CHAPTER-3

ANALYTICAL METHODS

CHAPTER 3: ANALYTICAL METHODS

3.1 Analytical method development and validation for estimation of DAR

Analytical methods (UV-spectrophotometric and RP-HPLC) were developed and validated to estimate Diacerein (DAR) in prepared formulations (i.e. nanosuspension and drug-cyclodextrin inclusion complex), *in vitro* and *in vivo* analysis. As DAR is completely metabolized by animals and humans into rhein (4,5-dioxy-9,10-dihydro-9,10-dioxy-2-antracencarboxylic acid), which is the active metabolite[1], hence quantitation of rhein in plasma was performed using RP-HPLC method for the *in vivo* study of DAR. Various UV-spectrophotometric[2-4], HPLC[5-11] and LCMS/MS[12,13] methods have been reported for estimation of DAR in formulations and in biological fluids.

3.1.1 Materials and reagents

DAR was obtained as gift sample from Wockhardt Research Centre, Aurangabad, Maharashtra, India. Fenofibrate was kindly gifted by Alembic Pharmaceutical Ltd. Vadodara, Gujarat. Rhein standard was purchased from Sigma-aldrich, India. Marketed formulation "Dycerin™",(Diacerein IP 50 mg, Glenmark Pharmaceuticals Ltd., Mumbai, India) was purchased from local pharmacy. Acetonitrile (HPLC Grade) and Methanol (HPLC Grade) were procured from Merck Chemicals, Mumbai, India. Dimethylsulfoxide (HPLC grade), Tetrahydrofuran (HPLC grade) Orthophosphoric acid (HPLC Grade), Glacial Acetic Acid (HPLC Grade), Ethyl Acetate (HPLC Grade) and Perchloric acid (HPLC Grade) were purchased from Spectrochem Chemicals (Mumbai, India). Potassium dihydrogen phosphate (AR grade), Sodium dihydrogen phosphate (AR grade), Ammonium acetate (AR grade), HCl (AR grade) and Sodium hydroxide (AR grade) were purchased from S.D. Fine Chemicals, Mumbai, India. Hank's balanced salt solution (HBSS) was purchased from Himedia, Mumbai. Purified HPLC grade water was obtained by filtering double distilled water through nylon filter paper 0.22 µm pore size and 47 mm diameter (Millipore, Bangalore, India). Human plasma was obtained free of cost from Suraktam Blood Bank, Vadodara, Gujarat.

3.1.2 Analytical methods for estimation of DAR by Ultraviolet (UV) Spectroscopy

A simple, sensitive and accurate UV method for estimation of actual amount of DAR from its formulations was developed.

3.1.2.1 Instrument and condition

UV-Visible double beam spectrophotometer (Shimadzu UV-1601, Japan) having ultraviolet rays as light source with fixed slit width (2 nm) coupled with a computer was

used for all absorbance measurements throughout the project, using 1.0 cm matched quartz cells.

3.1.2.2 Standard solution preparation

3.1.2.2.1 Standard stock solution

50 mg of DAR was weighed accurately and carefully transferred to the 50 mL volumetric flask. About 10 mL of Dimethylsulfoxide (DMSO) was added to the volumetric flask and sonicated for 2-3 minutes to dissolve the DAR. The volume was made up to the 50 mL mark with methanol to obtain standard stock solution (1000 µg/mL).

3.1.2.2.2 Working stock solution

An aliquot (2.5 mL) of standard stock solution of DAR was transferred to a 50 mL volumetric flask and volume was made up to the mark with methanol (MeOH) to get working stock solution (50 µg/mL).

The standard stock solution and working stock solution were stored at 2-8°C till further use.

3.1.2.3 Calibration curves of DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by Ultraviolet (UV) Spectroscopy

3.1.2.3.1 Calibration curve of DAR in methanol

The analysis was performed by first scanning DAR test solution (10 µg/mL) in methanol in the UV range between 200-400 nm against methanol as blank and determining its absorbance maxima (λ_{max}). Suitable aliquots of the working stock solution of DAR ranging from 0.2 mL to 3.6 mL were pipette out and transferred into 10 mL volumetric flasks and volume was made up to 10 mL mark with methanol to prepare final concentrations in the range of 1-18 µg/mL. Solutions were mixed well and their absorbances were measured at λ_{max} using methanol as blank. Calibration curve was constructed by plotting absorbance versus concentration of DAR and regression equation was calculated. The same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.1.2.3.2 Calibration curve of DAR in distilled water

The procedure was same as that of calibration curve of DAR in methanol. Distilled water was used as blank against range of concentrations (1-18 µg/mL) of DAR in distilled water. Calibration curve was constructed by plotting absorbance versus concentration of DAR and regression equation was calculated. The same procedure was repeated for

six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.1.2.3.3 Calibration curve of DAR in phosphate buffer pH-6.8

The procedure was same as that of calibration curve of DAR in methanol. Phosphate buffer pH-6.8 was used as blank against range of concentrations (1-18 µg/mL) of DAR in phosphate buffer pH-6.8. Calibration curve was constructed by plotting absorbance versus concentration of DAR and regression equation was calculated. Same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.1.2.3.4 Calibration curve in acetate buffer pH-4.5

The procedure was same as that of calibration curve of DAR in methanol. Acetate buffer pH-4.5 was used as blank against range of concentrations (1-18 µg/mL) of DAR in acetate buffer pH-4.5. Calibration curve was constructed by plotting absorbance versus concentration of DAR and regression equation was calculated. The same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.1.2.3.5 Calibration curve in 0.1N HCl

The procedure was same as that of calibration curve of DAR in methanol. Acetate buffer pH-4.5 was used as blank against range of concentrations (1-18 µg/mL) of DAR in acetate buffer pH-4.5. Calibration curve was constructed by plotting absorbance versus concentration of DAR and regression equation was calculated. The same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.1.2.4 Analytical method validation

The methods were validated for linearity, accuracy and precision.

3.1.2.4.1 Linearity

The linearity of an analytical method is its ability to elicit, test results that are directly, or by well-defined mathematical transformation, proportional to the concentration of the analyte in samples within a given range[14,15]. Linearity of a light absorption determination should be examined to ensure that Beer's law operates over the range of interest.

The linearity of the assay was determined by preparing the standard solutions using specific diluent to obtain final concentrations of DAR at 1, 2, 4, 6, 8, 10, 12, 14, 16 and 18

µg/mL (n=6) and absorbances were measured at λ_{max} of DAR (i.e. 257 nm) using diluent as blank. The calibration curve was built by plotting the drug concentrations versus their respective absorbance. The method was said to be linear for estimation of DAR if it R^2 was near to 1. Least square regression method was used to determine the regression coefficient, r and the equation for the best fitting line.

3.1.2.4.2 Accuracy

Accuracy refers to the closeness of an individual observation or mean to true value[16]. The "true" value is the result which would be observed in absence of error. Accuracy of the assay is defined as the percentage of the agreement between the measured value and the true value as follows[17,18]. The % relative error is calculated by using following formula:

$$\% \text{ Relative Error} = \frac{\text{True value} - \text{Measured value}}{\text{True value}} \times 100$$

To confirm the accuracy of proposed method, recovery study was performed. Recovery greater than 96 % with low SD justifies the accuracy of the method.

$$\% \text{ Recovery} = \frac{\text{Observed value}}{\text{Theoretical value}} \times 100$$

The intra-day and inter-day accuracy were determined by replicate analysis of the solutions of known concentrations of DAR at three quality control concentration (low – LQC, medium – MQC, and high – HQC) levels. The observed concentrations of the drug were then back calculated (from absorbance) using the equation of standard calibration curve and compared with the actual concentrations. The % relative error and % recovery were calculated.

3.1.2.4.3 Precision

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

relatively small. Thus, it is not unusual to find CV/%RSD of less than 1% for some analytical procedures[18-20].

The standard deviation is calculated from following formula given in equation below;

$$SD = \sqrt{\frac{\sum_{i=1}^{N}(x_i - \bar{x})^2}{(N-1)}}$$

Where, x_i is an individual measurement in a set,

$\bar{x}$ is the arithmetic mean of the set and

N is the total number of the replicated measurements taken in a set.

For the confirmation of precision, relative standard deviation (%RSD) can be calculated using following equation;

$$\%RSD = \frac{SD}{Mean} \times 100$$

For evaluation of the precision, within the day (intra-day) and between-day (inter-day) precision variability was performed at three concentration levels (5, 10 and 15 µg/mL) of DAR within linearity range. The experiments were repeated three times a day for *intra-day precision* and on three different days for *inter-day precision.*

Intra-day Precision

The suitable and accurate aliquots of working stock solutions of DAR were appropriately diluted using appropriate diluent to obtain three levels of concentrations at 5 µg/mL (LQC), 10 µg/mL (MQC) and 15 µg/mL (HQC). Six different sets of working stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at 257 nm against diluent as blank, for three times on the same day. All the solutions were prepared freshly each time. The % RSD was calculated.

Inter-day Precision

The suitable and accurate aliquots of working stock solutions of DAR were appropriately diluted using appropriate diluent to obtain three levels of concentrations at 5 µg/mL (LQC), 10 µg/mL (MQC) and 15 µg/mL (HQC). Six different sets of working stock solutions were prepared and diluted in the similar manner. The absorbance of samples was measured at 257 nm against diluent as blank, on three consecutive days. All the solutions were prepared freshly each day. The % RSD was calculated.

3.1.2.4.5 Limit of detection (LOD) and limit of Quantitation (LOQ)

The LOD and LOQ are quantitation parameters. There are several terms that have been used to define LOD and LOQ. In general, the LOD is taken as the lowest concentration of an analyte that can be detected, but not necessarily quantified, under the stated conditions of test. The LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of test[19,20].

LOD and LOQ are expressed as the concentration of analyte in the sample. The limit is usually expressed in terms of µg/mL, ng/mL, pg/mL, etc. LOD values are always specific for a particular set of experimental conditions. Anything that changes the sensitivity of a method, including instrument, sample preparation etc will change detection limits. The value of LOQ is almost 10 times higher than that of the blank.

Linear regression model can be used for the computation of LOD and LOQ. Therefore, the LOD and LOQ can be expressed as

$$\text{LOD} = \frac{3.3\sigma}{\bar{S}} \quad \text{and LOQ} = \frac{10\sigma}{\bar{S}}$$

Where, σ is the standard deviation of the response and

$\bar{S}$ is the mean of slope of the calibration curve.

The standard deviation of the response can be estimated by the standard deviation of either *y*-residuals, or *y*-intercepts, of regression lines. This method can be applied in all cases, and it is most applicable when the analysis method does not involve background noise. It uses a range of low values close to zero for calibration curve, and with a more homogeneous distribution will result in a more relevant assessment[21].

3.1.2.5 Results and Discussion

DAR yields a characteristic spectrum when scanned in the ultraviolet range between 200 nm and 400 nm. In all the media, DAR showed absorption maximum at 257 nm and this wavelength was chosen as analytical wavelength. Beer's law was obeyed between 1 and 18 µg/mL. Regression analysis was performed on the experimental data. Correlation coefficient for developed methods were found to be 0.9999 (in methanol), 0.9999 (in distilled water), 0.9999 (in phosphate buffer pH 6.8), 0.9999 (in acetate buffer pH 4.5) and 0.9999 (in 0.1N HCl). The value of correlation coefficient indicated the linear relationship between absorbance and concentration of DAR in each media.

Table 3.1 show the summary of calibration data for DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl.

Fig. 3.1(A), 3.2(A), 3.3(A), 3.4(A) and 3.5(A) show the overlay spectra of DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl, respectively (1-18 µg/mL).

Fig. 3.1(B), 3.2(B), 3.3(B), 3.4(B) and 3.5(B) indicate the calibration curves of DAR for linearity in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl, respectively (1-18 µg/mL).

Table 3.2 show the parameters indicating linearity for the used UV spectrophotometric method of analysis for DAR in methanol, distilled water, phosphate buffer pH 6.8, acetate buffer pH 4.5 and 0.1N HCl.

Table 3.3 and 3.4 demonstrate the intra-day and inter-day precision and accuracy for the DAR assay by UV spectroscopy, respectively. The low % RSD values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating accuracy of the method[19-21].

The interference studies with formulation excipients studies were carried out and no difference in absorbance was observed at 257 nm.

Table 3.5 represents the LOD and LOQ values for DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Table 3.1 Summary of calibration data for DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Conc. (µg/mL)	(Mean Absorbance ± SD)*				
	Methanol	Distilled Water	Phosphate Buffer pH-6.8	Acetate Buffer pH-4.5	0.1N HCl
1	0.111 ± 0.001	0.103 ± 0.001	0.106 ± 0.001	0.105 ± 0.002	0.101 ± 0.001
2	0.209 ± 0.002	0.196 ± 0.002	0.207 ± 0.002	0.209 ± 0.003	0.202 ± 0.002
4	0.397 ± 0.002	0.390 ± 0.001	0.394 ± 0.005	0.391 ± 0.002	0.384 ± 0.004
6	0.606 ± 0.003	0.590 ± 0.002	0.604 ± 0.003	0.602 ± 0.003	0.575 ± 0.005
8	0.809 ± 0.002	0.796 ± 0.003	0.798 ± 0.004	0.799 ± 0.003	0.782 ± 0.003
10	1.019 ± 0.002	0.979 ± 0.004	1.008 ± 0.002	0.991 ± 0.004	0.962 ± 0.005
12	1.211 ± 0.003	1.155 ± 0.004	1.207 ± 0.003	1.187 ± 0.004	1.154 ± 0.004
14	1.420 ±0.015	1.354 ±0.002	1.392 ±0.003	1.367 ±0.002	1.350 ±0.005
16	1.607 ± 0.002	1.535 ± 0.003	1.592 ± 0.002	1.575 ± 0.002	1.547 ± 0.002
18	1.806 ± 0.003	1.736 ± 0.002	1.778 ± 0.002	1.768 ± 0.003	1.732 ± 0.003

*mean of six determinations.

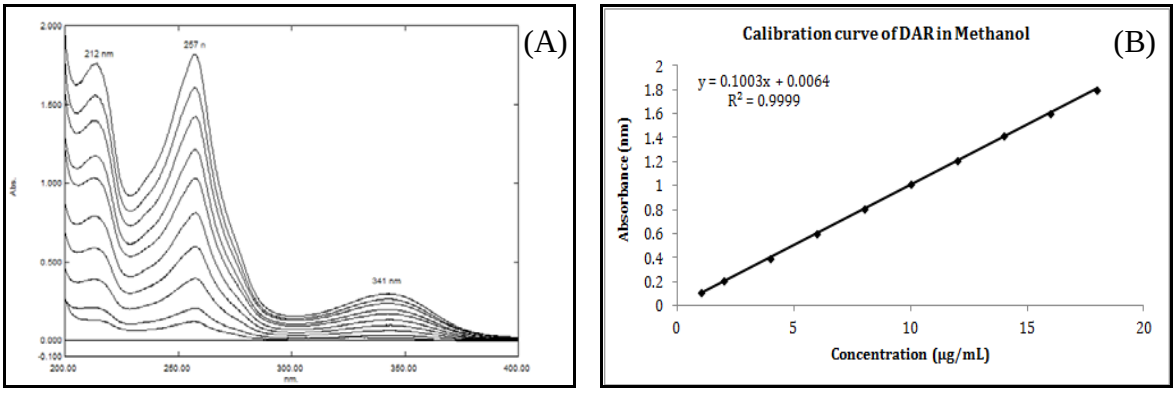


Fig. 3.1 (A) Overlay spectra and (B) Calibration curve of DAR in methanol.

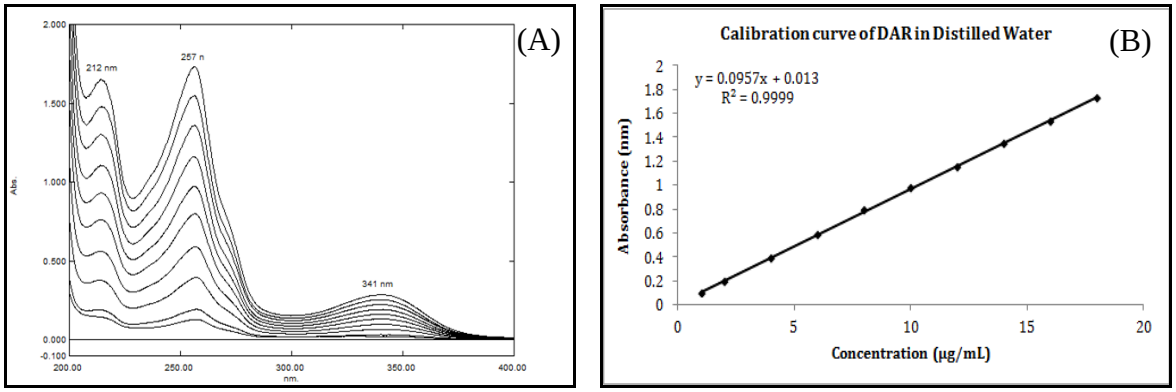


Fig. 3.2 (A) Overlay spectra and (B) Calibration curve of DAR in distilled water.

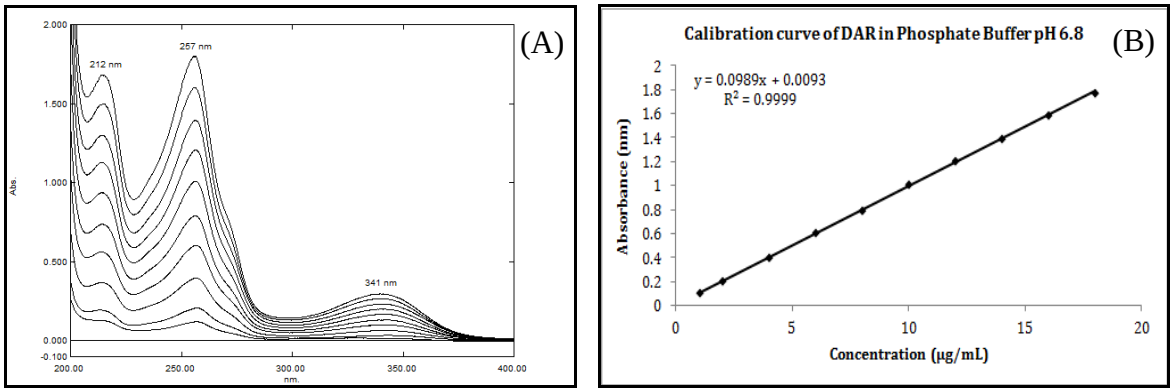


Fig. 3.3 (A) Overlay spectra and (B) Calibration curve of DAR in phosphate Buffer pH 6.8.

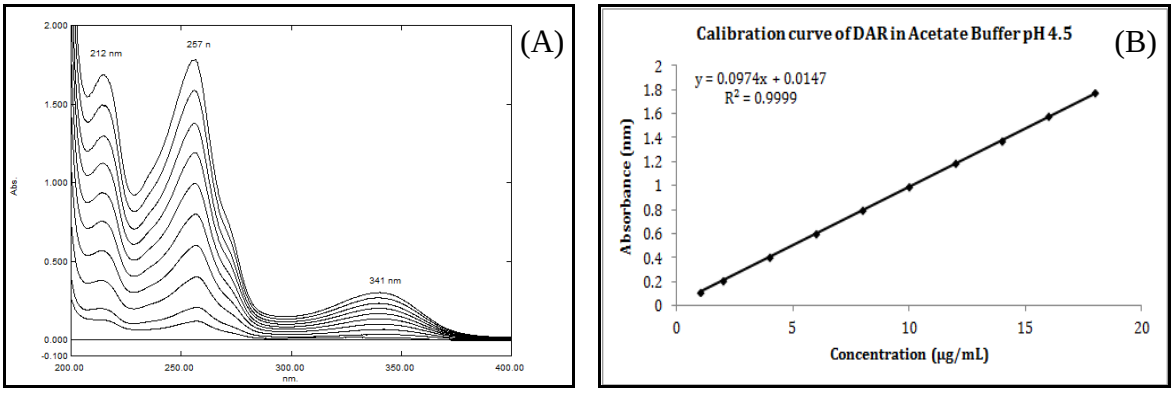


Fig. 3.4 (A) Overlay spectra and (B) Calibration curve of DAR in acetate buffer pH 4.5.

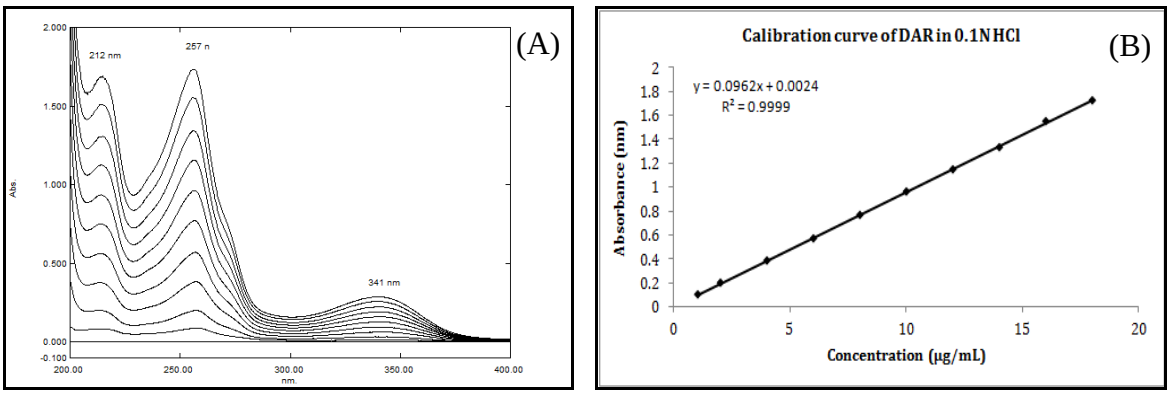


Fig. 3.5 (A) Overlay spectra and (B) Calibration curve of DAR in 0.1N HCl.

Table 3.2 Summary of linearity parameters for DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Linearity parameters	Media				
	Methanol	Distilled Water	Phosphate Buffer pH-6.8	Acetate Buffer pH-4.5	0.1N HCl
λ_{max} (nm)	257 nm				
Linearity range (µg/mL)	1-18 µg/mL				
Slope ± SD*	0.1003 ± 0.0003	0.0957 ± 0.0001	0.0989 ± 0.0004	0.0974 ± 0.0005	0.0962 ± 0.0001
Intercept ± SD*	0.0064 ± 0.002	0.013 ± 0.003	0.0093 ± 0.004	0.0147 ± 0.003	0.0024 ± 0.003
Correlation Coefficient (R^2)*	0.9999	0.9999	0.9999	0.9999	0.9999

*mean of six determinations.

Table 3.3 Summary of intra-day precision and accuracy for DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Media	Standard Concentration (µg/mL)		Precision§ (%)	Accuracy† (%)
	Actual	Observed*		
Methanol	5	5.023	0.61	100.47
	10	10.077	0.83	100.77
	15	15.087	0.67	100.58
Distilled water	5	5.057	0.64	101.13
	10	10.053	0.55	100.53
	15	15.017	0.73	100.11
Phosphate Buffer pH-6.8	5	5.07	0.86	101.40
	10	10.13	0.79	101.27
	15	15.06	0.20	100.42
Acetate Buffer pH-4.5	5	5.093	0.41	101.87
	10	10.123	0.15	101.23
	15	15.143	0.27	100.96
0.1N HCl	5	5.083	0.60	101.67
	10	10.106	0.64	101.07
	15	15.13	0.39	101.86

*mean of six determinations.
§ Expressed as % relative standard deviation (%RSD)
† Expressed as % recovery.

Table 3.4 Summary of inter-day precision and accuracy for DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Media	Standard Concentration (µg/mL)		Precision§ (%)	Accuracy† (%)
	Actual	Observed*		
Methanol	5	5.063	0.89	101.27
	10	10.037	0.70	100.37
	15	15.047	0.40	100.31
Distilled water	5	5.053	0.30	101.07
	10	10.034	0.51	100.33
	15	15.07	0.53	100.47
Phosphate Buffer pH-6.8	5	5.09	0.39	101.8
	10	10.17	0.25	101.17
	15	15.57	0.43	100.38
Acetate Buffer pH-4.5	5	5.07	0.69	101.46
	10	10.05	0.65	100.5
	15	15.036	0.33	100.24
0.1N HCl	5	5.04	0.59	100.8
	10	10.16	0.30	101.57
	15	15.17	0.30	101.07

*mean of six determinations.
§ Expressed as % relative standard deviation (%RSD)
† Expressed as % recovery.

Table 3.5 Summary of LOD and LOQ values for DAR in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Parameters	Media				
	Methanol	Distilled Water	Phosphate Buffer pH-6.8	Acetate Buffer pH-4.5	0.1N HCl
LOD* (µg/mL)	0.066	0.103	0.136	0.108	0.115
LOQ* (µg/mL)	0.199	0.313	0.413	0.326	0.348

*mean of six determinations.

3.1.2.6 Conclusion

The proposed methods were rapid, economical, accurate, precise and sensitive for the determination of DAR. These methods were later used for estimation of DAR in intermediate studies during formulation development of DAR.

3.1.3 Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method for estimation of DAR in formulations

HPLC analytical method was developed and validated to estimate DAR in developed formulations (nanosuspension and DAR-cyclodextrin inclusion complex).

3.1.3.1 Instrument and chromatographic conditions

Quantitative chromatography was performed on Shimadzu chromatographic system (Shimadzu Corporation, Kyoto, Japan) equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV UV/Visible detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 µl. Data acquisition and integration was performed using Spinchrome CFR software.

The chromatographic separation and quantitation was performed using a Phenomenex Hypersil BDS C18 (150 mm×4.6 mm i.d., 5 µm particle size) column attached with Phenomenex SecurityGuardStandard with C18 cartridge (4.0×3.0)mm. Separation was attained using a mobile phase consisting of acetonitrile and phosphoric acid buffer in the ratio of 50:50 (v/v), pumped at a flow rate of 1.0 ml min^{-1}. The eluent was monitored using UV detector at a wavelength of 257 nm. The column was maintained at ambient and an injection volume of 20 µL was used. The mobile phase was vacuum filtered through 0.22 µm nylon membrane filter followed by degassing in an ultrasonic bath prior to use.

Optimized chromatographic conditions

Column : Phenomenex Hypersil BDS C18, (150 x 4.6)mm, 5µm.

Flow : 1.0 ml/min

Wavelength	:	257 nm
Injection volume	:	20µl
Column oven temperature	:	Ambient
Runtime	:	10 mins

Injections of 20 µl were made for each sample concentration and chromatographed under the condition described above. The method was validated as per ICH guidelines.

3.1.3.2 Preparation of experimental solutions

3.1.3.2.1 Preparation of buffer solution

Added 14.7 mL Ortho-phosphoric acid in 35.3 mL of water and mixed well. Diluted 2 mL of this solution to 1000 ml with water and solution was filtered through 0.2µ nylon membrane filter.

3.1.3.2.2 Preparation of mobile phase

A mixture of Phosphoric acid buffer solution and Acetonitrile was prepared in the ratio of (50:50) v/v, mixed well and degassed.

Diluent: Mobile phase.

3.1.3.2.3 Preparation of standard stock solution

Accurately weighed and transferred 50mg of DAR working standard (API) into 50ml volumetric flask. Approximately 10 ml DMSO was added and sonicated to dissolve the DAR. Final volume was made up to the mark with diluent and mixed well. The concentration of resultant solution was 1000 µg/mL.

3.1.3.2.4 Preparation of working stock solution

Working stock solution was prepared by diluting 5 mL aliquot of standard stock solution to 50 mL with diluent in a 50 mL volumetric flask to produce a working stock solution of DAR having concentration of 100 µg/mL.

3.1.3.3 Calibration curve of DAR

Suitable aliquots of the working stock solution of DAR ranging from 0.05 mL to 4.0 mL were pipette out and transferred into 10 mL volumetric flasks and volume was made up to 10 mL mark with diluent to prepare final concentrations in the range of 0.5-40 µg/mL. Solutions were mixed well using vortex mixer before injecting in the HPLC. Injections of 20 µl were made for each concentration and chromatographed under the condition described above. Each of these drug solutions was injected three times into the column and the peak area and retention times were recorded. Calibration curve was constructed by plotting area under curve versus concentration of DAR and regression

equation was calculated. The same procedure was repeated for three times using freshly prepared working stock solution every time.

3.1.3.4 Validation of developed RP-HPLC method

Validation of developed RP-HPLC method was performed as per the recommendation of ICH (i.e. ICH guidelines Q2 (R1))[21]. The method was validated with respect to the parameters including system suitability, linearity, LOD, LOQ, precision, accuracy and selectivity.

3.1.3.4.1 System suitability

System suitability analysis was carried out by injecting six consecutive injections of solution having concentration at 20 µg/mL of DAR during the start of method validation and the start of each day. Various peak parameters like peak area, retention time, tailing factor, theoretical plates, were observed and recorded.

3.1.3.4.2 Linearity and range

Linearity study of HPLC detector response for determination of DAR was evaluated by analyzing a series of standard solutions of 10 different concentrations of DAR. Calibration curve constructed was linear over the concentration range of 0.5 – 40 µg/mL for DAR (i.e. 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40 µg/mL). Regression analysis has been carried out on resultant linearity graphs and regression equation and correlation coefficient were obtained. The same procedure was repeated for total three times. The % RSD values for respective peak areas of each DAR standards across the calibration concentration range were calculated and found to be less than 2.

3.1.3.4.3 Precision

Precision was estimated by the determination of the repeatability of the method. Repeatability was assessed using six determinations at each of three different test concentrations (5, 20 and 35 µg/mL -covering the specified range of the method), in a day for Intra-day precision and on three consecutive days for Inter-day precision. A volume of 20 µL of each test solution was injected into HPLC and chromatograms were recorded.

3.1.3.4.4 Accuracy:

Accuracy of the method was confirmed by recovery study from commercial capsule formulation (Dycerin™, Glenmark Pharmaceuticals Ltd., Mumbai, India, level claim-Diacerein IP 50 mg) of DAR at 3 level of standard addition (80%, 100%, and 120%) of

label claim. Intra-day and inter-day accuracy studies were performed and everytime six determinations at each level of the amounts were acquired.

3.1.3.4.5 Limit of detection (LOQ) and limit of quantitation (LOQ)

The LOQ and LOD were determined based on a signal to noise (S/N) ratios and were based on analytical responses of 10 and 3 times the background noise respectively. LOD and LOQ were experimentally verified by diluting known concentration of DAR until the average responses were approximately 3 or 10 times the standard deviation of the responses for six replicate determinations.

3.1.3.4.6 Robustness and ruggedness

To evaluate robustness and ruggedness of the method, few method conditions were deliberately varied. The conditions included variation of flow rate by ± 0.05 mL/min, percentage of acetonitrile in mobile phase by ± 1% v/v and acetonitrile of two different manufacturers, columns of two different brands and using two different instruments. Conditions were slightly changed at the three levels (-1, 0, +1); each time one parameter was changed from level (0), and the other factors remained at level (0). Six replicate injections of standard solutions were performed under little changes of above said chromatographic conditions.

3.1.3.4.7 Stability

Three different concentrations of DAR (i.e. 5, 20 and 35 µg/mL) in diluent were prepared from working stock solution of DAR and stored at room temperature and at 5 °C in refrigerator for 48 hours. Six replicates of each of the stored solutions were injected in to HPLC system.

3.1.3.5 Result and discussion

The RP-HPLC method was developed for the quantitation of DAR using a Phenomenex Hypersil BDS C18 (150 mm × 4.6 mm i.d., 5 µm particle size) column. Separation was attained using a mobile phase consisting of acetonitrile and phosphoric acid buffer in the ratio of 50:50 (v/v), pumped at a flow rate of 1.0 ml min^{-1}. The eluent was monitored using UV detector at a wavelength of 257 nm. The column was maintained at ambient and an injection volume of 20 µL was used.

System suitability study was performed with six replicate injections of standard solution of DAR (20µg/mL) in the HPLC system and retention time of DAR was observed at 5.2 ± 0.057 min. The % RSD for the area under curve of chromatographic peak of DAR was calculated and found to be 0.65 which indicated the minimal variation in peak area.

The asymmetry factor and theoretical plate counts were in agreement with the standard limits[20,21]. The system suitability parameters are summarized in Table 3.6.

Fig. 3.6(A) represents the overlay chromatogram of DAR in the linear range.The calibration curve of DAR constructed by plotting the observed peak area of DAR standards (y-axis) versus concentration of DAR (x-axis) and found to be linear in the range of 0.5-40 µg/mL (Table 3.7, Fig. 3.6(B)). The low value of correlation coefficient (0.9999) confirmed the linear relationship between peak area and concentration of DAR. The RSD based on the peak area for six injections were found to be less than 2.0 for calibration curve (Table 3.7). The slope and intercepts with low values of standard deviation were obtained (Table 3.8).

Table 3.3 System suitability parameters of RP-HPLC method for estimation of DAR.

Parameters	Observation
Peak area (mV.s)*	1388.76
% RSD of peak area	0.65
Retention Time (minutes) ± SD*	5.2± 0.057 min
Asymmetry ± SD*	1.012 ± 0.008
Theoretical plates ± SD*	6581 ± 15.62

* mean of six determinations.

Table 3.4 Calibration data of RP- HPLC method for estimation of DAR in formulations.

Concentration (µg/mL)	Mean peak area* (mV.s)	%RSD
0.5	34.458	0.97
1.0	67.259	1.04
5.0	353.615	0.76
10.0	662.9298	0.89
15.0	1018.416	0.71
20.0	1388.763	0.65
25.0	1713.417	0.61
30.0	2056.08	0.39
35.0	2402.1	0.33
40.0	2741.265	0.22

* mean of six determinations.

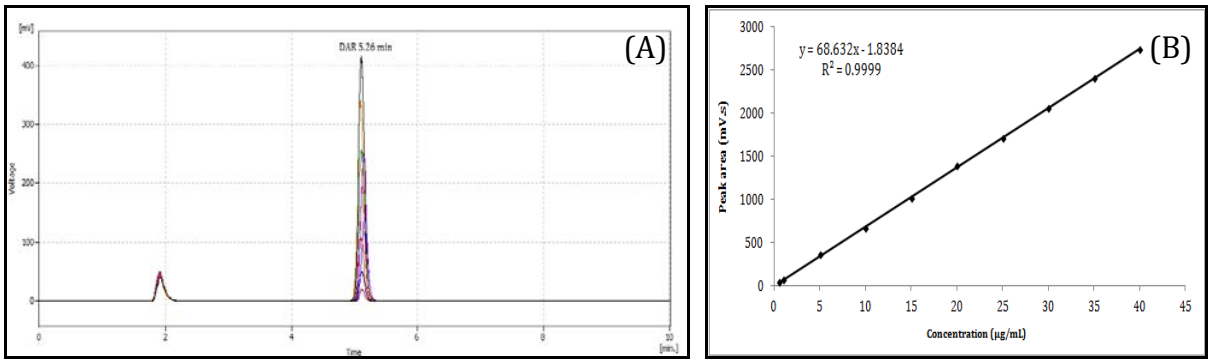


Fig. 3.6 (A) Overlay chromatogram and (B) Calibration curve of DAR in diluent.

Table 3.5 Summarized linearity parameters of RP-HPLC method for estimation of DAR in formulations.

Linearity Parameters	Results
Analytical wavelength (nm)	257
Linearity range (µg/mL)	0.5-40
Slope ± SD*	68.63 ± 0.13
Intercept ± SD*	-1.84 ± 1.01
Correlation coefficient (R^2)*	0.9999

* mean of six determinations.

The developed method was validated for its intra-day and inter-day precision (3 consecutive days, n=6) at three different test concentrations (5, 20 and 35 µg/mL - covering the specified linear range of the method). The average %RSD of intra-day and inter-day precision were found to be less than 1 which complies with the specified limit and confirm the precision of the method (Table 3.9).

Table 3.6 Intra-day and inter-day precision of RP-HPLC method for estimation of DAR in formulations.

Precision	Concentration Levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
Intra-day precision	5	5.12	0.81
	20	20.06	0.62
	35	35.58	0.51
Inter-day precision	5	5.08	0.60
	20	20.19	0.23
	35	35.48	0.92

* mean of six determinations at each level.

The intra-day and inter-day accuracy (3 consecutive days, n=6) of the proposed method was checked at 3 level of standard additions (80%, 100%, and 120%). No interference could be observed with the proposed method. The excellent recoveries of standard addition method with low %RSD justified the accuracy of the method (Table 3.10).

The LOQ and LOD were determined based on a signal to noise (S/N) ratios and were based on analytical responses of 10 and 3 times the background noise respectively. The LOQ was found to be 0.28 µg/ml with resultant % RSD of 0.84 % (n=6). The LOD was found to be 0.091 µg/ml (Table 3.11).

Robustness and ruggedness studies were performed for flow rate, mobile phase composition, acetonitrile of two different manufacturers, two different columns and two separate HPLC instruments. Results, presented in Table 3.12 indicate that the selected factors remained unaffected by small variation of these parameters. It was also found to that acetonitrile of different lots from the same manufacture has no significant influence

on the determination. Insignificant difference in asymmetric factor and less variability in retention time were observed.

Table 3.7 Intra-day and inter-day accuracy of RP-HPLC method for estimation of DAR in formulations.

Concentration of test solution used for standard addition=10 µg/mL					
Accuracy	**Excess drug added to test solution (%)**	**Actual concentration (µg/mL)**	**Estimated concentration* (µg/mL)**	**% Recovery* ±SD**	**%RSD**
Intra-day accuracy	0	10	10.14	101.43±0.05	0.49
	80	18	18.19	101.06±0.11	0.61
	100	20	20.08	100.38±0.10	0.52
	120	22	22.38	101.71±0.10	0.46
Inter-day accuracy	0	10	10.18	101.77±0.04	0.35
	80	18	18.34	101.91±0.13	0.71
	100	20	20.22	101.12±0.13	0.65
	120	22	22.19	100.86±0.09	0.40

* mean of six determinations at each level.

Table 3.8 LOD and LOQ of RP-HPLC method for estimation of DAR in formulations.

Parameters*	**Results**
LOD (µg/mL)	0.091
LOQ (µg/mL)	0.28

* mean of six determinations.

Table 3.9 Robustness and ruggedness of RP-HPLC method for the estimation of DAR in formulations.

Variable conditions	**Levels**	**Estimated Factors**	
		Mean Retention Time of DAR (min)*	**Mean Asymmetric factor of DAR peak***
Flow rate (mL/min)	0.95 mL/min	5.249	1.008
	1.0 mL/min	5.208	1.013
	1.05 mL/min	5.172	1.015
	Mean ± SD	5.207±0.037	1.012±0.004
% of ACN in mobile phase (%v/v)	49 % v/v	5.257	1.018
	50 % v/v	5.211	1.015
	51 % v/v	5.189	1.010
	Mean ± SD	5.219±0.035	1.014±0.004
ACN of different manufacturer	Merck, India	5.219	1.012
	Spectrochem, India	5.226	1.027
	Mean ± SD	5.222±0.005	1.019±0.011
Columns of different brands	Column 1	5.235	1.019
	Column 2	5.286	1.073
	Mean ± SD	5.261±0.036	1.046±0.038
Two different instrument	Instrument 1	5.215	1.026
	Instrument 2	5.278	1.017
	Mean ± SD	5.247±0.045	1.022±0.006

* mean of six determinations at each level.

Stability study for solution of DAR at three levels of concentration (stored at room temperature and at 5 °C in refrigerator for 48 hours) was performed. Six replicates of each concentration were injected in the HPLC system and %RSD for peak areas was calculated. . No significant decrease in estimated concentration and low %RSD values were indicating the stability of DAR in the sample solutions prepared in mobile phase (Table 3.13).

Table 3.10 Stability of DAR solutions used in RP-HPLC method for estimation of drug in formulations.

Storage conditions	Concentration levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
At room temperature for 48 hours	5	4.99	1.11
	20	20.08	0.52
	35	35.31	1.09
At 5 °C for 48 hours	5	5.03	0.80
	20	20.01	0.69
	35	35.10	0.53

* mean of six determinations at each level.

3.1.3.6 Conclusion

The developed RP-HPLC method for estimation of DAR was successfully validated for different parameters as per ICH guidelines. The results of the study showed that the proposed RP-HPLC was simple, rapid, economical, sensitive, accurate and precise and could be used for estimation of DAR in its new pharmaceutical dosage forms.

Developed RP-HPLC method was successfully adopted for the estimation of DAR in newly developed formulation (nanosuspension and cyclodextrin inclusion complex) and could be used for the estimation of DAR or its active metabolite (rhein) from matrix (plasma), required for pharmacokinetic study to estimate the amount of drug in systemic circulation. Further, the developed HPLC method could also be significantly used for determination of DAR in transport medium used in *in vitro* gastro-intestinal permeability study using Caco-2 cell model.

3.1.4 RP-HPLC method for estimation of DAR in transport buffer (HBSS-Hank's balanced salt solution) used for *in vitro* gastro-intestinal permeability study using Caco-2 cell model

3.1.4.1 Instrument and chromatographic conditions

Same as in Section 3.1.3.1

3.1.4.2 Preparation of experimental solutions

3.1.4.2.1 Preparation of DAR standard stock solution

Accurately weighed and transferred 50mg of DAR working standard (API) into 50ml volumetric flask. Approximately 10 ml DMSO was added and sonicated to dissolve the DAR. Final volume was made up to the mark with HBSS buffer and mixed well. The concentration of resulting solution was 1000 µg/mL.

3.1.4.2.2 Preparation of DAR working stock solution

An aliquot of 5.0 mL of standard stock solution of DAR was pipette out in a 50 mL volumetric flask and diluted up to the 50 mL mark with HBSS buffer to produce a working stock solution of DAR having concentration of 100 µg/mL.

3.1.4.3 Calibration curve of DAR in HBSS buffer

Suitable aliquots of the working stock solution of DAR ranging from 0.05 mL to 4.0 mL were pipette out and transferred into 10 mL volumetric flasks and volume was made up to 10 mL mark with HBSS to prepare final concentrations in the range of 0.5 – 40 µg/mL. Solutions were mixed well using vortex mixer before injecting in the HPLC. Injections of 20 µl were made for each concentration and chromatographed under the condition described earlier (Section 3.1.3.2). Each of these drug solutions was injected three times into the column and the peak area and retention times were recorded. Calibration curve was constructed by plotting area under curve versus concentration of DAR and regression equation was calculated. The same procedure was repeated for three times using freshly prepared working stock solution every time.

3.1.4.4 Validation of the RP-HPLC method for the determination of DAR in HBSS buffer

Method validation was performed based on both USP and International Conference on Harmonization (ICH) guidelines for the validation of analytical methods[20,21]. All samples used during validation were freshly prepared in HBSS buffer.

3.1.4.4.1 System suitability

System suitability analysis was carried out by injecting six consecutive injections of solution having concentration at 20 µg/mL of DAR during the start of method validation and the start of each day. Various peak parameters like peak area, retention time, tailing factor, theoretical plates, were observed and recorded.

3.1.4.4.2 Linearity and range

Linearity study was performed for estimation of DAR in transport buffer by analyzing a series of standard solutions of 10 different concentrations of DAR in HBSS buffer. Calibration curve plotted was found to be linear over the concentration range of 0.5 – 40 µg/mL for DAR (i.e. 0.5, 1, 5, 10, 15, 20, 25, 30, 35, 40 µg/mL). Regression analysis has been carried out on resultant linearity graphs and regression equation and correlation coefficient were calculated. The same experiment was again repeated for two more times. The % RSD values for respective peak areas of each DAR standards across the calibration concentration range were calculated and found to be less than 2.

3.1.4.4.3 Precision

Precision was estimated by the determination of the repeatability of the method. Repeatability was assessed using six determinations at each of three different test concentrations in HBSS buffer (10, 20 and 30 µg/mL -covering the specified range of the method), in a day for Intra-day precision and on three consecutive days for Inter-day precision. A volume of 20 µL of each test solution was injected into HPLC and chromatograms were recorded.

3.1.4.4.4 Accuracy

Accuracy of the method was confirmed by recovery study. Recovery was estimated using three level of DAR concentration in HBSS buffer (50%, 100% and 150% i.e. 10 µg/mL, 20 µg/mL and 30 µg/mL). Six replicates of each level of concentration were injected and chromatograms recorded. Intra-day and inter-day accuracy studies were performed and % recoveries were calculated for each level of concentration.

3.1.4.4.5 Limit of detection (LOQ) and limit of quantitation (LOQ)

Calibration curve was repeated for 3 times and the standard deviation (SD) of the intercepts was calculated. According to ICH recommendations, the approach based on the standard deviation (SD) of the response and the slope was used for determining the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were measured as follows.

LOD=3.3 * SD of response/slope of calibration curve

LOQ=10 * SD of response/slope of calibration curve

SD = Standard deviation of response/intercepts

The theoretical values were assessed practically.

3.1.4.4.6 Stability study of DAR in HBSS buffer

Three different concentrations of DAR in HBSS buffer (i.e. 10, 20 and 30 µg/mL) were prepared from working stock solution of DAR and stored at room temperature, at 37°C in an incubator and at 5°C in refrigerator for 48 hours for 48 hours. Six replicates of each of the stored solutions were injected in to HPLC system.

3.1.4.5 Result and discussion

Chromatographic conditions were same as described earlier in Section 3.1.3.2.

System suitability study was carried out with six replicate injections of standard solution of DAR in HBSS (20 µg/mL) in the HPLC system and retention time of DAR was observed at 5.2 ± 0.096 min. The % RSD for the area under curve of chromatographic peak of DAR was calculated and found to be 0.62 which indicated the minimal variation in peak area. The asymmetry factor and theoretical plate counts were in agreement with the standard limits[20,21]. Different system suitability parameters are summarized in Table 3.14.

Table 3.11 System suitability parameters of RP-HPLC method for estimation of DAR in HBSS buffer.

Parameters	Observation
Peak area*	1277.76
% RSD of peak area	0.62
Retention Time (minutes) ± SD*	5.2± 0.096 min
Asymmetry ± SD*	1.108 ± 0.042
Theoretical plates ± SD*	6042 ± 27

* mean of six determinations.

Fig. 3.7(A) represents the overlay chromatogram of DAR in the linear range. The calibration curve of DAR in HBSS buffer was obtained by plotting the peak area of DAR standards (y-axis) versus concentration of DAR (x-axis) and found to be linear in the range of 0.5-40 µg/mL (Table 3.16, Fig. 3.7(B)). The low value of correlation coefficient (1.0000) confirmed the linear relationship between peak area and concentration of DAR. The RSD based on the peak area for six injections were found to be less than 2.0 for calibration curve (Table 3.15). The slope and intercepts with low values of standard deviation were obtained (Table 3.16).

Table 3.12 Calibration data of RP-HPLC method for estimation of DAR in HBSS buffer.

Concentration (µg/mL)	Peak area* (mV.s)	%RSD
0.5	32.368	1.14
1.0	64.103	0.73
5.0	325.615	0.82
10.0	636.154	0.72
15.0	956.218	0.44
20.0	1277.765	0.62
25.0	1600.919	0.55
30.0	1923.609	0.42
35.0	2240.061	0.22
40.0	2565.671	0.48

* mean of six determinations.

Table 3.13 Summarized linearity parameters of RP-HPLC method for estimation of DAR in HBSS buffer.

Linearity Parameters	Results
Analytical wavelength (nm)	257
Linearity range (µg/mL)	0.5-40
Slope ± SD*	64.073±0.14
Intercept ± SD*	0.668±1.73
Correlation coefficient (R^2)*	1.0000

* mean of six determinations.

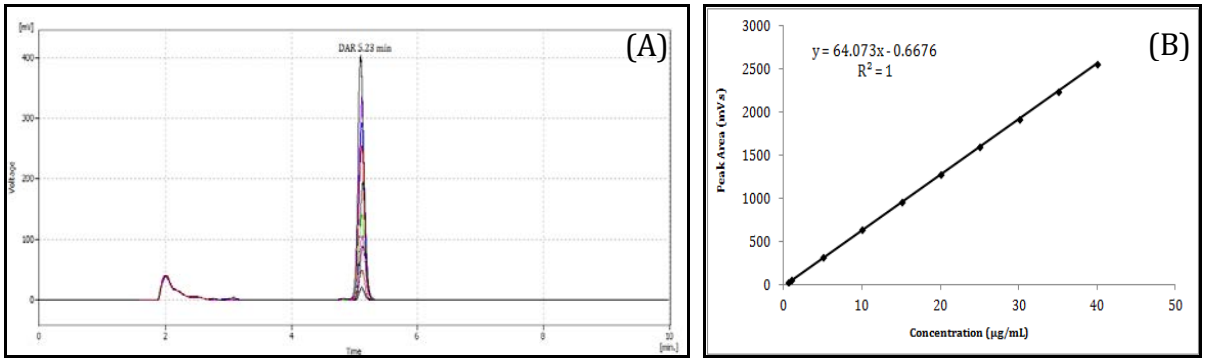


Fig. 3.7 (A) Overlay chromatogram and (B) Calibration curve of DAR in HBSS buffer.

The LOQ and LOD were determined for the estimation of DAR in HBSS buffer and summarized in Table 3.17.

Table 3.17 LOD and LOQ of RP-HPLC method for estimation of DAR in HBSS buffer.

Parameters*	Results
LOD (µg/mL)	0.089
LOQ (µg/mL)	0.27

* mean of six determinations.

The developed method was validated for its intra-day and inter-day precision (3 consecutive days, n=6) at three different test concentrations (10, 20 and 30 µg/mL - covering the specified linear range of the method). The average %RSD of intra-day and

inter-day precision were found to be less than 2 which complies with the standard guidelines and confirmed the precision of the method (Table 3.18).

Table 3.18 Intra-day and inter-day precision of RP-HPLC method for estimation of DAR in HBSS buffer.

Precision	Concentration Levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
Intra-day precision	10	10.12	0.30
	20	20.10	0.71
	30	30.58	0.59
Inter-day precision	10	10.18	0.69
	20	20.21	0.47
	30	30.24	0.38

* mean of six determinations at each level.

The intra-day and inter-day accuracy (3 consecutive days, n=6) of the proposed HPLC method was checked at 3 level of DAR concentration in HBSS buffer (50%, 100% and 150% i.e. 10 µg/mL, 20 µg/mL and 30 µg/mL). No interference could be observed with the proposed method. The excellent recoveries of standard addition method with low %RSD justified the high accuracy of the proposed method (Table 3.19).

Table 3.19 Intra-day and inter-day accuracy of RP-HPLC method for estimation of DAR in HBSS buffer.

Accuracy	Concentration Level (%)	Actual concentration (µg/mL)	Observed concentration* (µg/mL)	% Recovery* ±SD	%RSD
Intra-day accuracy	50	10	10.16	101.63	0.85
	100	20	20.14	100.70	0.75
	150	30	30.42	101.41	0.50
Inter-day accuracy	50	10	10.10	101.03	1.09
	100	20	20.26	101.30	0.36
	150	30	30.02	100.08	0.93

* mean of six determinations at each level.

Stability study for solution of DAR at three levels of concentration in HBSS buffer (stored at room temperature, at 37°C in an incubator and at 5°C in refrigerator for 48 hours) was performed. Six replicates of each concentration were injected in the HPLC system and %RSD for peak areas was calculated. No accountable decrease in DAR concentration and low %RSD values were indicating the stability of DAR in the sample solutions prepared in HBSS buffer (Table 3.20).

Table 3.14 Stability of DAR solution in HBSS buffer using RP-HPLC method.

Storage conditions	Concentration levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
At room temperature for 48 hours	10	10.02	1.08
	20	20.01	0.46
	30	30.13	1.23
At 37°C for 48 hours	10	9.99	0.51
	20	19.98	0.98
	30	30.04	0.62
At 5°C for 48 hours	10	10.01	0.56
	20	20.22	1.24
	30	30.21	0.92

* mean of six determinations at each level.

3.1.4.6 Conclusion

The proposed RP-HPLC method for estimation of DAR was successfully validated for different parameters as per USFDA and ICH guidelines. The results of the study showed that the developed RP-HPLC was simple, rapid, economical, sensitive, accurate and precise and could be used for estimation of DAR in transport buffer (HBSS-Hank's balanced salt solution) used for *in vitro* gastro-intestinal permeability study using Caco-2 cell model).

3.1.5 Bioanalytical RP-HPLC method for estimation of rhein (an active metabolite of DAR) in plasma

3.1.5.1 Instrument and chromatographic conditions

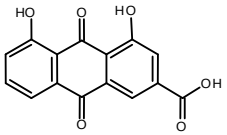
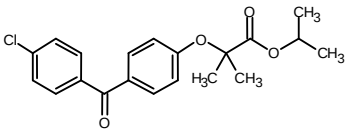
Same as in Section 3.1.3.1

3.1.5.2 Selection of internal standard (ISTD)

During the maturation of modern bio-analysis the use of internal standard has become an integral part of bioanalytical methods. The main purpose of utilizing internal standard is to compensate any variation other than that related to the amount of analyte present in a sample, such as the variability in dilutions, evaporation, degradation, recovery, adsorption, injection and detection. Changes in the system which occur throughout the analysis due to matrix effect can also be managed by the use of internal standard into each sample at a constant level. Hence internal standard should be added in the sample processing as early as possible. Internal standard should have the same or similar physicochemical properties as of analyte, which means that they should have approximately similar partition coefficients, molecular weights and should be as structurally similar to each other, as possible[22-24].

As described earlier, after oral administration of DAR, it has been completely metabolized in rhein before reaching in systemic circulation. Rhein is an active metabolite of DAR. The internal standard was selected as per the physico-chemical properties of rhein for this study. Fenofibrate (FNB)[25,26], has somewhat similar physicochemical properties as rhein[27,28] therefore FNB was selected as ISTD for the pharmacokinetic study of DAR (Table 3.21).

Table 3.15 Physicochemical properties of rhein and FNB.

Properties	Rhein	Fenofibrate (FNB)
Log P	4.58	4.86
Water solubility (mg/mL)	1.5×10^{-2}	7.07×10^{-4}
Log S	-4.27	-5.7
pKa	3.7 ± 0.8	4.9
Molecular formula	$C_{15}H_8O_6$	$C_{20}H_{21}ClO_4$
Molecular weight (Dalton)	284.032	360.83
Molecular structure		

3.1.5.3 Preparation of experimental solutions

3.1.5.3.1 Preparation of rhein standard stock solution

Accurately weighed and transferred 50mg of purified rhein into 50ml volumetric flask. Approximately 10 ml DMSO was added and sonicated to dissolve the rhein. Final volume was made up to the mark with methanol and mixed well. The concentration of resulting solution was 1000 µg/mL.

3.1.5.3.2 Preparation of rhein working stock solutions (spiking solutions)

Aliquot ranging from of 0.05 mL to 3.0 mL of standard stock solution of rhein was pipette out in separate 10 mL volumetric flasks and diluted up to the mark with methanol to produce working stock solutions of rhein having concentration of 5, 25, 50, 100, 200 and 300 µg/mL.

3.1.5.3.3 Preparation of FNB (ISTD) working stock solution

Accurately weighed and transferred 50 mg FNB (ISTD) in a 50 ml volumetric flask. About 35 ml of methanol added and sonicated to dissolve the content. Volume was made up to the mark with methanol and vortex-mixed. The concentration of resulting solution was at 1000 µg/mL. (1st Stock)

An aliquot of 5.0 mL was pipette out from 1st Stock and transferred in a 50 ml volumetric flask. Volume was made up to the mark with methanol and vortex-mixed to produce the working stock solution of FNB at 100 µg/mL.

3.1.5.3.4 Preparation of calibration curve standard solutions of rhein in plasma

An aliquot of 0.1 mL was pipette out from each of the working stock solutions of rhein and transferred in separate 5 ml screw capped tubes and 4.9 mL of human plasma was added in each tube to yield rhein concentrations of 0.1, 0.5, 1.0, 2.0, 4.0, 6.0 µg/mL.

Liquid-liquid extraction method was adopted for the extraction of rhein from plasma. The procedure of the same is as follows[6]:

- 0.25 ml of each plasma sample was transferred to separate prelabelled ria vials.
- 25 µl of FNB (ISTD) working stock solution (100 µg/mL) was added to all samples except plasma blank and vortexed for about 30 seconds.
- Then 25 µl of 3.5% perchloric acid (prepared by 20 times dilution of 70% perchloric acid) was added to all samples and vortex-mixed for 60 seconds.
- 2 ml of ethyl acetate was added to all samples and vortexed for 5 min.
- The samples were centrifuged at 4000 rpm at 4 °C for 10 min.
- Organic layer was carefully transferred to prelabelled ria vials after freezing the aqueous layer at -20°C and the samples were evaporated to dryness at 40 °C under the gentle stream of nitrogen.
- The dried residues were further reconstituted with 50 µl of mobile phase and vortexed for 30 seconds.

Six replicates of 20 µL volume of each concentration of calibration curve standard solutions were injected in the HPLC system under the chromatographic conditions described earlier in Section 3.1.3.1. The same procedure was repeated for three times. Fig. 3.8 (A) represents the overlay chromatogram of rhein calibration curve standard solutions recovered from plasma in the range of 0.1-6.0 µg/mL.

3.1.5.3.5 Preparation of un-extracted standard rhein samples

100 µl of respective spiking solutions of rhein were transferred in separate pre-labeled ria vials and 500 µl of FNB (ISTD) working stock solution (100 µg/mL) was added in the respective vials and vortex-mixed. Volume was made up to 1000 µl by adding another 400 µl of mobile phase and vials were vortexed for about 30 seconds.

3.1.5.4 Validation of bioanalytical RP-HPLC method for estimation of rhein in plasma

The proposed method was validated for estimation of rhein in plasma as per the standard guidelines[17-21].

3.1.5.4.1 System suitability study

System suitability analysis was carried out by injecting 20 µL of each six consecutive injections of extracted plasma sample having concentration at 2 µg/mL of rhein during the start of method validation and the start of each day. Various peak parameters like peak area, retention time, tailing factor, theoretical plates, were observed and recorded.

3.1.5.4.2 Linearity and range

The calibration curve was constructed for linearity of rhein in plasma. The concentrations of standards were calculated by plotting rhein concentration on x-axis and area ratio (drug/ISTD) on y-axis. Calibration curve obtained was found to be linear over the concentration range of 0.1 – 6.0 µg/mL for rhein (i.e. 0.1, 0.5, 1.0, 2.0, 4.0, 6.0 µg/mL). Regression analysis has been carried out on resultant linearity graphs and regression equation and correlation coefficient were calculated. The same experiment was again repeated for two more times. The percent recoveries and % RSD values for each rhein standards across the calibration concentration range were calculated.

3.1.5.4.3 Precision

Precision was estimated by the determination of the repeatability of the method. Repeatability was assessed using six determinations at each of three different test concentrations of rhein (0.1, 2 and 6 µg/mL -covering the specified range of the method), in a day for Intra-day precision and on three consecutive days for Inter-day precision. A volume of 20 µL of each test solution was injected into HPLC and chromatograms were recorded.

3.1.5.4.4 Accuracy

Accuracy of the method was confirmed by recovery study. The recovery of rhein was determined by comparing the response of three quality control plasma samples of rhein (i.e. 0.1, 2 and 6 µg/mL) with the response of identical rhein standards prepared in the mobile phase which did not undergo sample pre-treatment. Six replicates of each level of concentration were injected and chromatograms recorded. Intra-day and inter-day accuracy studies were performed and % recoveries were calculated for each level of

concentration.

3.1.5.4.5 Absolute Recovery

Absolute recovery of rhein from plasma was determined by comparing three levels of QC concentration at 0.1, 2 and 6 µg/mL of both un-extracted rhein standard samples and extracted plasma samples of rhein. Both types of samples were pre-spiked with ISTD. A 20 µl volume of each six replicates of both type of samples were injected in the HPLC system and chromatogram recorded. The % mean recovery and % RSD for rhein and ISTD were calculated.

3.1.5.4.6 Stability

The stability of rhein in plasma was determined using two concentration level of quality control samples (i.e. 0.1 and 6 µg/mL). The freeze and thawed stability of above said QC samples were tested after third freeze-thawed cycle, where the first storage of 24 hrs at -70°C was followed by two additional periods of 12 hrs. The stability was determined by comparing the mean of calculated concentration of drug from the three freeze-thawed samples with that of freshly thawed quality control samples.

The bench top stability was determined at room temperature using two concentration level of QC samples (i.e. 0.1 and 6 µg/mL) by comparing the mean of calculated concentrations from the freshly thawed QC samples of those were kept on bench top for about 6 hrs.

A volume of 20 µl of six replicates of each level of concentration were injected in the LC system and chromatograms recorded. The % mean concentrations and %RSD of both QC samples were calculated.

3.1.5.5 Result and discussion

System suitability study was carried out with six replicate injections of middle QC sample (i.e. 2 µg/mL) in the HPLC system and retention time of rhein and FNB were observed at 7.11 ± 0.05 min 4.64 ± 0.06 min respectively. The % RSD for the area under curve of chromatographic peak of rhein was calculated and found to be 0.28 which indicated the minimal variation in peak area. The asymmetry factor and theoretical plate counts were in agreement with the standard limits[17,18,20,21]. Different system suitability parameters are summarized in Table 3.22.

The calibration curve of rhein in plasma was obtained by plotting the peak area ratio of rhein standards and FNB (ISTD) on y-axis versus concentration of rhein on x-axis. Table

3.23, represents the calibration data for the estimation of rhein in plasma using RP-HPLC. Fig. 3.8(A) represents the overlay chromatogram of rhein in the linear range. The calibration curve was found to be linear in the range of 0.1–6.0 µg/mL for rhein (Fig. 3.8(B)). The slope and intercepts with low values of standard deviation and correlation coefficient value near 1.0 confirmed the linear relationship between peak area and concentration of rhein (Table 3.25). The percent recoveries and % RSD values for each rhein standards across the calibration concentration range were calculated and found to be in agreement with the acceptance criteria. The precision and mean accuracy observed for the calibration curve standards of rhein were ranged from 0.53% to 1.15% and 98.45% to 100.56% respectively, which are within acceptance limit of 2.0 for precision and 85 to 115% for accuracy. (Table3.24)

Table 3.16 System suitability parameters of RP-HPLC method for rhein in plasma.

Parameters	Observation
Peak area*	175.26
% RSD of peak area	0.28
Retention Time (minutes) ± SD*	7.11 ± 0.05 min
Asymmetry ± SD*	1.05 ± 0.04
Theoretical plates ± SD*	8253 ± 12.72

* mean of six determinations.

Table 3.17 Calibration data of RP-HPLC method for estimation of rhein in plasma.

Concentration (µg/mL)	Rhein Peak area*±SD (mV.s)	FNB (ISTD) Peak area*±SD (mV.s)	Area ratio*±SD (Rhein/FNB)
0.1	10.104±0.101	264.119±1.029	0.038±0.001
0.5	45.485±0.416	264.668±0.658	0.172±0.003
1.0	88.506±0.365	265.257±0.786	0.334±0.004
2.0	175.626±0.498	265.908±0.828	0.661±0.003
4.0	348.332±0.695	264.039±0.517	1.319±0.007
6.0	518.008±1.189	264.430±0.833	1.959±0.007

*mean of six determinations.

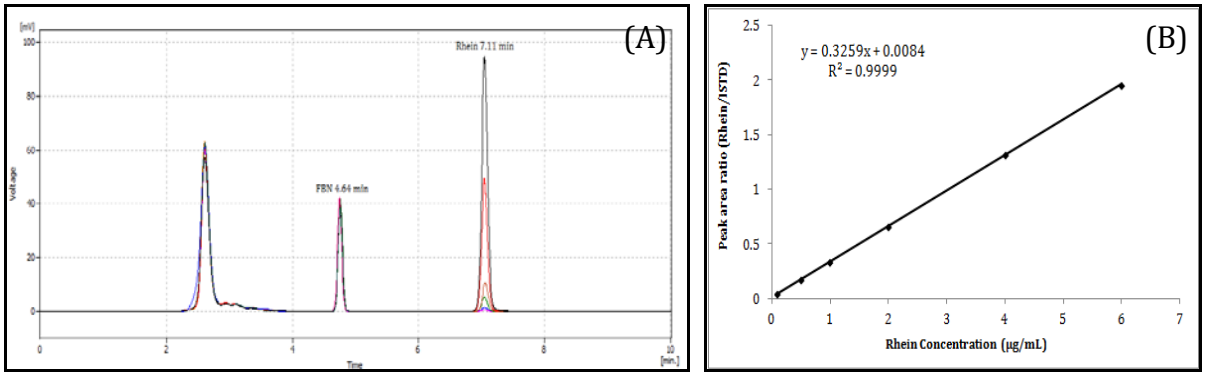


Fig. 3.8 (A) Overlay chromatogram and (B) Calibration curve of rhein in plasma.

Table 3.18 Back calculation of extracted concentration of rhein in plasma by recorded peak area of calibration curve standards.

Actual concentration (µg/mL)	Calculated Concentration* (µg/mL)	%RSD (n=6)	%Accuracy (n=6)
0.1	0.098	1.09	98.45
0.5	0.502	1.15	100.32
1.0	0.998	0.93	99.82
2.0	2.001	0.87	100.05
4.0	4.022	0.53	100.56
6.0	5.985	0.76	99.75

*mean of six determinations.

Table 3.19 Summarized linearity parameters of RP-HPLC method for estimation of rhein in plasma.

Linearity Parameters	Results
Analytical wavelength (nm)	257
Linearity range (µg/mL)	0.1 - 6.0
Slope ± SD*	0.3259 ± 0.001
Intercept ± SD*	0.0084 ± 0.003
Correlation coefficient (R^2)*	0.9999

* mean of six determinations.

The developed method was validated for its intra-day and inter-day precision (3 consecutive days, n=6) at three different test concentrations (0.1, 2.0 and 6.0 µg/mL - covering the specified linear range of the method). The average %RSD of intra-day and inter-day precision were found to be in the range of 1.14% to 5.12% which complies with the standard acceptance limit of 15.0% and confirmed the precision of the method (Table 3.26).

Table 3.20 Intra-day and inter-day precision of RP-HPLC method for estimation of rhein in plasma.

Precision	Concentration Levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
Intra-day precision	0.1	0.103	1.89
	2.0	2.035	3.02
	6.0	6.025	1.39
Inter-day precision	0.1	0.104	5.12
	2.0	2.054	2.04
	6.0	6.082	1.14

* mean of six determinations at each level.

The intra-day and inter-day accuracy (3 consecutive days, n=6) of the proposed HPLC method was checked at 3 level of rhein concentration in plasma (i.e. 0.1 µg/mL, 2.0 µg/mL and 6.0 µg/mL). No interference could be observed with the proposed method.

The acceptable recoveries of rhein in quality control samples with low %RSD justified the high accuracy of the proposed method (Table 3.27).

Table 3.21 Intra-day and inter-day accuracy of RP-HPLC method for estimation of rhein in plasma.

Accuracy	Actual concentration (µg/mL)	Observed concentration* (µg/mL)	% Recovery*	%RSD
Intra-day accuracy	0.1	0.103	102.63	3.14
	2.0	2.066	103.29	3.65
	6.0	6.075	101.25	1.97
Inter-day accuracy	0.1	0.102	101.77	2.55
	2.0	2.033	101.65	2.91
	6.0	6.122	102.03	0.83

* mean of six determinations at each level.

Absolute recoveries of rhein and FNB (ISTD) from plasma were determined. The obtained results are summarized in Table 3.28. The % recoveries (extraction efficiency) of rhein and ISTD from plasma for all the QC samples were more than 90% which is in agreement with the standard guidelines.

Table 3.22 Absolute recovery of rhein and FNB (ISTD) from plasma.

Analyte	Concentration Level (µg/mL)	% Recovery*	%RSD
Rhein	0.1	93.72	1.47
	2.0	95.88	2.19
	6.0	95.11	1.83
FNB (ISTD)	0.1	96.19	1.15
	2.0	97.42	3.06
	6.0	97.89	0.94

* mean of six determinations at each level.

The results of stability of rhein in plasma are shown in Table 3.29. The mean % recoveries of rhein in both QC samples used for stability studies were found within the acceptance limit of 85% to 115%.

Table 3.23 Stability of rhein in plasma used in RP-HPLC method.

Stability conditions	Concentration levels (µg/mL)	Estimated concentration* (µg/mL)	% Recovery	% RSD
Freeze-thaw stability	0.1	0.099	99.94	3.22
	6.0	6.044	100.73	2.94
Bench-top stability	0.1	0.100	100.62	4.74
	6.0	6.113	101.88	1.59

* mean of six determinations at each level.

3.1.5.6 Conclusion

The proposed RP-HPLC method for estimation of rhein in plasma was successfully validated for different parameters as per USFDA and ICH guidelines. The results of the study showed that the developed RP-HPLC was simple, sensitive, accurate and precise and could be used as bioanalytical method for the estimation of DAR in pharmacokinetic study of developed formulations.

3.2 Analytical method development and validation for estimation of Febuxostat (FBX)

Febuxostat (FBX) is another drug chosen for this project. Analytical methods were developed and validated to estimate FBX in developed formulations (i.e. nanosuspension and drug-cyclodextrin inclusion complex), *in vitro* and *in vivo* analysis. Various UV-spectrophotometric[29-31], HPLC[32-36] and LCMS/MS[37] methods are reported for the estimation of FBX in bulk drug and in its formulations. Reports stated that HPLC and LCMS/MS techniques have been efficiently used for the estimation of FBX in plasma (*in vivo* analysis)[38-42].

3.2.1 Materials and reagents

FBX was kindly gifted by Lupin Pharmaceuticals Ltd, Aurangabad, Maharashtra, India. Indomethacin was obtained as a gift sample from Cadila Healthcare Ltd., Ahmedabad, Gujarat. Marketed formulation "Febustat*40", (Febuxostat 40 mg, Abbott Healthcare Pvt. Ltd., Mumbai India) was purchased from local pharmacy. Acetonitrile (HPLC Grade) and Methanol (HPLC Grade) were procured from Merck Chemicals, Mumbai, India. Dimethylsulfoxide (HPLC grade), Triethylamine (TEA) (HPLC grade) Orthophosphoric acid (HPLC Grade), Glacial Acetic Acid (HPLC Grade) and Methyl tertiary butyl ether (MTBE) (HPLC Grade) were purchased from Spectrochem Chemicals (Mumbai, India). Potassium dihydrogen phosphate (AR grade), Sodium dihydrogen phosphate (AR grade), Ammonium acetate (AR grade), HCl (AR grade) and Sodium hydroxide (AR grade) were purchased from S.D. Fine Chemicals, Mumbai, India. Hank's balanced salt solution (HBSS) was purchased from Himedia, Mumbai. Purified HPLC grade water was obtained by filtering double distilled water through nylon filter paper 0.22 µm pore size and 47 mm diameter (Millipore, Bangalore, India). Human plasma was obtained free of cost from Suraktam Blood Bank, Vadodara, Gujarat.

3.2.2 Analytical methods for estimation of FBX by Ultraviolet (UV) Spectroscopy

Various simple, sensitive and accurate UV methods were developed for estimation of actual amount of FBX from its formulations.

3.2.2.1 Instrument and condition

Refer Section 3.1.2.1.

3.2.2.2 Standard solution preparation

3.2.2.2.1 Standard stock solution of FBX

50 mg of FBX was weighed accurately and carefully transferred to the 50 mL volumetric flask. About 35 mL of methanol was added to the volumetric flask and sonicated for 2-3 minutes to dissolve the FBX. The volume was made up to the 50 mL mark with methanol to obtain standard stock solution (1000 µg/mL).

3.2.2.2.2 Working stock solution of FBX

An aliquot (2 mL) of standard stock solution of FBX was transferred to a 50 mL volumetric flask and volume was made up to the mark with methanol (MeOH) to get working stock solution (40 µg/mL).

The standard stock solution and working stock solution were stored at 2-8°C till further use.

3.2.2.3 Calibration curves of FBX in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by Ultraviolet (UV) Spectroscopy

3.2.2.3.1 Calibration curve of FBX in methanol

The analysis was performed by first scanning FBX test solution (8 µg/mL) in methanol in the UV range between 200-400 nm against methanol as blank and determining its absorbance maxima (λ_{max}). Suitable aliquots of the working stock solution of FBX ranging from 0.25 mL to 4.0 mL were pipette out and transferred into 10 mL volumetric flasks and volume was made up to 10 mL mark with methanol to prepare final concentrations in the range of 1-16 µg/mL. Solutions were mixed well and their absorbances were measured at λ_{max} using methanol as blank. Calibration curve was constructed by plotting absorbance versus concentration of FBX and regression equation was calculated. The same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.2.2.3.2 Calibration curve of FBX in distilled water

Same procedure, as described in previous section was followed using distilled water as diluent. Calibration curve was constructed by plotting absorbance versus concentration of FBX and regression equation was calculated. The same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.2.2.3.3 Calibration curve of FBX in phosphate buffer pH-6.8

Same procedure, as described in previous section was followed using phosphate buffer pH-6.8 as diluent. Calibration curve was constructed by plotting absorbance versus concentration of FBX and regression equation was calculated. The same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.2.2.3.4 Calibration curve of FBX in acetate buffer pH-4.5

Same procedure, as described in previous section was followed using acetate buffer pH-4.5 as diluent. Calibration curve was constructed by plotting absorbance versus concentration of FBX and regression equation was calculated. The same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.2.2.3.5 Calibration curve of FBX in 0.1N HCl

Same procedure, as described in previous section was followed using 0.1N HCl as diluent. Calibration curve was constructed by plotting absorbance versus concentration of FBX and regression equation was calculated. The same procedure was repeated for six times using freshly prepared working stock solution every time. The method was validated for linearity, accuracy and precision.

3.2.2.4 Analytical method validation

The methods were validated for linearity, accuracy and precision as per standard guidelines[19-21] using respective diluents as blank.

3.2.2.5 Results and discussion

FBX yields a characteristic spectrum when scanned in the ultraviolet range between 200 nm and 400 nm. In all the media, FBX showed absorption maximum at 315 nm and this wavelength was chosen as analytical wavelength. Beer's law was obeyed between 1 and 16 µg/mL. Regression analysis was performed on the experimental data. Correlation coefficient for developed methods were found to be 0.9999 (methanol), 0.9999

(distilled water), 0.9998 (phosphate buffer pH 6.8), 0.9999 (acetate buffer pH 4.5) and 0.9998 (0.1N HCl). The value of correlation coefficient indicated the linear relationship between absorbance and concentration of FBX in each media (Table 3.31)

Table 3.30 show the summary of calibration data for FBX in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl.

Fig. 3.9(A), 3.10(A), 3.11(A), 3.12(A) and 3.13(A) show the overlay spectra of FBX standards for linearity in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl, respectively.

Fig. 3.9(B), 3.10(B), 3.11(B), 3.12(B) and 3.13(B) indicate the linearity graph of FBX standards for linearity in Methanol, Distilled Water, Phsphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl, respectively.

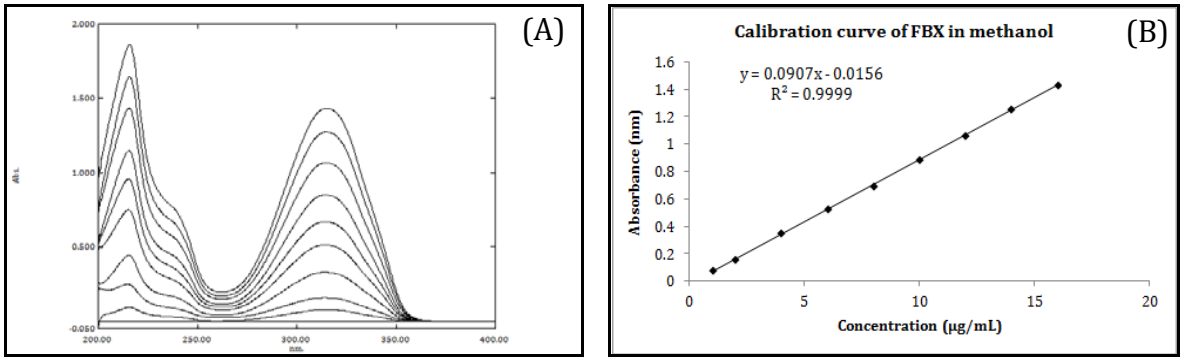


Fig. 3.9 (A) Overlay spectra and (B) Calibration curve of FBX in Methanol.

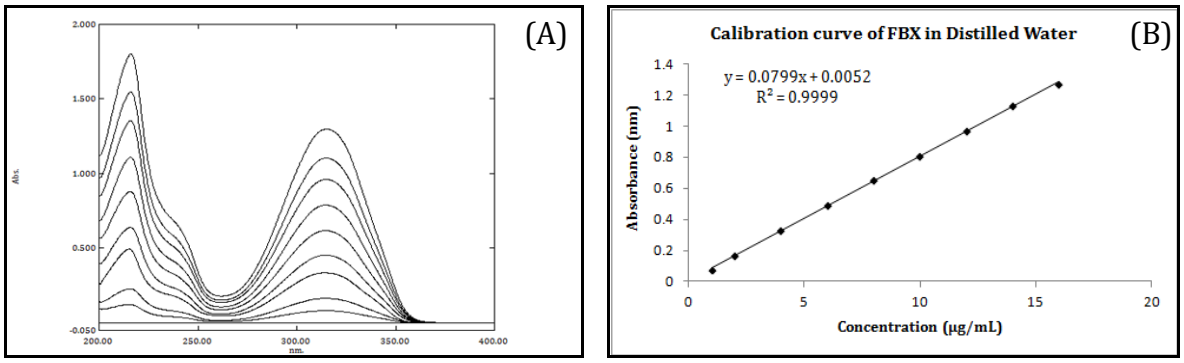


Fig. 3.20 (A) Overlay spectra and (B) Calibration curve of FBX in Distilled water.

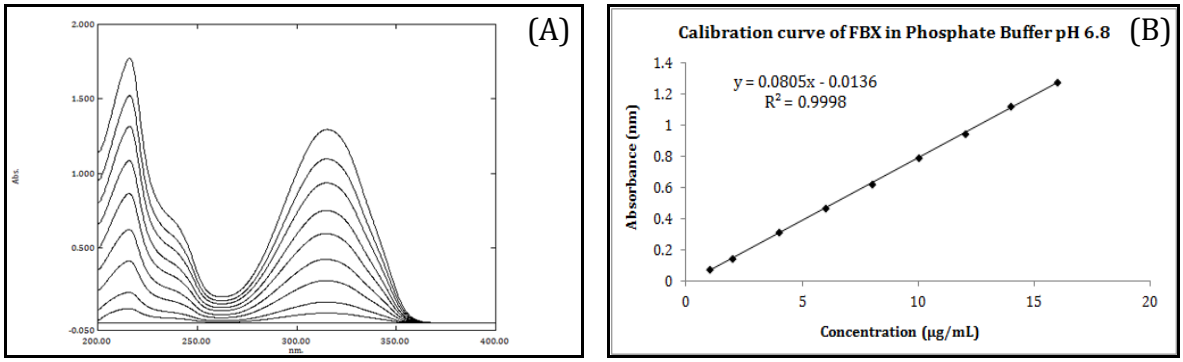


Fig. 3.31 (A) Overlay spectra and (B) Calibration curve of FBX in Phosphate Buffer pH 6.8.

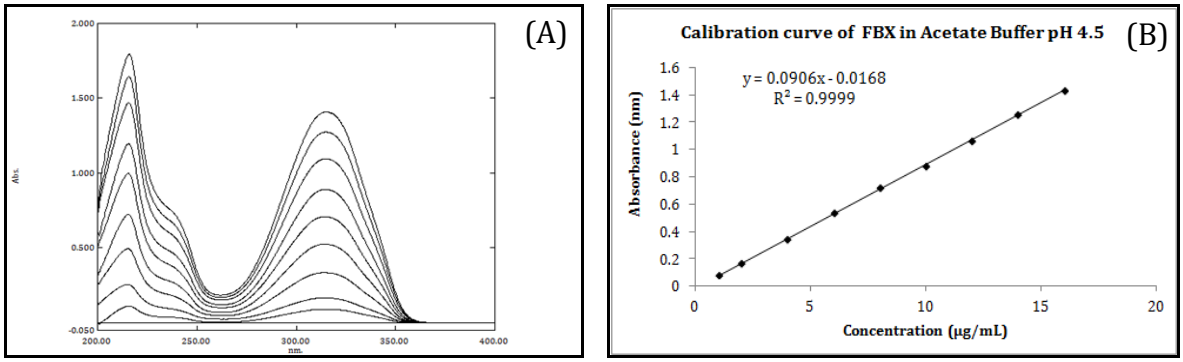


Fig. 3.42 (A) Overlay spectra and (B) Calibration curve of FBX in Acetate Buffer pH 4.5.

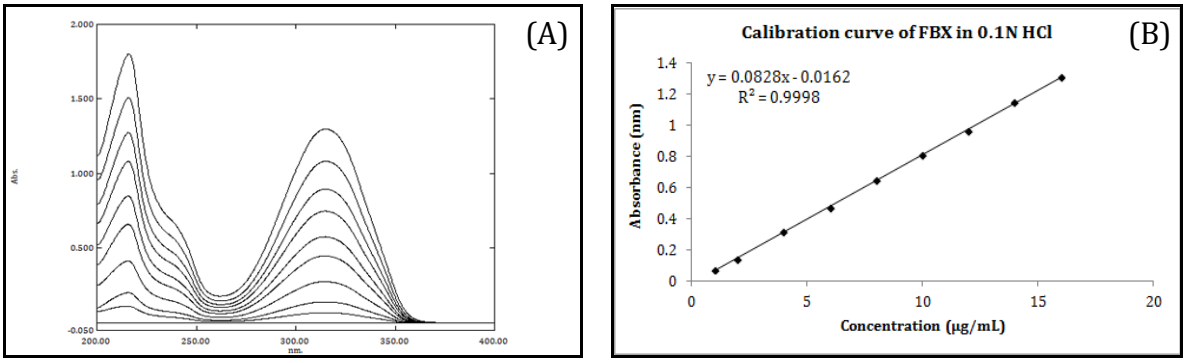


Fig. 3.53 (A) Overlay spectra and (B) Calibration curve of FBX in 0.1N HCl.

Table 3.31 show the parameters indicating linearity for the used UV spectrophotometric method of analysis for FBX in methanol, distilled water, phosphate buffer pH 6.8, acetate buffer pH 4.5 and 0.1N HCl. Table 3.32 represents the LOD and LOQ values for FBX in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy. Table 3.33 and 3.34 demonstrate the intra-day and inter-day precision and accuracy for the FBX assay by UV spectroscopy, respectively. The low % RSD values indicate precision of the method. No significant difference between the amount of drug added (actual) and observed concentration was noticed indicating

accuracy of the method[19-21].

The interference studies with formulation excipients studies were carried out and no difference in absorbance was observed at 315 nm.

Table 3.24 Summary of calibration data for FBX in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Conc. (µg/mL)	(Mean Absorbance ± SD)*				
	Methanol	**Distilled Water**	**Phosphate Buffer pH-6.8**	**Acetate Buffer pH-4.5**	**0.1N HCl**
1	0.080±0.002	0.075±0.001	0.072±0.001	0.078±0.001	0.075±0.001
2	0.160±0.003	0.164±0.002	0.148±0.002	0.165±0.002	0.146±0.002
4	0.353±0.005	0.331±0.002	0.310±0.001	0.339±0.005	0.318±0.003
6	0.534±0.002	0.486±0.003	0.468±0.003	0.530±0.005	0.472±0.006
8	0.693±0.005	0.651±0.004	0.619±0.006	0.715±0.007	0.648±0.004
10	0.892±0.012	0.807±0.003	0.793±0.004	0.877±0.005	0.811±0.007
12	1.070±0.008	0.968±0.006	0.947±0.005	1.065±0.007	0.967±0.009
14	1.256±0.012	1.127±0.006	1.118±0.004	1.258±0.007	1.149±0.007
16	1.439±0.006	1.273±0.021	1.278±0.007	1.435±0.017	1.314±0.006

*mean of six determinations.

Table 3.25 Summary of linearity parameters for FBX in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Linearity parameters	Media				
	Methanol	**Distilled Water**	**Phosphate Buffer pH-6.8**	**Acetate Buffer pH-4.5**	**0.1N HCl**
λ_{max}	315 nm				
Linearity range	1-16 µg/mL				
Slope ± SD*	0.0906 ±0.0003	0.0799 ±0.0006	0.0805 ±0.0004	0.0906 ±0.0005	0.0961 ±0.0002
Intercept ± SD*	0.0155 ±0.0006	0.0052 ±0.0033	0.0136 ±0.0009	0.0168 ±0.0017	0.0038 ±0.0014
Correlation Coefficient (R^2)*	0.9999	0.9999	0.9998	0.9999	0.9998

*mean of six determinations.

Table 3.32 Summary of LOD and LOQ values for FBX in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Parameters	Media				
	Methanol	**Distilled Water**	**Phosphate Buffer pH-6.8**	**Acetate Buffer pH-4.5**	**0.1N HCl**
LOD* (µg/mL)	0.021	0.136	0.039	0.066	0.055
LOQ* (µg/mL)	0.063	0.413	0.121	0.201	0.166

*mean of six determinations.

Table 3.33 Summary of intra-day precision and accuracy for FBX in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Media	Standard Concentration (µg/mL)		Precision§ (%)	Accuracy† (%)
	Actual	Observed*		
Methanol	2	2.037	1.02	101.83
	8	8.15	0.44	101.88
	16	16.15	0.33	100.94
Distilled water	2	2.016	1.25	100.80
	8	8.11	1.69	101.38
	16	16.25	1.61	101.56
Phosphate Buffer pH-6.8	2	2.056	1.01	102.83
	8	8.133	1.35	101.67
	16	16.123	0.56	100.77
Acetate Buffer pH-4.5	2	2.003	0.58	100.17
	8	8.090	1.24	101.13
	16	16.117	1.49	100.73
0.1N HCl	2	2.027	1.59	101.33
	8	8.043	0.95	100.54
	16	16.20	1.52	101.25

*mean of six determinations.
§ Expressed as % relative standard deviation (%RSD)
† Expressed as % recovery.

Table 3.34 Summary of inter-day precision and accuracy for FBX in Methanol, Distilled Water, Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1N HCl by UV spectroscopy.

Media	Standard Concentration (µg/mL)		Precision§ (%)	Accuracy† (%)
	Actual	Observed*		
Methanol	2	2.046	1.49	102.33
	8	8.126	0.87	101.58
	16	16.137	1.05	100.85
Distilled water	2	2.033	1.02	101.67
	8	8.036	1.12	100.46
	16	16.21	0.51	101.31
Phosphate Buffer pH-6.8	2	2.043	1.57	102.17
	8	8.206	0.93	102.58
	16	16.216	1.16	101.35
Acetate Buffer pH-4.5	2	1.996	1.53	99.83
	8	8.053	0.75	100.67
	16	16.133	1.60	100.83
0.1N HCl	2	2.023	1.51	101.16
	8	8.090	1.24	101.13
	16	16.083	1.26	100.52

*mean of six determinations.
§ Expressed as % relative standard deviation (%RSD)
† Expressed as % recovery.

3.2.2.6 Conclusion

The proposed methods were rapid, economical, accurate, precise and sensitive for the determination of FBX. These methods were later used for estimation of FBX in intermediate studies during formulation development of FBX.

3.2.3 Reverse Phase High Performance Liquid Chromatography (RP-HPLC) methods for estimation of FBX in formulations

3.2.3.1 Instrument and chromatographic conditions

Instrument description was same as described in Section 3.1.3.1.

The chromatographic separation and quantitation was performed using a Phenomenex Hypersil BDS C18 (150 mm×4.6 mm i.d., 5 µm particle size) column attached with Phenomenex SecurityGuardStandard with C18 cartridge (4.0×3.0)mm. Separation was attained using a mobile phase consisting of acetonitrile and 0.05M ammonium acetate buffer (pH-5.0) in the ratio of 50:50 (v/v) with 0.1% of triethylamine, pumped at a flow rate of 1.0 ml min^{-1}. The eluent was monitored using UV detector at a wavelength of 315 nm. The column was maintained at ambient and an injection volume of 20 µL was used. The mobile phase was vacuum filtered through 0.22 µm nylon membrane filter followed by degassing in an ultrasonic bath prior to use.

Optimized chromatographic conditions:

Column	:	Phenomenex Hypersil BDS C18, (150 x 4.6)mm, 5µm.
Flow	:	1.0 ml/min
Wavelength	:	315 nm
Injection volume	:	20µl
Column oven temperature	:	Ambient
Runtime	:	10 mins

Injections of 20 µl were made for each sample concentration and chromatographed under the condition described above. The method was validated as per ICH guidelines.

3.2.3.2 Preparation of experimental solutions

3.2.3.2.1 Preparation of 0.05M acetate buffer solution pH-5.0

Accurately weighed and dissolved 3.85 gm ammonium acetate in 1000 mL double distilled water and pH-5.0 was adjusted with glacial acetic acid. Resultant buffer solution was filtered through 0.2µ nylon membrane filter.

3.2.3.2.2 Preparation of mobile phase

A mixture of 0.05M acetate buffer solution pH-5.0 and acetonitrile was prepared in the

ratio of (50:50) v/v with 0.1% triethylamine (TEA), mixed well and degassed in an ultrasonic bath prior to use.

3.2.3.2.3 Diluent: Mobile phase.

3.2.3.2.4 Preparation of standard stock solution

Accurately weighed and transferred 50mg of FBX working standard (API) into 50ml volumetric flask. Approximately 25 ml methanol was added and sonicated to dissolve the solid content of FBX. Final volume was made up to the mark with methanol and mixed well. The concentration of resultant solution was 1000 µg/mL.

3.2.3.2.5 Preparation of working stock solution

Working stock solution was prepared by diluting 5 mL aliquot of standard stock solution to 50 mL with diluent in a 50 mL volumetric flask to produce a working stock solution of FBX having concentration of 100 µg/mL.

3.2.3.3 Calibration curve of FBX

Suitable aliquots of the working stock solution of FBX ranging from 0.1 mL to 3.0 mL were pipette out and transferred into 10 mL volumetric flasks and volume was made up to 10 mL mark with diluent to prepare final concentrations in the range of 1-30 µg/mL. Solutions were mixed well using vortex mixer before injecting in the HPLC. Injections of 20 µl were made for each concentration and chromatographed under the condition described above. Each of these drug solutions was injected three times into the column and the peak area and retention times were recorded. Calibration curve was constructed by plotting area under curve versus concentration of FBX and regression equation was calculated. The same procedure was repeated for three times using freshly prepared working stock solution every time.

3.2.3.4 Validation of developed RP-HPLC method for estimation of FBX

Validation of developed RP-HPLC method was performed as per the recommendations of USP and ICH (i.e. ICH guidelines Q2 (R1))[20,21]. The method was validated with respect to the parameters including system suitability, linearity, LOD, LOQ, precision, accuracy and robustness (**Section 3.1.3.5**).

3.2.3.5 Result and discussion

The RP-HPLC method was developed for the quantitation of FBX using a Phenomenex Hypersil BDS C18 (150 mm × 4.6 mm i.d., 5 µm particle size) column. Separation was attained using a mobile phase consisting of acetonitrile and 0.05M ammonium acetate buffer (pH-5.0) in the ratio of 50:50 (v/v) with 0.1% of triethylamine, pumped at a flow

rate of 1.0 ml min^{-1}. The eluent was monitored using UV detector at a wavelength of 315 nm. The column was maintained at ambient and an injection volume of 20 µL was used. System suitability study was performed with six replicate injections of standard solution of FBX (15 µg/mL) in the HPLC system and retention time of FBX was observed at 4.18±0.03 min. The % RSD for the area under curve of chromatographic peak of FBX was calculated and found to be 0.83 which indicated the minimal variation in peak area. The asymmetry factor and theoretical plate counts were in agreement with the standard limits[20,21]. Various system suitability parameters are summarized in Table 3.35.

Table 3.26 System suitability parameters for FBX using developed RP-HPLC method.

Parameters	Observation
Peak area (mV.s)*	773.692
% RSD of peak area	0.83
Retention Time (minutes) ± SD*	4.18±0.03
Asymmetry ± SD*	1.63±0.02
Theoretical plates ± SD*	7593.22±11.66

* mean of six determinations.

The calibration curve of FBX constructed by plotting the observed peak area of FBX standards (y-axis) versus concentration of FBX (x-axis) and found to be linear in the range of 1-30 µg/mL (Table 3.37, Fig. 3.14(B)). Fig. 3.14(A) represents the overlay chromatogram of FBX in the linear range. The low value of correlation coefficient (0.9999) confirmed the linear relationship between peak area and concentration of FBX. The RSD based on the peak area for six injections were found to be less than 2.0 for calibration curve (Table 3.36). The slope and intercepts with low values of standard deviation were obtained (Table 3.37).

Table 3.27 Calibration data of RP-HPLC method for estimation of FBX in formulations.

Concentration (µg/mL)	Mean peak area (mV.s)	%RSD
1	49.616	1.64
2.5	140.357	0.92
5.0	259.03	1.33
10.0	517.083	0.99
15.0	772.613	0.89
20.0	1026.167	1.03
25.0	1293.463	0.46
30.0	1543.517	0.28

* mean of six determinations.

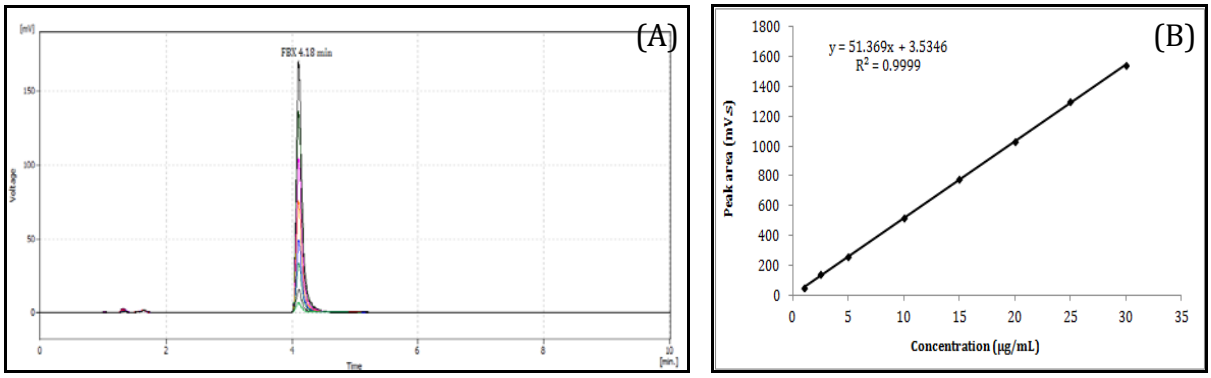


Fig. 3.14 (A) Overlay chromatogram and (B) Calibration curve of FBX in mobile phase.

Table 3.28 Summarized linearity parameters of RP-HPLC method for estimation of FBX in formulations.

Linearity Parameters	Results
Analytical wavelength (nm)	315
Linearity range (µg/mL)	1-30
Slope ± SD*	51.36±0.15
Intercept ± SD*	3.53±1.82
Correlation coefficient (R^2)*	0.9999

* mean of six determinations.

The developed method was validated for its intra-day and inter-day precision (3 consecutive days, n=6) at three different test concentrations (1, 15 and 30 µg/mL - covering the specified linear range of the method). The average %RSD of intra-day and inter-day precision were found to be less than 2 which complies with the specified limit and confirm the precision of the method (Table 3.38).

Table 3.29 Intra-day and inter-day precision of RP-HPLC method for estimation of FBX in formulations.

Precision	Concentration Levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
Intra-day precision	1	1.02	1.49
	15	15.29	0.94
	30	30.52	0.66
Inter-day precision	1	1.00	1.66
	15	15.13	0.96
	30	30.71	0.73

* mean of six determinations at each level.

The intra-day and inter-day accuracy (3 consecutive days, n=6) of the proposed method was checked at 3 level of standard addition (80%, 100%, and 120%). No interference could be observed with the proposed method. The excellent recoveries of standard addition method with low SD and %RSD justified the high accuracy of the proposed method (Table 3.39).

Table 3.30 Intra-day and inter-day accuracy of RP-HPLC method for estimation of FBX in formulation.

Concentration of test solution used for standard addition=10 µg/mL					
Accuracy	**Excess drug added to test solution (%)**	**Actual concentration (µg/mL)**	**Estimated concentration* (µg/mL)**	**% Recovery* ±SD**	**%RSD**
Intra-day accuracy	0	10	10.14	101.43±0.08	0.79
	80	18	18.36	101.98±0.13	0.73
	100	20	20.38	101.88±0.28	1.35
	120	22	22.18	100.80±0.17	0.84
Inter-day accuracy	0	10	10.06	100.63±0.18	1.77
	80	18	18.21	101.17±0.09	0.48
	100	20	20.19	100.95±0.19	0.93
	120	22	22.29	101.32±0.18	0.79

* mean of six determinations at each level.

The LOQ and LOD were determined based on a signal to noise (S/N) ratios and were based on analytical responses of 10 and 3 times the background noise respectively. The LOD and LOQ were found to be 0.12 µg/ml and 0.35 µg/ml respectively (Table 3.40).

Table 3.31 LOD and LOQ of RP-HPLC method for estimation of FBX in formulation.

Parameters*	**Results**
LOD (µg/mL)	0.12
LOQ (µg/mL)	0.35

* mean of six determinations.

Robustness and ruggedness studies were performed for pH of buffer, flow rate, mobile phase composition, acetonitrile of two different manufacturers and two separate HPLC instruments. A 20 µl volume of each six replicates of QC concentration (10 µg/mL) were injected in the chromatographic system for every variation. Results, presented in Table 3.41 indicate that the selected factors remained unaffected by small variation of these parameters. It was also found to that acetonitrile of different lots from the same manufacture has no significant influence on the determination. Insignificant difference in asymmetric factor and less variability in retention time were observed.

Table 3.32 Robustness and ruggedness of RP-HPLC method for estimation of FBX in formulation.

Variable conditions	Levels	Estimated Factors	
		Mean Retention Time of FBX (min)*	**Mean Asymmetric factor of FBX peak***
pH of buffer	4.9	4.229	1.618
	5.0	4.182	1.623
	5.1	4.188	1.635
	Mean ± SD	4.19±0.03	1.63±0.01
Flow rate (mL/min)	0.95 mL/min	4.229	1.661
	1.00 mL/min	4.188	1.639
	1.05 mL/min	1.172	1.625
	Mean ± SD	4.19±0.03	1.64±0.02
% of ACN in mobile phase (%v/v)	49 % v/v	4.271	1.627
	50 % v/v	4.186	1.647
	51 % v/v	4.164	1.618
	Mean ± SD	4.21±0.06	1.63±0.01
ACN of different manufacturer	Merck, India	4.189	1.642
	Spectrochem, India	4.216	1.627
	Mean ± SD	4.20±0.02	1.63±0.01
Two different instrument	Instrument 1	4.185	1.621
	Instrument 2	4.258	1.647
	Mean ± SD	4.22±0.05	1.63±0.02

* mean of six determinations.

Stability study for solution of FBX was performed using three levels of QC concentration (i.e. 1, 15 and 30 µg/mL) at room temperature and at 5 °C in refrigerator for 48 hours. Six replicates of each concentration were injected in the HPLC system and %RSD for peak areas was calculated. No significant decrease in estimated concentration and low %RSD values were indicating the stability of FBX in the sample solutions prepared in mobile phase (Table 3.42)

Table 3.33 Stability of FBX solutions used in RP-HPLC method for estimation of drug in formulations.

Storage conditions	Concentration levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
At room temperature for 48 hours	1	1.08	1.85
	15	15.10	1.65
	30	30.94	1.37
At 5 °C for 48 hours	1	1.03	1.12
	15	15.06	1.18
	30	30.55	1.11

* mean of six determinations at each level.

3.2.3.6 Conclusion

The developed RP-HPLC method for estimation of FBX was successfully validated for different parameters as per ICH guidelines. The results of the study showed that the developed RP-HPLC was simple, rapid, economical, sensitive, accurate and precise and could be used for estimation of FBX in its new pharmaceutical dosage forms.

Developed RP-HPLC method was successfully applied for the estimation of FBX in newly developed formulation (nanosuspension and cyclodextrin inclusion complex) and could be used for the estimation of FBX from matrix (plasma), required for pharmacokinetic study to estimate the amount of drug in systemic circulation. Further, the developed HPLC method could also be significantly used for determination of FBX in transport medium used for *in vitro* gastro-intestinal permeability study using Caco-2 cell model.

3.2.4 RP-HPLC method for estimation of FBX in transport buffer (HBSS-Hank's balanced salt solution) used for in vitro gastro-intestinal permeability study using Caco-2 cell model

3.2.4.1 Instrument and chromatographic conditions

Same as described in **Section 3.2.3.2.**

3.2.4.2 Preparation of experimental solutions

3.2.4.2.1 Preparation of FBX standard stock solution

Accurately weighed and transferred 10mg of FBX working standard (API) into 10ml volumetric flask. Approximately 5 ml DMSO was added and sonicated to dissolve the FBX. Final volume was made up to the mark with DMSO and mixed well. The concentration of resulting solution was 1000 µg/mL.

3.2.4.2.2 Preparation of FBX working stock solution

An aliquot of 2.5 mL of standard stock solution of FBX was pipette out in a 25 mL volumetric flask and diluted up to the 25 mL mark with HBSS buffer to produce a working stock solution of FBX having concentration of 100 µg/mL.

3.2.4.3 Calibration curve of FBX in HBSS buffer

Suitable aliquots of the working stock solution of FBX ranging from 0.05 mL to 3.0 mL were pipette out and transferred into 10 mL volumetric flasks and volume was made up to 10 mL mark with HBSS to prepare final concentrations in the range of 0.5 – 30 µg/mL. Solutions were mixed well using vortex mixer before injecting in the HPLC. Injections of 20 µl were made for each concentration and chromatographed under the condition described earlier. Each of these drug solutions was injected three times into

the column and the peak area and retention times were recorded. Calibration curve was constructed by plotting area under curve versus concentration of FBX and regression equation was calculated. The same procedure was repeated for three times using freshly prepared working stock solution every time.

3.2.4.4 Validation of the RP-HPLC method for the determination of FBX in HBSS buffer

Method validation was performed based on both USP and International Conference on Harmonization (ICH) guidelines for the validation of analytical methods[20,21]. Method was validated for the parameters such as system suitability, linearity and range, precision, accuracy. LOD and LOQ and stability studies of FBX in HBSS buffer, as described in Section 3.1.3.5.

Freshly prepared quality control samples in HBSS buffer at different concentrations of FBX were used for the validation of this method.

3.2.4.5 Result and discussion

Chromatographic conditions were same as described earlier in Section 3.2.3.2.

System suitability study was carried out with six replicate injections of standard solution of FBX in HBSS (15 µg/mL) in the HPLC system and retention time of FBX was observed at 4.22 ± 0.03 min. The % RSD for the area under curve of chromatographic peak of FBX was calculated and found to be 1.47 which indicated the minimal variation in peak area. The asymmetry factor and theoretical plate counts were in agreement with the standard limits[20,21]. Different system suitability parameters are summarized in Table 3.43.

Table 3.34 System suitability parameters of RP-HPLC method for estimation of FBX in HBSS buffer.

Parameters	Observation
Peak area*	752.096
% RSD of peak area	1.47
Retention Time (minutes) ± SD*	4.22±0.03
Asymmetry ± SD*	1.69±0.02
Theoretical plates ± SD*	7575.44±12.69

* mean of six determinations.

The calibration curve of FBX in HBSS buffer was obtained by plotting the peak area of FBX standards (y-axis) versus concentration of FBX (x-axis) and found to be linear in the range of 0.5-30 µg/mL (Table 3.45, Fig. 3.15 (B)). Fig. 3.15 (A) represents the overlay chromatogram of FBX in the linear range. The low value of correlation

coefficient (0.9999) confirmed the linear relationship between peak area and concentration of FBX. The RSD based on the peak area for six injections were found to be less than 2.0 for calibration curve (Table 3.44). The slope and intercepts with low values of standard deviation were obtained (Table 3.45).

Table 3.35 Calibration data of RP-HPLC method for estimation of FBX in HBSS buffer.

Concentration (µg/mL)	Peak area* (mV.s)	%RSD
0.5	29.161	1.23
1.0	47.58	1.64
2.5	135.374	0.92
5.0	240.696	1.33
10.0	503.931	0.99
15.0	748.627	0.89
20.0	1004.428	1.03
25.0	1248.497	0.46
30.0	1490.515	0.79

* mean of six determinations.

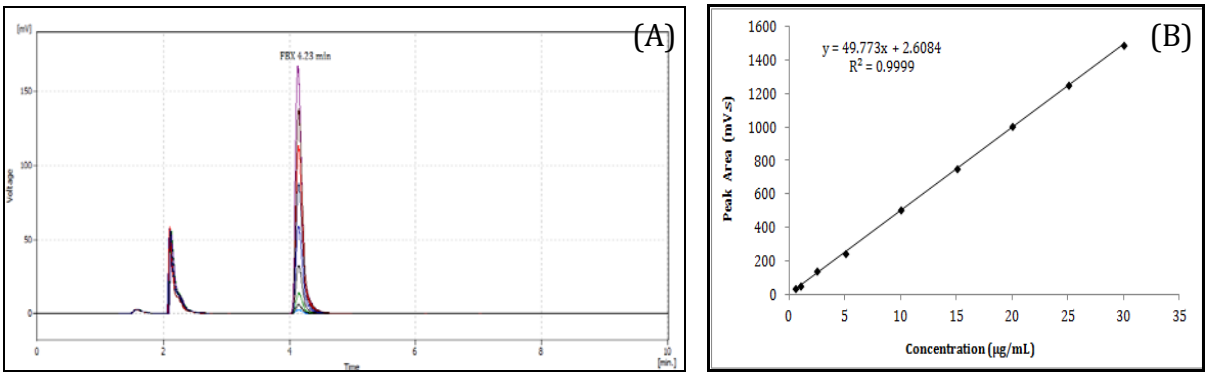


Fig. 3.15 (A) Overlay chromatogram and (B) Calibration curve of FBX in HBSS buffer.

Table 3.36 Summarized linearity parameters of RP-HPLC method for estimation of FBX in HBSS buffer.

Linearity Parameters	Results
Analytical wavelength (nm)	315
Linearity range (µg/mL)	0.5-30
Slope ± SD*	49.777±0.196
Intercept ± SD*	2.608±0.469
Correlation coefficient (R^2)*	0.9999

* mean of six determinations.

The developed method was validated for its intra-day and inter-day precision (3 consecutive days, n=6) at three different test concentrations (1, 15 and 30 µg/mL - covering the specified linear range of the method). The average %RSD of intra-day and inter-day precision were found to be less than 2 which complies with the standard guidelines and confirmed the precision of the method (Table 3.46).

Table 3.37 Intra-day and inter-day precision of RP-HPLC method for estimation of FBX in HBSS buffer.

Precision	Concentration Levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
Intra-day precision	1	0.99	1.54
	15	15.36	0.57
	30	30.82	0.47
Inter-day precision	1	1.01	1.30
	15	15.20	1.28
	30	30.38	0.53

* mean of six determinations at each level.

The intra-day and inter-day accuracy (3 consecutive days, n=6) of the proposed HPLC method was checked at 3 level of FBX concentration in HBSS buffer (50%, 100% and 150% i.e. 10 µg/mL, 20 µg/mL and 30 µg/mL). No interference could be observed with the proposed method. The excellent recoveries of standard addition method with low SD and %RSD justified the high accuracy of the proposed method (Table 3.47).

Table 3.38 Intra-day and inter-day accuracy of RP-HPLC method for estimation of FBX in HBSS buffer.

Accuracy	Concentration Level (%)	Actual concentration (µg/mL)	Observed concentration* (µg/mL)	% Recovery* ±SD	%RSD
Intra-day accuracy	50	10	10.15	101.53±0.07	0.66
	100	20	20.27	101.37±0.17	0.82
	150	30	30.21	100.69±0.32	1.04
Inter-day accuracy	50	10	10.09	100.97±0.06	0.64
	100	20	20.34	101.68±0.19	0.96
	150	30	30.48	101.59±0.21	0.65

* mean of six determinations at each level.

The LOQ and LOD were determined for the estimation of FBX in HBSS buffer and summarized in Table 3.48.

Table 3.39 LOD and LOQ of RP-HPLC method for estimation of FBX in HBSS buffer.

Parameters*	Results
LOD (µg/mL)	0.031
LOQ (µg/mL)	0.094

* mean of six determinations.

Stability study for solutions of FBX in HBSS buffer was performed at three levels of concentration (i.e. 1, 15 and 30 µg/mL), which were stored at room temperature, at 37°C in an incubator and at 5°C in refrigerator for 48 hours. Six replicates of each concentration were injected in the HPLC system and %RSD for peak areas was calculated. No accountable decrease in FBX concentration, low %RSD and absence of

any additional peak in chromatogram indicating the stability of FBX in the sample solutions prepared in HBSS buffer (Table 3.49).

Table 3.40 Stability of FBX solutions used in RP-HPLC method for estimation of drug in HBSS buffer.

Storage conditions	Concentration levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
At room temperature for 48 hours	1	0.98	1.02
	15	15.46	0.75
	30	29.92	1.34
At 37°C for 48 hours	1	1.03	0.93
	15	15.85	0.79
	30	30.51	1.12
At 5°C for 48 hours	1	1.02	0.54
	15	15.77	0.32
	30	30.75	0.66

* mean of six determinations at each level.

3.2.4.6 Conclusion

The proposed RP-HPLC method for estimation of FBX was successfully validated for different parameters as per USFDA and ICH guidelines. The results of the study showed that the developed RP-HPLC was simple, rapid, economical, sensitive, accurate and precise and could be used for estimation of FBX in transport buffer (HBSS-Hank's balanced salt solution) used for *in vitro* gastro-intestinal permeability study using Caco-2 cell model.

3.2.5 Bioanalytical RP-HPLC method for estimation of FBX in plasma

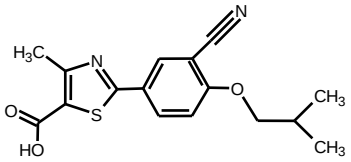
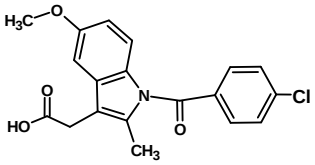
3.2.5.1 Instrument and chromatographic conditions

Same as described in Section 3.2.3.2.

3.2.5.2 Selection of internal standard (ISTD)

The internal standard was selected as per the physico-chemical properties of FBX for this study. Indomethacin (INDM)[43,44] has similar physicochemical properties as Febuxostat[45,46] therefore FNB was selected as ISTD for the pharmacokinetic study of FBX (Table 3.50).

Table 3.41 Physicochemical properties of FBX and INDM.

Properties	FBX	INDM
Log P	3.8	4.2
Water solubility (mg/mL)	1.83×10^{-2}	2.4×10^{-3}
Log S	-4.2	-5.2
pKa	3.08	3.8
Molecular formula	$C_{16}H_{16}N_2O_3S$	$C_{19}H_{16}ClNO_4$
Molecular weight (Dalton)	316.374	357.787
Molecular structure		

3.2.5.3 Preparation of experimental solutions

3.2.5.3.1 Preparation of FBX 1st standard stock solution

Accurately weighed and transferred 10mg of FBX into 10ml volumetric flask. Approximately 5 ml DMSO was added and sonicated to dissolve the FBX. Final volume was made up to the mark with DMSO and mixed well. The concentration of resulting solution was 1000 µg/mL.

3.2.5.3.2 Preparation of FBX 2nd standard stock solution

An aliquot of 5 mL from 1st standard stock solution of FBX was pipette out and transferred in a 10 ml volumetric flask. The volume was made up to the mark with methanol and vortex-mixed to produce 2nd standard stock solution of FBX (500 µg/mL)

3.2.5.3.3 Preparation of FBX working stock solutions (spiking solutions)

Aliquot ranging from of 0.05 mL to 2.5 mL of standard stock solution of FBX was pipette out in separate 5 mL volumetric flasks and diluted up to the 5 mL mark with methanol to produce working stock solutions of rhein having concentration of 5, 25, 50, 100, 150, 200 and 250 µg/mL.

3.2.5.3.4 Preparation of INDM (ISTD) working stock solution

Accurately weighed and transferred 10 mg INDM (ISTD) in a 100 ml volumetric flask. About 50 ml of methanol added and sonicated to dissolve the content. Volume was made up to the mark with methanol and vortex-mixed. The concentration of resulting solution was at 100 µg/mL. (1st Stock)

An aliquot of 0.5 mL was pipette out from 1st Stock and transferred in a 10 ml volumetric flask. Volume was made up to the mark with distilled water and vortex-mixed to produce the working stock solution of INDM of 5 µg/mL.

3.2.5.3.5 Preparation of calibration curve standard solutions of FBX in plasma

An aliquot of 0.1 mL was pipette out from each of the working stock solutions of FBX and transferred in separate 5 ml screw capped tubes and 4.9 mL of human plasma was added in each tube to yield FBX concentrations of 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL.

Liquid-liquid extraction method was adopted for the extraction of FBX from plasma. The procedure of the same is as follows:

- 0.25 ml of each plasma sample was transferred to separate prelabelled ria vials.
- 50 µl of INDM (ISTD) working stock solution (5 µg/mL) was added to all samples except plasma blank and vortexed for about 30 seconds.
- 25 µl of glacial acetic acid was added to all the tubes and vortex-mixed for about 30 seconds.
- Then 2 ml of methyl tertiary-butyl ether (MTBE) was added to all samples and vortexed for 5 min.
- The samples were centrifuged at 4000 rpm at 4 °C for 10 min.
- Organic layer was transferred to prelabelled ria vials after freezing the aqueous layer and the samples were evaporated to dryness at 40 °C under the gentle stream of nitrogen.
- The dried residues were further reconstituted with 50 µl of mobile phase and vortexed for 30 seconds.

Six replicates of 20 µL volume of each concentration of calibration curve standard solutions were injected in the HPLC system under the chromatographic conditions described earlier. The same procedure was repeated for three times. Fig. 3.16(A) represents the overlay chromatogram of FBX calibration curve standard solutions recovered from plasma in the range of 0.1-5.0 µg/mL.

3.2.5.3.6 Preparation of un-extracted standard FBX samples

100 µl of respective spiking solutions of FBX were transferred in separate pre-labeled ria vials and 100 µl of INDM (ISTD) working stock solution (5 µg/mL) was added in the

respective vials and vortex-mixed. Volume was made up to 1000 µl by adding another 800 µl of mobile phase and vials were vortexed for about 30 seconds.

3.2.5.4 Validation of bioanalytical RP-HPLC method for estimation of FBX in plasma

The proposed method was validated for estimation of FBX in plasma as per the standard guidelines[17-21]. The method was validated with respect to the parameters including system suitability, linearity, precision and accuracy. The stability of extracted samples and absolute recovery of FBX from plasma were also checked (Section 3.1.5.5).

3.2.5.5 Result and discussion

System suitability study was carried out with six replicate injections of middle QC sample (i.e. 2 µg/mL) in the HPLC system and retention time of FBX and INDM were observed at 4.22±0.04 min and 6.8±0.05 min respectively. The % RSD for the area under curve of chromatographic peak of FBX was calculated and found to be 0.83 which indicated the minimal variation in peak area. The asymmetry factor and theoretical plate counts were in agreement with the standard limits[17,18,20,21] (Table 3.51).

Table 3.42 System suitability parameters of RP-HPLC method for estimation of FBX in plasma.

Parameters	Observation
Peak area*	88.23
% RSD of peak area	0.83
Retention Time (minutes) ± SD*	4.22 ± 0.04 min
Asymmetry ± SD*	1.65 ± 0.06
Theoretical plates ± SD*	7553 ± 9.87

* mean of six determinations.

The calibration curve of FBX in plasma was obtained by plotting the peak area ratio of FBX standards and INDM (ISTD) on y-axis versus concentration of FBX on x-axis. Table 3.52 represents the calibration data for the estimation of FBX in plasma using RP-HPLC. Fig. 3.16(A) represents the overlay chromatogram of FBX in the linear range. The calibration curve was found to be linear in the range of 0.1–5.0 µg/mL for FBX (Fig. 3.16(B)). The slope and intercepts with low values of standard deviation and correlation coefficient value near 1.0 confirmed the linear relationship between peak area and concentration of FBX (Table 3.54). The percent recoveries and % RSD values for each FBX standards across the calibration concentration range were calculated and found to be in agreement with the acceptance criteria. The precision and mean accuracy observed for the calibration curve standards of FBX were ranged from 0.48% to 1.75%

and 100.2% to 109.4% respectively, which are within acceptance limit of 2.0 for precision and 85 to 115% for accuracy (Table 3.53).

Table 3.43 Calibration data of RP-HPLC method for estimation of FBX in plasma.

Concentration (µg/mL)	FBX Peak area*±SD (mV.s)	INDM Peak area*±SD (mV.s)	Area ratio*±SD (FBX/INDM) (N=6)
0.1	3.047±0.02	132.05±2.23	0.023±0.001
0.5	21.79±0.16	130.12±0.52	0.167±0.001
1.0	46.26±0.51	132.37±1.48	0.349±0.006
2.0	88.23±0.83	130.99±0.18	0.673±0.005
3.0	133.01±1.16	130.67±0.62	1.023±0.009
4.0	184.87±1.73	131.71±1.88	1.404±0.026
5.0	233.46±2.14	132.32±2.64	1.764±0.009

*mean of six determinations.

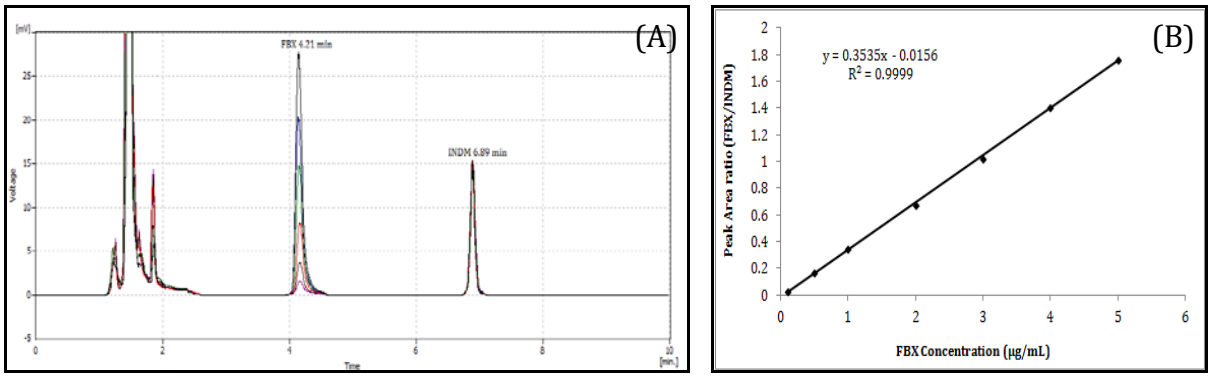


Fig. 3.16 (A) Overlay chromatogram and (B) Calibration curve of FBX in plasma.

Table 3.44 Back calculation of extracted concentration of FBX in plasma by recorded peak area of calibration curve standards.

Actual concentration (µg/mL)	Calculated Concentration* (µg/mL)	%RSD (n=6)	%Accuracy (n=6)
0.1	0.109	1.27	109.4
0.5	0.518	0.44	103.6
1.0	1.033	1.75	103.3
2.0	2.072	0.79	103.6
3.0	3.125	0.93	104.2
4.0	4.02	1.82	100.4
5.0	5.04	0.48	100.7

*mean of six determinations.

Table 3.45 Summarized linearity parameters of RP-HPLC method for estimation of FBX in plasma.

Linearity Parameters	Results
Analytical wavelength (nm)	315
Linearity range	0.1 - 5.0
Slope ± SD*	0.3536 ± 0.001
Intercept ± SD*	0.0153 ± 0.003
Correlation coefficient (R^2)*	0.9999

* mean of six determinations.

The developed method was validated for its intra-day and inter-day precision (3 consecutive days, n=6) at three different test concentrations (0.1, 2.0 and 5.0 µg/mL - covering the specified linear range of the method). The average %RSD of intra-day and inter-day precision were found to be in the range of 1.59% to 4.63% which complies with the standard acceptance limit of 15.0% and confirmed the precision of the method (Table 3.55).

Table 3.46 Intra-day and inter-day precision of RP-HPLC method for estimation of FBX in plasma.

Precision	Concentration Levels (µg/mL)	Estimated concentration* (µg/mL)	% RSD
Intra-day precision	0.1	0.101	2.58
	2.0	2.017	4.63
	5.0	5.149	1.85
Inter-day precision	0.1	0.099	3.14
	2.0	2.081	1.67
	5.0	5.458	1.59

* mean of six determinations at each level.

The intra-day and inter-day accuracy (3 consecutive days, n=6) of the proposed HPLC method was checked at 3 level of FBX concentration in plasma (i.e. 0.1 µg/mL, 2.0 µg/mL and 5.0 µg/mL). No interference could be observed with the proposed method. The acceptable recoveries of FBX in quality control samples with low %RSD justified the high accuracy of the proposed method (Table 3.56).

Table 3.47 Intra-day and inter-day accuracy of RP-HPLC method for estimation of FBX in plasma.

Accuracy	Actual concentration (µg/mL)	Observed concentration* (µg/mL)	% Recovery*	%RSD
Intra-day accuracy	0.1	0.104	104.63	2.14
	2.0	2.039	101.95	1.87
	5.0	5.12	102.4	3.27
Inter-day accuracy	0.1	0.102	101.97	2.89
	2.0	2.103	105.15	2.04
	5.0	5.19	103.8	1.46

* mean of six determinations at each level.

Absolute recoveries of FBX and INDM (ISTD) from plasma were determined. The obtained results are summarized in Table 3.57. The % recoveries (extraction efficiency) of FBX and INDM (ISTD) from plasma for all the QC samples were more than 85% which is in agreement with the standard guidelines.

Table 3.57 Absolute recovery of FBX and INDM (ISTD) from plasma.

Analyte	Concentration Level (µg/mL)	Extraction efficiency* (%)	%RSD
FBX	0.1	91.83	2.87
	2.0	91.68	2.15
	5.0	92.64	1.69
INDM (ISTD)	0.1	88.49	1.17
	2.0	89.18	2.65
	5.0	88.26	1.58

* mean of six determinations at each level.

The results of stability of FBX in plasma are shown in Table 3.58. The mean % recoveries of FBX in both QC samples (0.1 and 5.0 µg/mL) used for stability studies were found within the acceptance limit of 85% to 115%.

Table 3.48 Stability of FBX in plasma used in RP-HPLC method.

Stability conditions	Concentration levels (µg/mL)	Estimated concentration* (µg/mL)	% Recovery	% RSD
Freeze-thaw stability	0.1	0.101	101.27	1.25
	5.0	5.142	102.84	2.31
Bench-top stability	0.1	0.098	98.67	2.87
	5.0	5.013	100.26	3.04

* mean of six determinations at each level.

3.2.5.6 Conclusion

The proposed RP-HPLC method for estimation of FBX in plasma was successfully validated for different parameters as per USFDA and ICH guidelines. The results of the study showed that the developed RP-HPLC was simple, sensitive, accurate and precise and could be used as bioanalytical method for the estimation of FBX in pharmacokinetic study of developed formulations.

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