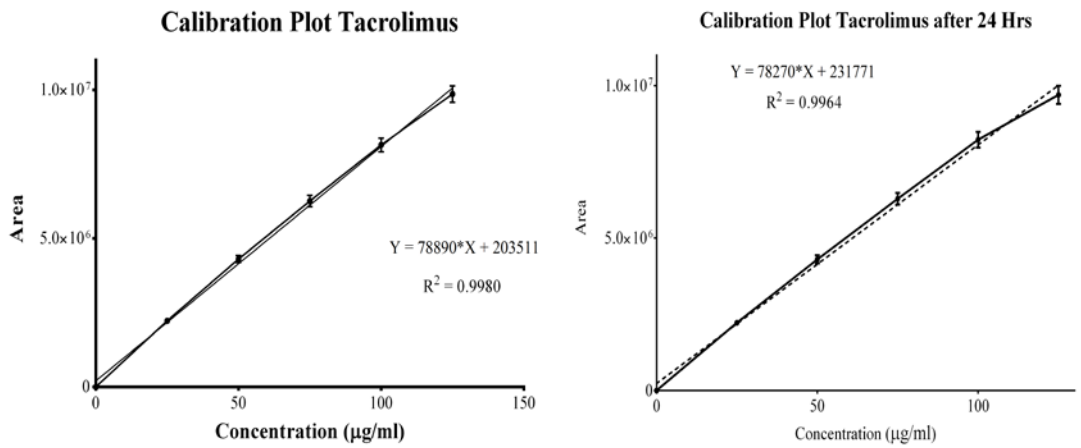
3.6.1.1. Validation of HPLC method of Tacrolimus

Regression analysis of calibration data showed a positive correlation between concentration of TAC and peak area values with a good linearity (R^2 =9980). obtained result proved that selected TAC concentration range of 25 to 125 µg/ml and obeys the Beer law.

Table 3.8: Mean area of TAC obtained using HPLC

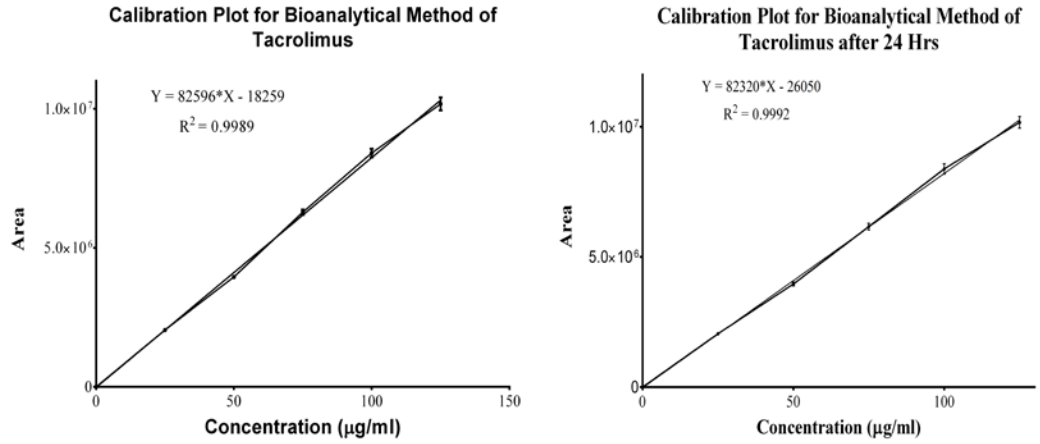
Sr. No.	Concentration (µg/ml)	Mean Peak Area	
		Initial	After 24 hrs
Analytical Method			
1	0	0	0
2	25	2227123 ± 82550.08	2226894 ± 82960.3
3	50	4306748 ± 120200.4	4310371 ± 118310.7
4	75	6260048 ± 188653.5	6259370 ± 180894.4
5	100	8149058 ± 230059.9	8151973 ± 238907.2
6	125	9862008 ± 275121.3	9863630 ± 289837.3
Bioanalytical Method			
1	0	0	0
2	25	2046508 ± 55911.19	2047698 ± 60056.48
3	50	3955676 ± 66509.27	3957996 ± 69357.62
4	75	6280334 ± 96620.93	6161818 ± 127142.2
5	100	8410201 ± 154712.7	8380775 ± 195204.5
6	125	10171091 ± 232813.9	10165374 ± 226091.4

Mean ± SD (n=3)



(A) (B)

Figure 3.2: Calibration plot of TAC for analytical method (A) at 0 Hr and (B) after 24 Hr using HPLC analytical method



(A) (B)

Figure 3.3: Calibration plot of TAC for bioanalyical method (A) at 0 Hr and (B) after 24 Hr using HPLC analytical method

No significant change was observed in the peak area values (Table 3.8, Fig. 3.2 & 3.3) when the same standard TAC solutions were measured after storage for 24 hrs at room temperature which indicated stability of standard TAC solutions over a period of time.

Table 3.9 captures the LOD and LOQ values of HPLC methods for TAC estimation in analytical samples. The LOD values were found well below the concentration range selected for calibration indicating the sensitivity of methods for accurate detection of TAC present in standard solutions.

Table 3.9: Sensitivity evaluation of HPLC method of TAC

Method	Slope of line	SD of line	LOD (µg/ml)	LOQ (µg/ml)
Analytical	76448.33	129689.11	5.60	16.96
Bioanalytical	82814.67	74585.85	2.97	9.01

Intraday and interday precision data under the same operating conditions are summarized in Table 3.10. The results were found to be precise over the selected time interval as the % RSD values obtained for the HPLC methods were within the acceptable range (< 2%).

Table 3.10: Intraday and interday precision analysis of HPLC methods of TAC

Conc. prepared (µg/ml)	Intraday precision				Interday precision			
	Observed conc. (µg/ml)	Mean Area	SD	% RSD	Observed conc. (µg/ml)	Mean Area	SD	% RSD
Analytical method								
25	26.07	2260456.3	38787.22	1.72	25.36	2216907	13448.62	0.61
75	75.93	6193381.7	105423.12	1.70	75.58	6280388.3	92645.31	1.48
125	124.67	9805341.3	188170.7	1.92	125.54	1005770 5	185140.51	1.84
Bioanalytical method								
25	25.40	2079841.7	26645.11	1.28	24.87	2081031	28553.17	1.37
75	75.69	6280333	96620.9	1.5	75.01	6148484	116475.	1.8

		.7	3	4		.7	85	9
125	123.43	1018109 1	196903. 39	1.9 3	124.61	1006537 4	154092. 2	1.5 3

The mean % recovery and % RSD values for low, medium and high concentration are summarized in Table 3.11. The developed HPLC method exhibits high accuracy as the obtained mean % recovery values are near to 100% with low %RSD (% RSD < 2 %).

Table 3.11: Accuracy evaluation of HPLC methods of TAC by standard addition technique

Drug spiked (%)	Initial concentration (µg/ml)	Recovery			
		µg/ml	% Recovery	Mean % Recovery	% RSD
Analytical Method					
80	20	35.83	99.52	100.47	1.98
		36.99	102.74		
		35.69	99.14		
100	20	40.94	102.35	100.11	2.00
		39.40	98.49		
		39.79	99.47		
120	20	43.89	99.76	100.83	1.07
		44.83	101.90		
		44.37	100.84		
Bioanalytical Method					
80	20	35.94	99.83	100.47	1.92
		35.62	98.95		
		36.95	102.64		
100	20	39.41	98.53	99.58	0.97
		40.17	100.43		
		39.92	99.80		

120	20	43.46	98.76	99.10	1.05
		44.12	100.27		
		43.24	98.28		

3.6.2. Estimation of Tacrolimus using LC-MS

Typical chromatograms obtained from LC-MS analysis using C18 column is shown in Fig. 3.4. Sharp, symmetric peaks were observed with average retention time of 0.7 min using mass detector and 0.3 ml/min flow rate.

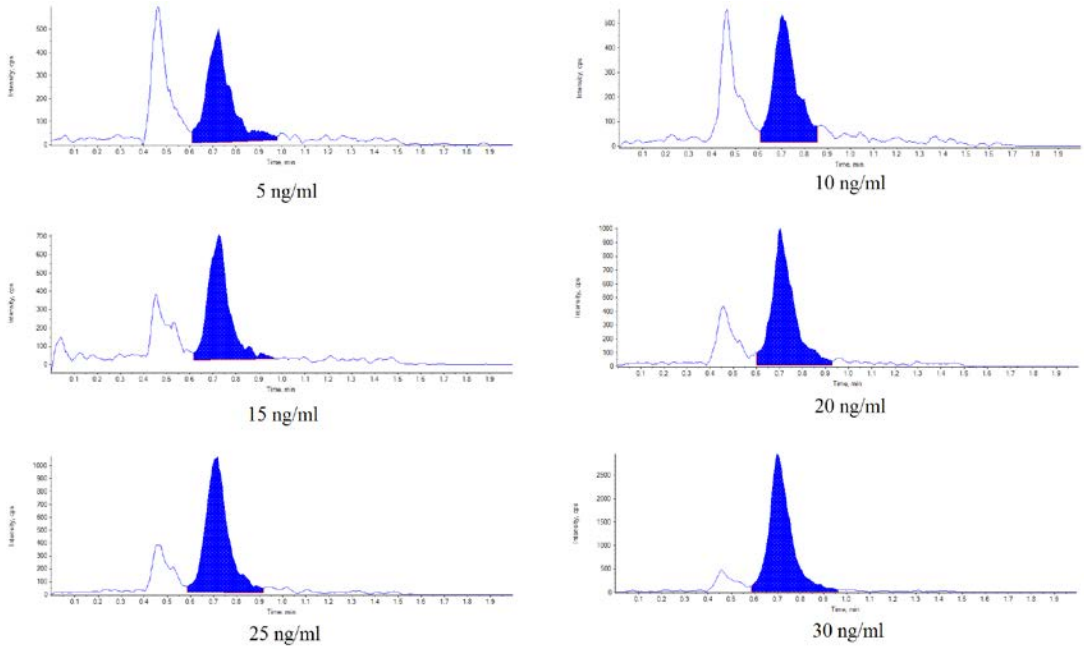


Figure 3.4: Typical LC-MS plot of TAC (2-30 ng/ml)

The peak area values corresponding to selected concentration range of TAC for both the methods are given in Table 3.12 and calibration plots for the same are illustrated in Fig. 3.5.

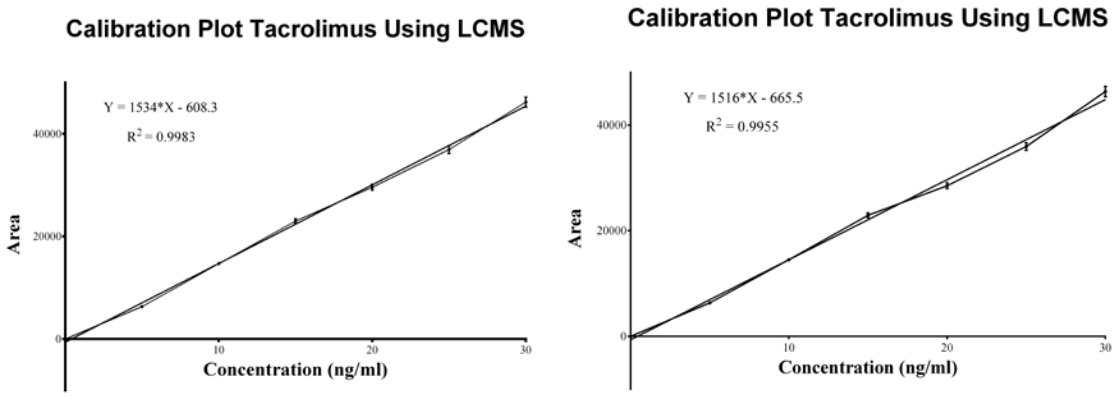
3.6.2.1. Validation of LCMS method of Tacrolimus

Regression analysis of calibration data showed a positive correlation between concentration of TAC and peak area values with a good linearity (R^2 =9980). Obtained result proved that selected TAC concentration range of 5 to 30 ng/ml and obeys the Beer law.

Table 3.12: Mean area of TAC obtained using LCMS

Sr. No.	Concentration (ng/ml)	Mean Area	
		Initial	After 24 hrs
1	0	0	0
2	5	6333.33 ± 110.15	6330 ± 130
3	10	14700 ± 264.58	14500 ± 200
4	15	23000 ± 458.26	22900 ± 435.89
5	20	29600 ± 556.78	28500 ± 529.15
6	25	36933.33 ± 757.19	35933.33 ± 737.15
7	30	46200 ± 964.37	46333.33 ± 971.25

Mean ± SD (n=3)



(A) (B)

Figure 3.5: Calibration plot of TAC (A) at 0 Hr and (B) after 24 Hr using LCMS analytical method

No significant change was observed in the peak area values (Table 3.12, Fig. 3.5) when the same standard TAC solutions were measured after storage for 24 hrs at room temperature which indicated stability of standard TAC solutions over a period of time.

Table 3.13 captures the LOD and LOQ values of LCMS methods for TAC estimation in analytical samples. The LOD values were found well below the concentration range selected for calibration indicating the sensitivity of methods for accurate detection of TAC present in standard solutions.

Table 3.13: Sensitivity evaluation of LCMS method of TAC

Method	Slope of line	SD of line	LOD (ng/ml)	LOQ (ng/ml)
1	1557.90	465.26	0.99	2.99

Intraday and interday precision data under the same operating conditions are summarized in Table 3.14. The results were found to be precise over the selected time interval as the % RSD values obtained for the LCMS methods were within the acceptable range (< 2%).

Table 3.14: Intraday and interday precision analysis of LCMS methods of TAC

Concentration prepared (ng/ml)	Concentration observed							
	Intraday precision				Interday precision			
	Observed conc. (ng/ml)	Mean Area	SD	% RSD	Observed conc. (ng/ml)	Mean Area	SD	% RSD
5	4.81	6333.33	110.15	1.74	4.88	6293.33	45.09	0.72
15	15.19	23000	458.26	1.99	15.54	23100	300	1.30
30	30.30	46200	964.37	2.09	30.23	45900	916.53	2.00

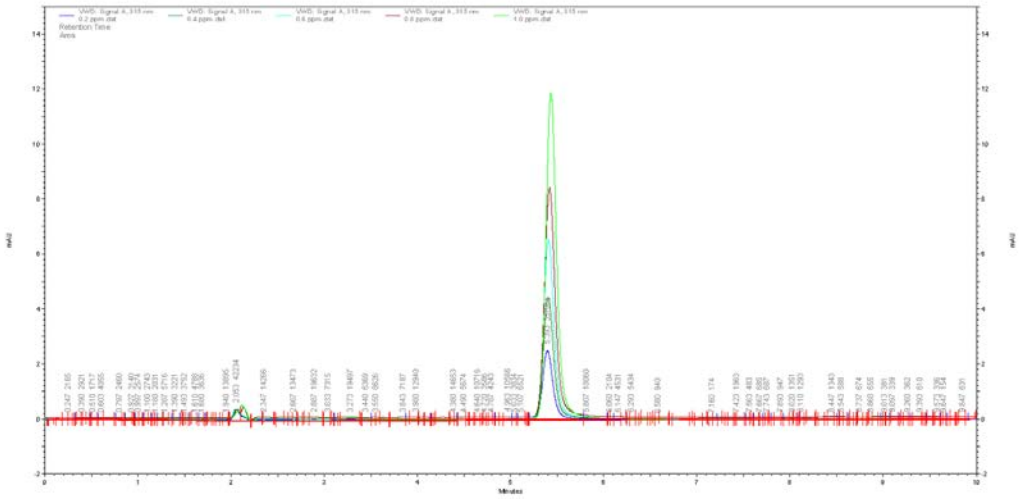
The mean % recovery and % RSD values for low, medium and high concentration are summarized in Table 3.15. The developed LCMS method exhibits high accuracy as the obtained mean % recovery value are near to 100% with low RSD (% RSD < 2 %).

Table 3.15: Accuracy evaluation of LCMS methods of TAC by standard addition technique

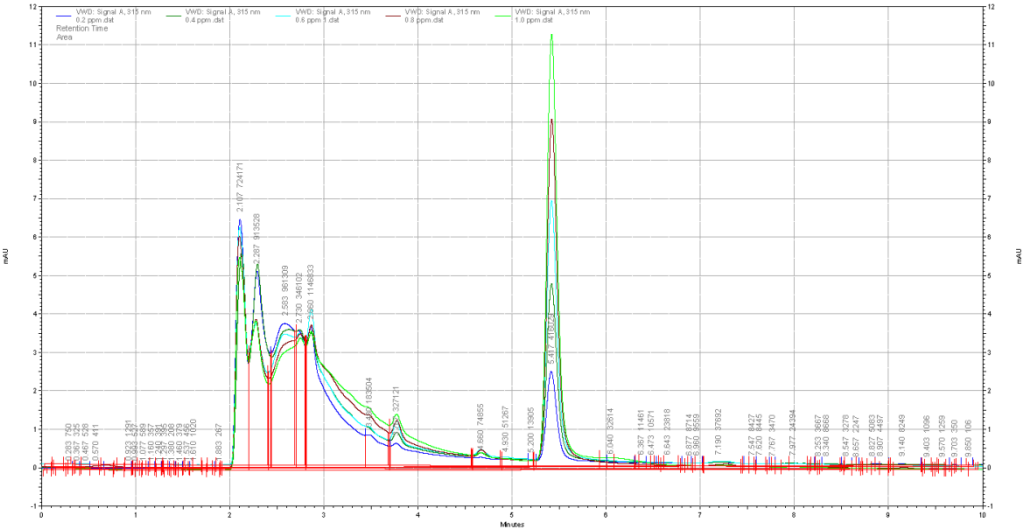
Drug spiked (%)	Initial concentration (ng/ml)	Recovery			
		ng/ml	% Recovery	Mean % Recovery	% RSD
80	2	3.62	100.66	99.35	1.35
		3.53	97.99		
		3.58	99.41		
100	2	3.96	98.94	97.44	1.37
		3.88	97.01		
		3.85	96.37		
120	2	4.33	98.41	98.55	1.26
		4.39	99.87		
		4.28	97.39		

3.6.3. Estimation of Febuxostat using HPLC

Typical chromatograms obtained from RP-HPLC analysis using C18 column is given in Fig. 3.6. Sharp, symmetric peaks were observed with average retention time of 5.39 min at 315 nm detection wavelength and 1 ml/min flow rate in both methods.



(A)



(B)

Figure 3.6: Overly plot of FBX using HPLC (0.2-1.0 µg/ml) (A) for analytical method (B) for bioanalytical method

The peak area values corresponding to selected concentration range of FBX for both the methods are given in Table 3.16 and calibration plots for the same are illustrated in Fig. 3.7.

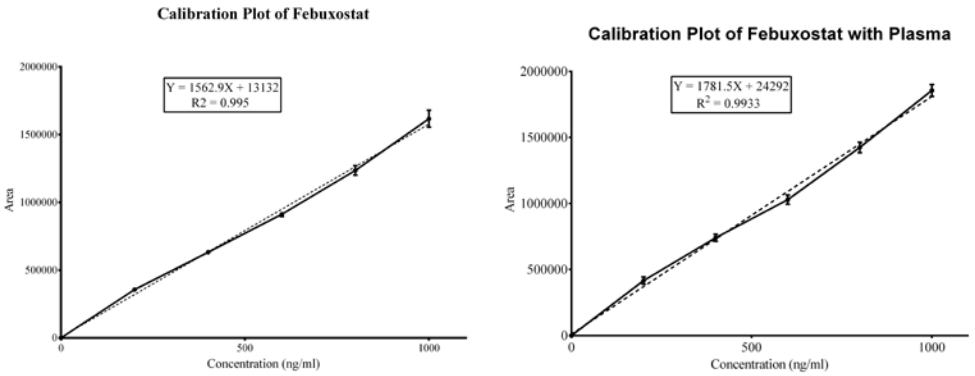
3.6.3.1. Validation of HPLC method of Febuxostat

A regression analysis of calibration data showed positive correlation between concentration of FBX and peak area values with a good linearity

(R^2=1). The result reflected that Beer's law was followed for selected FBX concentration in range of 0.2 to 1.0 µg/ml by analytical method and same range also selected for bioanalytical method.

Table 3.16: Peak area data of FBX at 0 and 24 h for calibration and stability

Concentration (ng/ml)	Peak area			
	Analytical Method		Bioanalytical Method	
	Initial	After 24 Hr	Initial	After 24 Hr
200	356459 ± 5539.35	357920 ± 6183.17	416485 ± 26896.9	417094 ± 27349.04
400	632290 ± 11226.66	640927 ± 10348.09	740336 ± 26360.67	743917 ± 25863.88
600	912120 ± 16653.39	913317 ± 15168.67	1028746 ± 35159	1029180 ± 361177.6
800	1236003 ± 34915.82	1234973 ± 351089.13	1424502 ± 39531.83	1501992 ± 40017
1000	1617510 ± 62104.93	1618376 ± 63760.74	1855908 ± 44744.34	1860781 ± 480118.37



(A) **(B)**

Figure 3.7: Calibration plot for FBX using HPLC (A) Analytical method (B) Bioanalytical method

No significant change was observed in the peak area values (Table 3.16, Fig. 3.7) when the same standard FBX solutions were measured after storing

them for 24 hours at room temperature which indicated that FBX standard solutions are stable over the period of analysis.

Table 3.17 captures the LOQ and LOD values of HPLC methods for FBX estimation in analytical samples. A LOD values were found well below the concentration range selected for calibration indicating the sensitivity of methods for accurate detection of FBX present in standard solutions.

Table 3.17: Sensitivity evaluation of developed HPLC method of FBX

Method	Slope of line	SD of line	LOD (ng/ml)	LOQ (ng/ml)
Analytical	1562.90	14956.10	31.58	95.69
Bioanalytical	1781.50	43350.33	33.60	101.83

Intraday and interday precision data under the same operating conditions are summarized in Table 3.18. The results were found to be precise over the selected time interval as the % RSD values obtained for the HPLC methods were within the acceptable range (< 2%).

Table 3.18: Intraday and interday precision analysis of HPLC methods of FBX

Conc. prepared (ng/ml)	Intraday precision				Peak Area observed Interday precision			
	Conc. observed (ng/ml)	Mean Area	SD	% RSD	Conc. observed (ng/ml)	Mean Area	SD	% RSD
Analytical Method								
200	213.27	346459.33	5539.35	1.60	207.71	357072	5256.16	1.47
600	588.00	912120.67	16653.39	1.83	587.49	914658.3	11860.89	1.30
1000	1005.21	1664177	29237.1	1.76	1021.13	1699059.	13406.2	0.79

			2			3	2	
Bioanalytical Method								
200	210.57	411088.7	5467.70	1.33	204.23	416462.7	3103.03	0.75
600	591.28	1024322	8476.42	0.83	593.12	1030938	11365.07	1.10
1000	1012.71	1188435	13792.87	1.16	1006.39	1190507	12600.91	1.06

Mean % recovery and % RSD values for low, medium and high concentration are summarized in Table 3.19. Developed HPLC method was highly accurate as the mean % recovery values, were found to be near to 100% with low RSD (% RSD < 2 %).

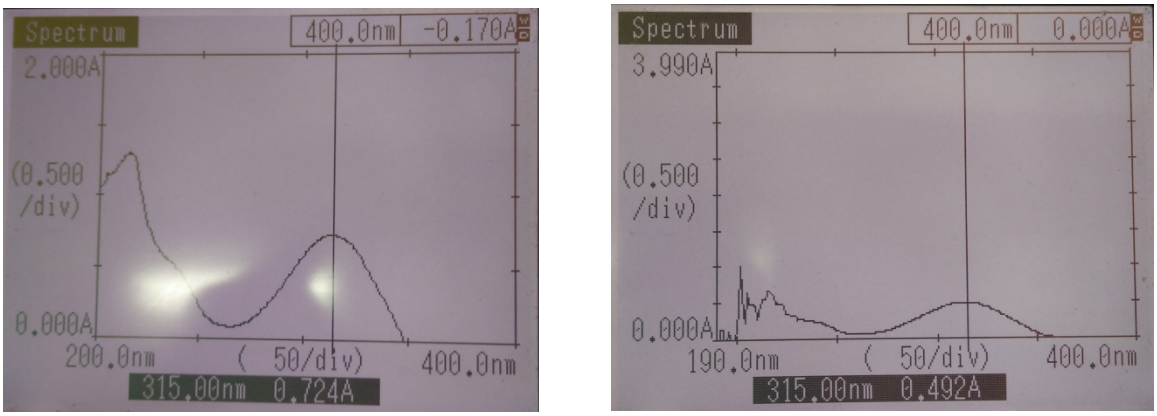
Table 3.19: Accuracy evaluation of HPLC methods of FBX by standard addition technique

Drug spiked (%)	Initial concentration (ng/ml)	Recovery			
		ng/ml	% Recovery	Mean % Recovery	% RSD
Analytical Method					
80	200	365.83	101.62	100.26	1.19
		359.16	99.77		
		357.82	99.39		
100	200	399.72	99.93	100.09	1.37
		406.14	101.53		
		395.20	98.80		
120	200	427.19	97.09	98.10	1.14
		430.86	97.92		
		436.93	99.30		
Bioanalytical Method					
80	200	355.26	98.68	99.88	1.74
		356.71	99.08		
		366.76	101.88		

100	200	397.90	99.48	99.61	1.21
		403.53	100.88		
		393.94	98.48		
120	200	444.45	101.01	99.78	1.07
		436.45	99.19		
		436.16	99.13		

3.6.4. Estimation of Febuxostat using UV spectrophotometric method

The UV spectrum scans of 10 µg/ml standard solutions of FBX in ACN:MOH (9:1) and phosphate buffer pH 7.4 is shown in Fig. 3.8. From the spectrum it can be concluded that in both solvents, FBX has a peak absorbance at a wavelength of 315 nm, so this wavelength is selected as the analytical wavelength for quantitative and qualitative purpose. The calibration data and calibration curves of FBX in selected solvents are given in Table 3.20 and Fig. 3.9, respectively.

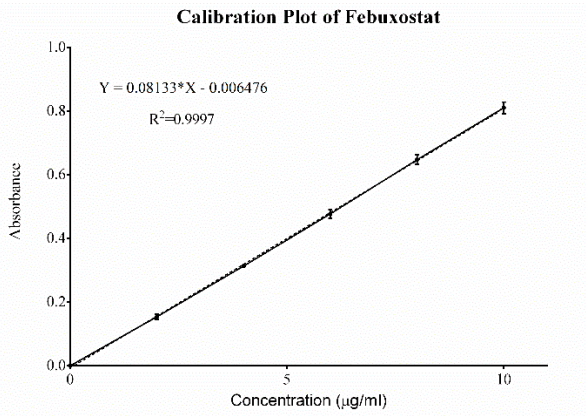


(A) (B)

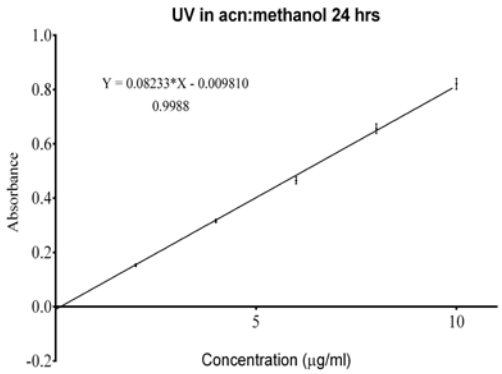
Figure 3.8: UV absorption spectra of FBX in (A) ACN:MOH (9:1) (B) Phosphate buffer pH 7.4

Table 3.20: Absorbance data of FBX in ACN:MOH (9:1) and Phosphate buffer pH 7.4 at 0 and 24 Hrs for calibration and stability

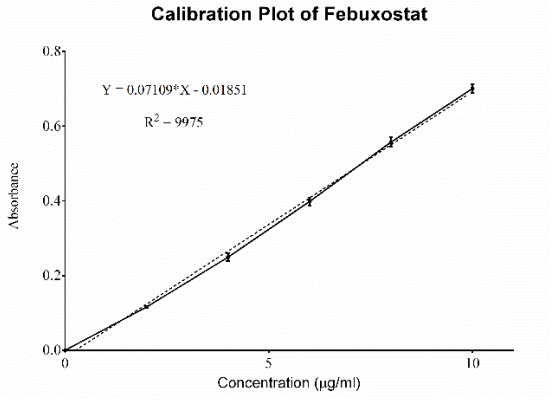
Sr No.	Concentration (µg/ml)	Absorbance	
		In: ACN:MOH (9:1)	In: Phosphate buffer pH 7.4
After 0 Hr (initial)			
1	0	0 ± 0.000	0 ± 0.000
2	2	0.154 ± 0.008	0.116 ± 0.002
3	4	0.313 ± 0.004	0.249 ± 0.004
4	6	0.477 ± 0.037	0.398 ± 0.008
5	8	0.647 ± 0.015	0.557 ± 0.005
6	10	0.810 ± 0.028	0.700 ± 0.002
After 24 Hrs			
1	0	0 ± 0.000	0 ± 0.000
2	2	0.153 ± 005	0.113 ± 0.004
3	4	0.316 ± 0.006	0.246 ± 0.012
4	6	0.465 ± 0.012	0.4003 ± 0.013
5	8	0.656 ± 0.018	0.553 ± 0.016
6	10	0.821 ± 0.020	0.712 ± 0.019

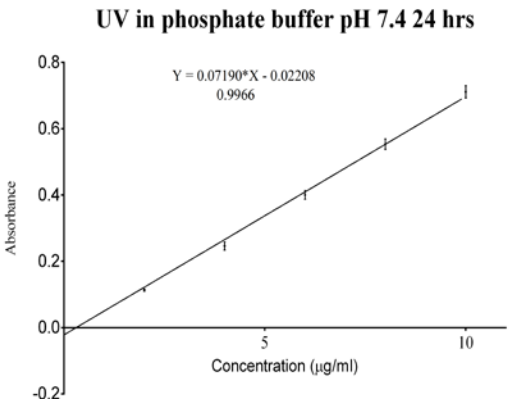


(A)



(B)





(C) (D)

Figure 3.9: Calibration curves of FBX in (A) ACN:MOH, (B) ACN:MOH after 24 hrs, (C) phosphate buffer pH 7.4 and (B) Phosphate buffer pH 7.4 after 24 hr

3.6.4.1. Validation of UV spectrophotometric methods for Febuxostat

The regression analysis showed a positive correlation between concentration of FBX and absorbance values at the respective λ_{max} with a good linearity ($R^2 \geq 0.997$, in both solvents). These results reflected that Beer's law was followed for selected FBX concentration ranges of 0 to 10 µg/ml in both solvents. Measurement of same standard solutions after storing the samples for 24 hours at room temperature didn't show any major variation in the absorbance values (Table 3.20, Fig. 3.9) indicating the stability of FBX in both solvents over the period of analysis.

Table 3.21 captures the LOD and LOQ values of UV spectrophotometric methods for FBX in both solvents. The LOD and LOQ values were found well below the concentration range selected for calibration indicating the sensitivity of methods for accurate quantification of drug present in their standard solutions.

Table 3.21: Sensitivity evaluation of UV methods of FBX in ACN:MOH (9:1) and Phosphate buffer pH 7.4

Solvents	Slope of line	SD of line	LOD (µg/ml)	LOQ (µg/ml)

ACN:MOH (9:1)	0.0823	0.013	0.524	1.587
Phosphate buffer pH 7.4	0.0748	0.009	0.415	1.257

Intraday and interday precision data under the same operating conditions are summarized in Table 3.22. Results were found to be precise over the selected time interval as the % RSD values obtained for these UV spectrophotometric methods were within the acceptable range (< 2%) (15).

Table 3.22: Intraday and interday precision analysis of UV spectrophotometric methods of FBX

Solvents	Conc. prepared (µg/ml)	Intraday precision				Interday precision			
		Observed conc. (µg/ml)	Abs.	SD	%RSD	Observed conc. (µg/ml)	Abs.	SD	%RSD
ACN:MOH	2	2.00	0.1562	0.003	1.76	1.99	0.154	0.003	1.79
	6	5.95	0.476	0.003	0.65	5.88	0.4746	0.004	0.80
	10	10.07	0.8089	0.004	0.45	10.1	0.8149	0.004	0.33
Phosphate buffer pH 7.4	2	1.93	0.116	0.002	1.42	1.94	0.112	0.002	1.99
	6		0.399	0.008	2.04	5.92	0.394	0.007	1.75
	10		0.7004	0.002	0.32	10.03	0.6948	0.009	1.26

Mean % recovery and % RSD values for lower, intermediate and higher concentration are summarized in Table 3.23 for both solvents. The developed spectrophotometric methods were highly accurate given to the mean % recovery

values which were near to 100% with low relative standard deviation (% RSD ≤ 2.0 %) (15).

Table 3.23: Accuracy evaluation of UV methods of FBX by standard addition technique

Drug spiked (%)	Initial concentration (µg/ml)	Recovery			
		µg/ml	% Recovery	Mean % Recovery	% RSD
ACN:MOH (9:1)					
80	2	3.60	99.9	100.02	1.74
		3.67	101.83		
		3.54	98.35		
100	2	3.97	99.36	99.78	0.86
		4.03	100.76		
		3.97	99.21		
120	2	4.47	101.63	101.26	1.48
		4.38	99.61		
		4.51	102.54		
Phosphate buffer pH 7.4					
80	2	3.47	96.49	98.78	2.00
		3.60	100.06		
		3.59	99.80		
100	2	3.83	95.68	97.08	1.28
		3.90	97.51		
		3.92	98.05		
120	2	4.33	98.46	99.05	0.68
		4.35	98.89		
		4.39	99.78		

The absorption spectra of the standard FBX solution in ACN:MOH::9:1 and phosphate buffer pH 7.4 were compared with that achieved for formulation prototype (Drug + Excipients).

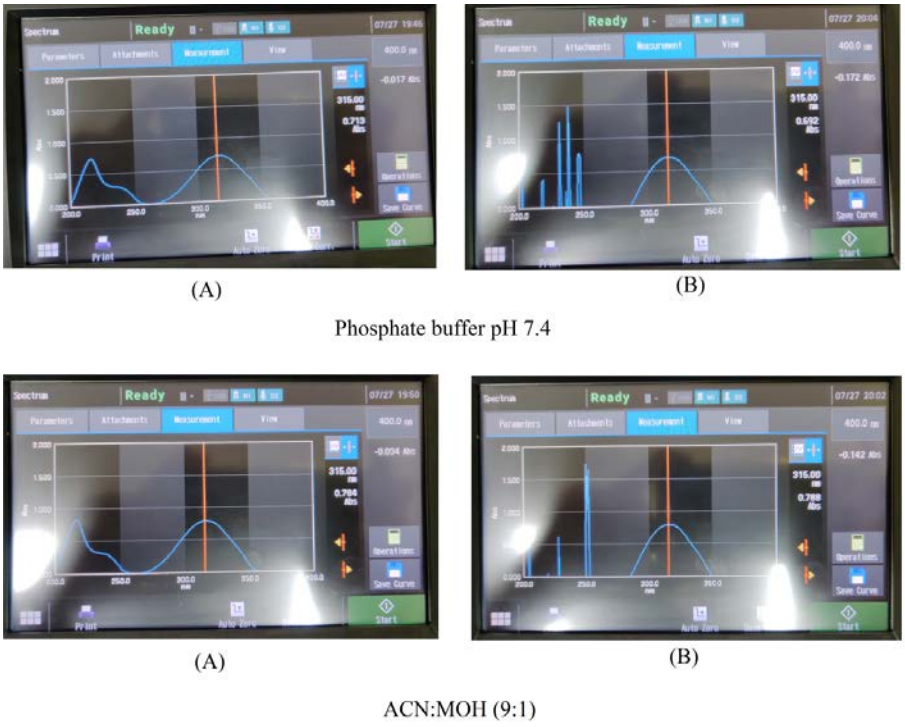


Figure 3.10: Absorption spectra of FBX (A) without blank cubosomal dispersion (B) in presence of cubosomal dispersion + Lactose to demonstrate specificity of analytical methods

As shown in Fig. 3.10, no change in position or intensity of the drug's peak was observed in formulation prototypes of cubosomes as compared to standard FBX solutions indicating the absence of any interference by formulation components at analytical wavelength. The absence of any overlapping or extraneous peaks in excipient mixtures at analytical wavelengths further suggested the specificity of the methods.

3.7. Conclusion

UV spectrophotometric, HPLC as well as LCMS methods for quantification of TAC and FBX in different in vivo, ex vivo and in vitro

experimental samples were successfully developed. Validation revealed that all the methods were linear, robust, sensitive, precise, accurate and specific.

3.8. References

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