

Chapter 10**HPLC METHOD DEVELOPMENT AND
VALIDATION FOR FEXINIDAZOLE****10.1 EXPERIMENTALS****10.1.1 Instruments Utilised**

The Shimadzu-HPLC system LC-20-AT-system with LC-Solution and Peak chrom software with both PDA & UV detector. Stationary phase column in reverse phase has been used C-18-Hypersil-BDS & -ODS-250 x 4.6 mm, 5 micron size has been selected.

Systronics UV-visible spectrophotometer was used along with other Shimadzu UV 1800 spectrophotometer & Systronics UV for the wavelength maxima estimation. FTIR Spectrometer Shimadzu 8400 series has been utilised for identification of drugs standard samples. Melting point apparatus Labtronics was used for melting point determinations. Veego VDA-8D Microprocessor Based Dissolution Test for dissolution testing

Wist Temperature Chamber was used for drying the drug samples and thermal degradation study. Ultra-sonicator Lab Branson ultrasonic's corporation was utilised. Digital pH meter labtronics was utilised. Veego VDA-8D Microprocessor Based Dissolution Test for dissolution testing Photostability Test Chamber Sanwood SM-LHH-GSD-UV Series was utilised. Electronic analytical balance AUX-220 Shimadzu has been used. Borosil glass-wares volumetric flasks measuring cylinder pipettes of analytical were used. 0.22 and 0.45 μm nylon Millipore filters caps were used.

10.1.2 Materials and Reagents Utilised

The chemicals used working reference standard drug Fexinidazole FEXI samples of solisom & torrent pharma has been utilised. Acetonitrile, Methanol, potassium dihydrogen ortho phosphate, orthophosphoric acid, used analytical HPLC Merck grade. H_2O_2 , HCl, NaOH analytical grade of Rankem used. Milli-Q pure water is utilized.

10.1.3 Identification of Standard Drug Sample

10.1.3.1 Melting Point Determination

The working standard drug Fexinidazole FEXI was identified by melting point determination. Melting point apparatus used was made of Labtronics™ Melting Point Apparatus. The melting points observed for the standard drug samples are shown in the Table 7.1.

Drug	Observed Melting Range	Standard Melting Range
FEXI	111.6 °C	110-120 °C

Table 7.1: Melting Points of FEXI

10.1.3.2 FTIR Spectral Determination for Identification Standard drug samples FEXI

The pure active pharmaceutical working standard drug substances FEXI as scanned between 400-4000 cm^{-1} in FTIR Spectrometer Shimadzu 8400 series. The drug dry powder samples were made die pressed pellets with KBr and the FTIR spectra were determined shown in Fig 7.1 for FEXI. The principal IR peaks recorded and observed for the drug are shown in Table 7.2, for FEXI.

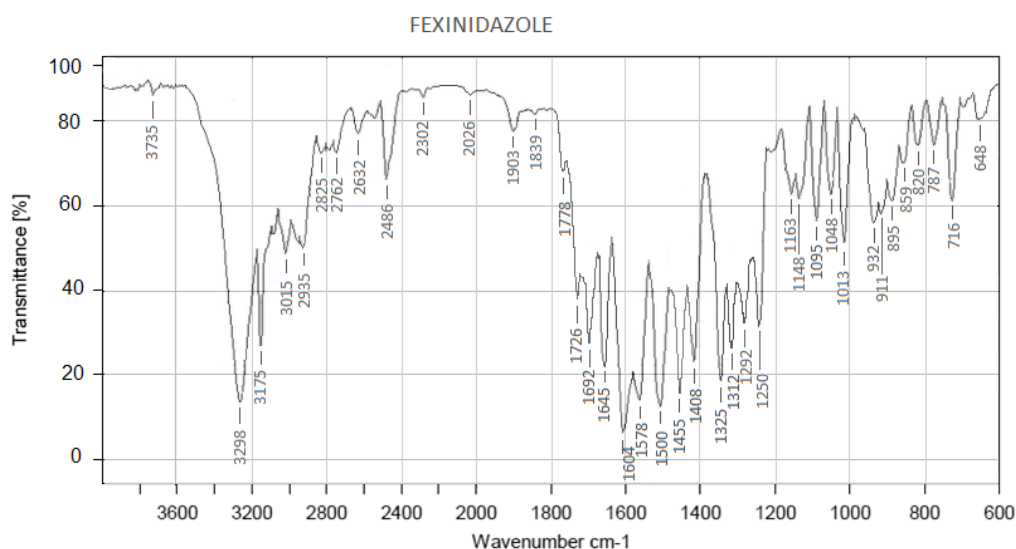


Figure 7.1: FTIR Spectra of Fexinidazole FEXI

FEXINIDAZOLE					
Energy (Cm ⁻¹)	Band Assignment	Peak Intensity	Energy (Cm ⁻¹)	Band Assignment	Peak Intensity
1550-1500	N-O Nitro	13.69	1749-1792 1275-1200	C-O (Ether)	67.38 31.43
1250-1020 3300-3400	C-N C-N 3 ⁰ N-CH ₃	29.56 17.65	705-570 725-655	C-S	61.24 81.07
3000-2800	C-H Methyl group	75.29 49.16 48.31	2000-1650 1455	C-H Aromatic	18.76
1690-1640	C=N	21.52	1317 1089-1034	S-CH ₃ S-Ar	27.32 62.36
1500-1700 1650-1580	N-H	12.46 17.76	1647 1600-1553	C=C (Aromatic) C=C Imidazole	21.37

Table 7.2: FTIR Interpretation of Fexinidazole FEXI

10.1.4 Preparation of Solutions

10.1.4.1 Preparation of standard solutions of FEXI

The standard stock soln. individual drugs prepared in 80:20 ACN : Methanol solvent mixture. 60mg of FEXI was dissolved in solvent mixture and made upto 100ml soln with same solvent to give 600 µg/ml standard stock solution of FEXI. From the above stock solutions of, 6ml from each was taken and diluted upto 100ml in to give FEXI 36 µg/ml drug standard Final solution.

10.1.4.2 Preparation of Sample Solutions

WINTHROP™ each tablet contains FEXI 600mg of Fexinidazole was taken and Dissolved in 50ml ACN : Methanol (80:20), sonicated, filtered and make up to 100ml (Stock solutionA) [6000 ug/ml FEXI]

From the Stock solution A, 6ml was taken, diluted with mobile phase upto 100ml to give Solution B [360ug/ml FEXI]

From the Solution B, 1ml was taken, diluted with mobile phase upto 10ml to give Final Solution C [36ug/ml FEXI] used for analysis.

10.1.4.3 Preparation of Optimized Mobile Phase

The mobile phase made by taking 65:35 ratio, 0.05M Phosphate buffer : ACN of pH 4.5. The phosphate buffer was prepared by accurately weighing 6.8gm KH_2PO_4 (MW. 136) in 1000ml HPLC grade milli-Q system purified water. The pH adjusted by 1% OPA Ortho-phosphoric acid. After filtration it was sonicated and the 1% OPA was prepared by taking (1.176ml) of 85%w/v orthophosphoric acid (MW 98) in 100ml HPLC grade water.

10.1.5 Selection of Wavelength for Detection

The Final standard solns of FEXI 36 $\mu\text{g/ml}$, scanned in 200 - 400 nm in UV-visible double beam spectrophotometer at a medium at scanning speed. The overlain spectra shown in Fig. 7.2 of FEXI 36 $\mu\text{g/ml}$, was taken in 20:80 Methanol : ACN and the 255.5nm wavelength was selected for estimation in the detection during the HPLC analysis.

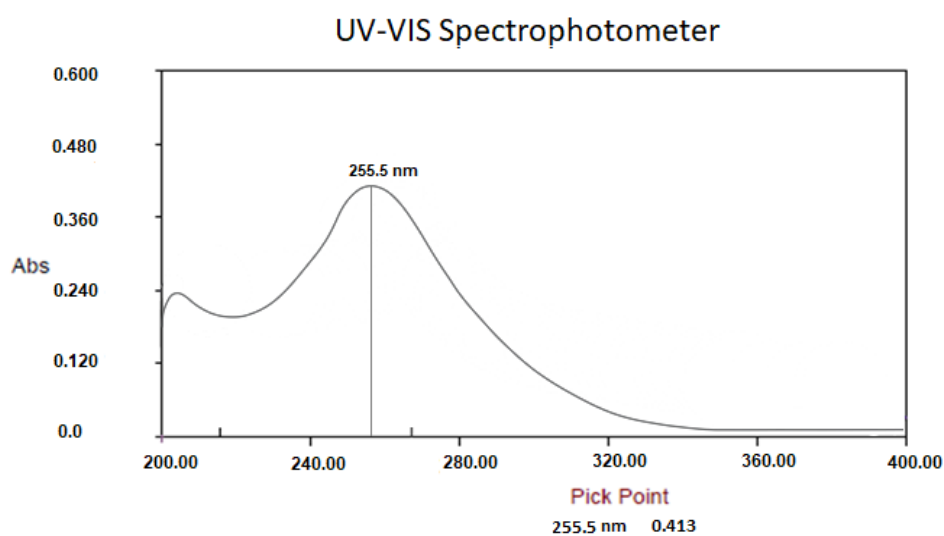


Figure 7.2: UV Spectra Overlay of FEXI

10.1.6 Selection and Optimization of Mobile phase

For the detection analysis of the FEXI drug in the working standard solutions by the HPLC method had been carried out in reverse phase by using polar solvents in mobile phase. The various trials with different mobile phase's has been carried out for the detection and separation of the drugs was carried out shown in Table 7.3

Sr No	Mobile Phase	pH	Ratio (v/v)	Retention Time (min) FEXI	REMARK
1	ACN : Methanol	-	80:20	-	No peak detected
2	ACN : Methanol	-	50:50	-	No peak detected
3	ACN : Methanol	-	20:80	-	No peak detected
4	0.05 M Phosphate buffer : ACN	7	80:20	12.36	Tailing in peak, Longer run time
5	0.05 M Phosphate buffer : ACN	7	70:30	9.24	Tailing in peak, Asymmetry in peak Tailing factor -3.46
6	0.05 M Phosphate buffer : ACN	6	60:40	6.17	Tailing in peak, Asymmetry in peak Tailing factor - 2.73
7	0.05 M Phosphate buffer : ACN	5.5	50:50	4.58	Tailing in peak, Asymmetry in peak - 2.58
8	0.05 M Phosphate buffer : ACN	5	40:60	3.27	Tailing in peak, Asymmetry in peak - 2.33
9	0.05 M Phosphate buffer : ACN Selected Mobile Phase	4.5	35:65	2.45	No tailing Good symmetry, Tailing factor- 1.34

Table 7.3: Trials for Selection of Mobile Phase for FEXI

10.1.7 Optimized Chromatographic Conditions

Optimized chromatographic conditions for developed HPLC analytical method are shown below-

Parameters	Conditions
Stationary Phase Coloumn	C18 Hypersil BDS 250 x 4.6mm , 5 micron
Mobile phase	Phosphate buffer : ACN 65:35 pH- 4.5
Flow rate	1ml/minl
Injection volume	20ul
Temp	Ambient Lab Temperature
Detection Wavelength	255.5nm
Retention Times (min)	FEXI- 2.45

Table 7.4: Optimized Chromatographic Conditions for FEXI

10.2 METHOD VALIDATION

10.2.1 Linearity (Calibration Curve)

The working standard and sample solutions of FEXI 9, 18, 27, 36, 45, 54ug/ml, prepared in the serial dilutions for drug, for conc. range, linearity, validation parameters and same con. ranges were used for the stability forced degradation studies. The calibration curves has been generated by plotting graph of peak area vs conc. for the drugs, and the regression equations, correlation coefficient R^2 value and the, Limit of Detection (LOD) & Limit of Quantification (LOQ) had been calculated .

10.2.2 Specificity and Selectivity

The selectivity and specificity parameters are utilised in selective detection particular analyte which are in the matrix or along with other substances without any interventions. 36ug/ml of FEXI was injected individually, and blank mobile phase as well as sample solutions from dosage form were compared to check the specificity & selectivity. Selectivity is a type of a qualitative determination of analytes, while the specificity is applied for both qualitative as well as quantitative estimations. The developed method must be selective and highly specific for the analyte for which the method is intended to use, even in presence of impurities or any other degraded products, additives, excipients, reagents or other substances.

10.2.3 Accuracy (Recovery Studies)

Accuracy is one of the important validation parameter which describes the trueness-exactness of the test results in accordance with the true values. The accuracy studies has been performed by doing the drug recovery studies of deliberately added working standard drugs from the sample, n=3 samples taken for drug FEXI at 50%, 100% & 150% had performed at each level to the pre-analysed samples. The amount of drug-substance added and amount of drug-substance recovered were calculated from the sample peak area and total peak area and the % Recovery had been calculated.

10.2.4 Precision

10.2.4.1 Repeatability (n=6)

The repeatability study has been performed by repeatedly n=6 sample standards injected 36ug/ml of FEXI, and the area response of drugs was obtained and the %RSD had been calculated

10.2.4.2 Intraday Precision (n=3)

The intraday precision was performed by using the 9, 36, 54 ug/ml of FEXI was used, and the solutions were repeatedly injected analysed by HPLC three times on same day, obtained results calculated into the terms of %RSD.

10.2.4.3 Interday Precision (n=3)

The interday precision was performed by using the 9, 36, 54 ug/ml of FEXI was used, and the solutions were repeatedly injected analysed by HPLC three times in different days obtained results calculated into the terms of %RSD.

10.2.5 LOD and LOQ

The LOD Limit of Detection has been obtained from 5 set of the calibration curves performed in the linearity-range studies, the LOD is calculated as $LOD = 3.3 \times SD/Slope$

LOQ Limit of Quantitation has been obtained from the same 5 set of the calibration curves performed as per the linearity-range studies, the LOD is calculated as $LOD = 10 \times SD/slope$

10.3 APPLICATION OF DEVELOPED ANALYTICAL METHOD AS A ASSAY METHOD FOR MARKETED FORMULATION

The developed analytical HPLC method is applied in the estimation-analysis of WINTHROP™ each tablet contains FEXI 600mg of Fexinidazole was taken and Dissolved in 50ml ACN : Methanol (80:20) , sonicated, filtered and make up to 100ml (Stock solutionA) [6000 ug/ml FEXI]. From the Stock solution A, 6ml was taken, diluted with mobile phase upto 100ml to give Solution B [360ug/ml FEXI]. From the Solution B, 1ml was taken, diluted with mobile phase upto 10ml to give Final Solution C [36ug/ml FEXI] used for analysis, were prepared, n=3 samples, analysed by the developed HPLC method.

The standard stock soln. individual drugs prepared in 80:20 ACN : Methanol solvent mixture. 60mg of FEXI was dissolved in solvent mixture and made upto 100ml soln with same solvent to give 600 µg/ml standard stock solution of FEXI .From the above stock solutions of, 6ml from each was taken and diluted upto 100ml in to give FEXI 36 µg/ml drug standard Final solution, were prepared and analysed by HPLC and the % purity or % label claim was estimated by comparing the area & calculating from regression equation, for working standard drug and marketed formulation.

10.4 APPLICATION OF DEVELOPED METHOD IN DISSOLUTION STUDIES

The dissolution method has been developed & performed on the marketed formulation WINTHROP™ solid oral tablet dosage form, developed HPLC method has been utilised for qualitative, quantitative and % drug release, and % Cumulative drug release estimation.

10.4.1 Dissolution Medium

The dissolution medium was prepared by using 0.05M K₂HPO₄ phosphate buffer with 1% SLS Sodium lauryl sulphate pH adjusted to 6.8. [6.8gm KH₂PO₄ (MW 136) in 1000ml dist water with 10gm SLS (1%) And adjust pH 6.8 with 1% OPA, nearly the pH was found between range of 7-8.5, so it was adjusted to pH - 6.8 with with 1% OPA] [1 % OPA : (1.176ml) of orthophosphoric acid (85%w/v) (MW 98) in 100ml dist water]

10.4.2 Dissolution Instrument & Procedure

USP type-2 Paddle (Veego VDA-8D Dissolution Test Apparatus) was used.

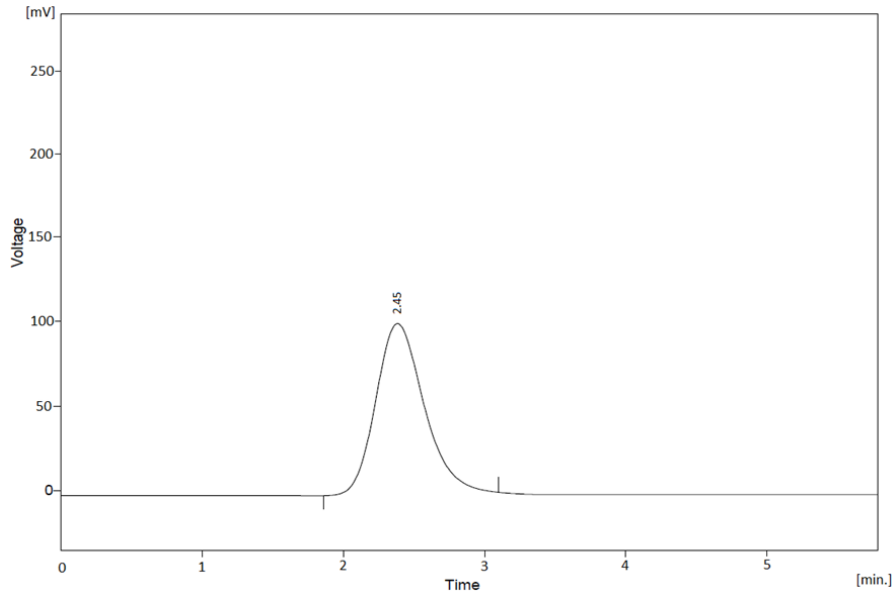
Bath Volume- 900ml, Bath Temp-37°C±0.5, Paddle RPM-50.

Each tablet WINTHROP™ containing FEXI 600mg was kept in dissolution medium n=6 and 5ml of the samples were withdrawn into solutions bath from the dissolution bath at sampling time intervals of 10, 20, 30, 40, 50 & 60mins, and bath volume was maintained 900ml with dissolution medium. [0.666mg/ml or 666.66ug/ml of FEXI at proposed 100% Release]. The 5ml withdrawn sample filtered through nylon 0.20u filter cap and was made upto 10ml with (80:20 ACN:Methanol) to give Stock Soln [333.33ug/ml of FEXI at 100% release]. From above stock soln, 1.5ml was taken and made upto 10ml with mobile phase, the Final dilution *Sample Soln-B* used in analysis by HPLC. [50ug/ml of FEXI at proposed 100% Release]

10.5 RESULTS & DISCUSSIONS

10.5.1 Method Development

The developed analytical HPLC method found to be reliable, accurate.,- precise for analysis and quality control testing for FEXI in pure form, in marketed tablet dosage form's. The method is advantageous as the low cost solvents are used, good resolution and separation has been achieved, as well as the peak symmetry tailing factor are in greater acceptable limits. The isocratic mode adds the advantage of simplicity of the developed method. Method consists of the optimized mobile phase Phosphate buffer:ACN (65 : 35) pH - .5, flow rate 1ml / min , detection wavelength at 255.5nm. Excipients in the marketed formulation does not affect in the resolution, separations as well do not have any interfering peaks. The average retention time was found to be FEXI -2.45 minutes. The chromatogram of the drugs are shown below.



Result Table (Uncal - FEXI -standard) phosphate buffer pH - 3.5 : ACN : 35 : 65

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Peak Purity
1	2.452	1046.715	96.573	100.000	0.999
Total		1046.715	96.573	100.000	

Column Performance Table (From - FEXI- standard)

	Reten. Time	Asymmetry [-]	Efficiency [th.pl]	Resolution [-]
1	2.452	1.346	3872	-

Figure 7.3: Chromatogram of Standard FEXI

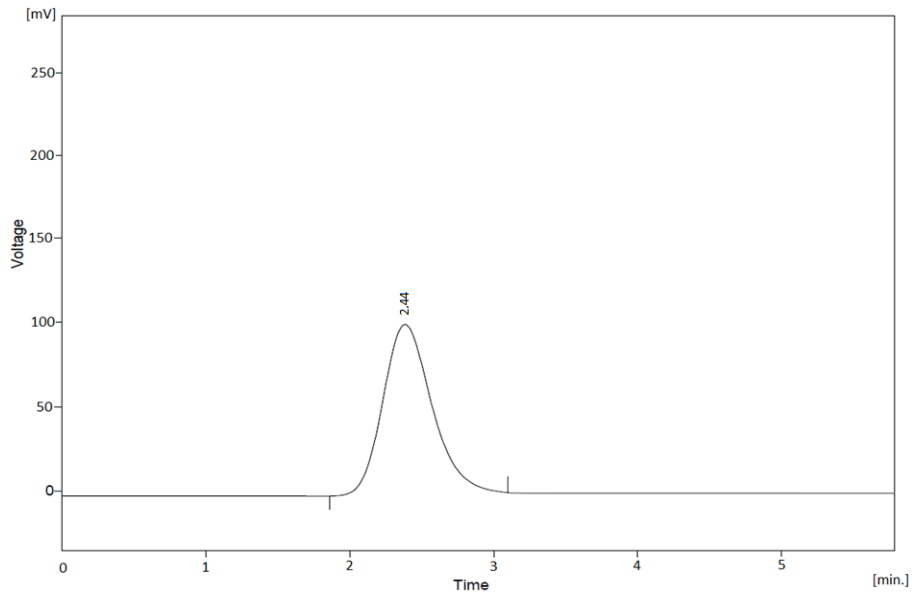


Figure 7.4: Chromatogram of Sample FEXI

10.5.2 Method Validation

10.5.2.1 Specificity

Developed method is specific and selective as the no other peaks of, mobile phase or any excipients impurities were interfering or overlapping in the chromatograms.

The method effectively analyses the drug in pure form as well as in the marketed formulations with accuracy, and has reproducible results for individual drugs as well as for the combined formulation analysis.

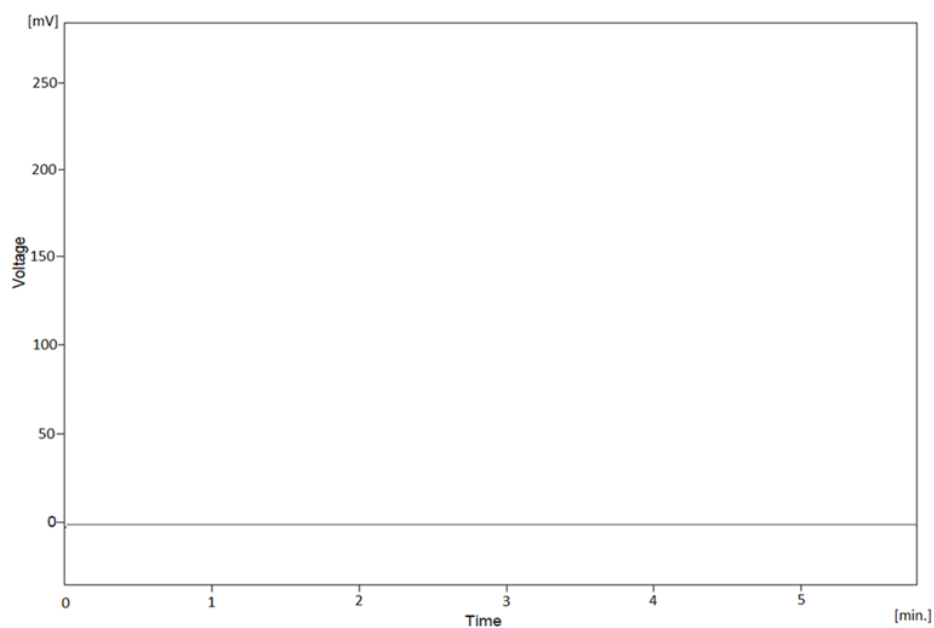


Figure 7.5: Blank Chromatogram

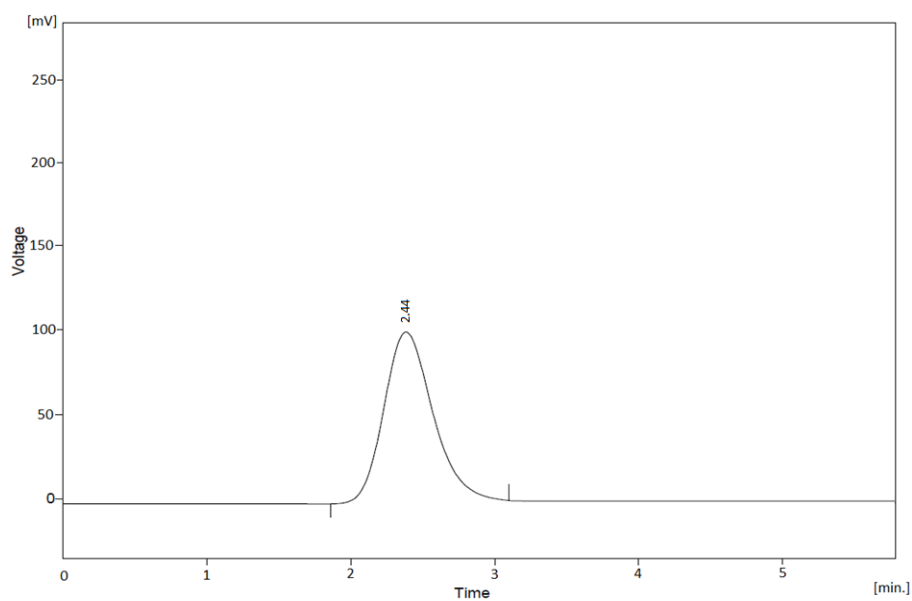


Figure 7.6: Chromatogram of Sample Standard FEXI

10.5.2.2 Linearity and Range (n = 5)

Drugs FEXI Linearity has been followed in a particular concentration ranges of 9, 18, 27, 36, 45, 54ug/ml. The linearity showing overlain chromatogram had been generated and the calibration curve been plotted of peak area vs conc. and straight line eqn. and correlation coefficient had been calculated.

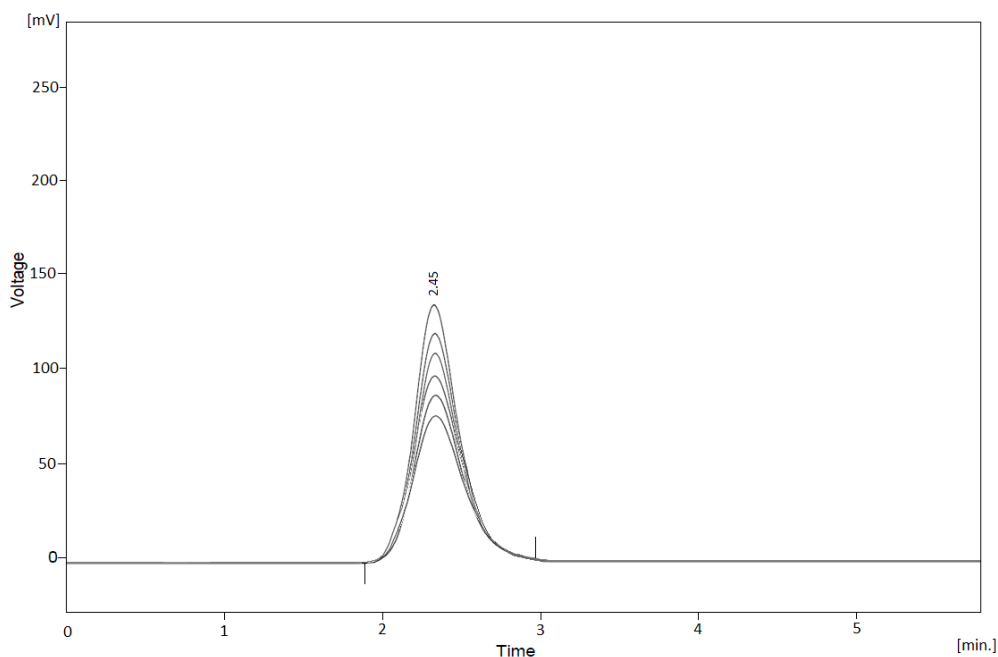


Figure 7.7: Overlain Chromatogram of Linearity for FEXI

(x) Conc. µg/ml	(y) Area
9	258.64
18	521.41
27	782.15
36	1046.71
45	1296.63
54	1565.29
STD ERROR	4.04
Slope	28.96
LOD	0.46
LOQ	1.39

Table 7.5: Linearity data of FEXI

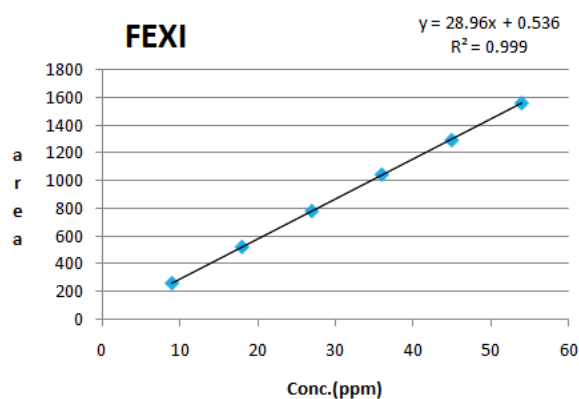


Figure 7.8: Calibration Curve for FEXI

10.5.2.3 Accuracy (Recovery Studies) (n = 3)

The accuracy has been done by performing the recovery studies of the working standard drug from the pre-analysed sample of the drug FEXI. The recovered drug from the samples has been calculated as % Recovery is been reported in the table below.

Drug	Amt of Sample Taken (μg)	% Amt of Std Added	Spiked Std Drug Amount (μg)	Spiked Amt Recovered Mean (μg)	% Recovery	% Mean Recovery
FEXI	36	50	18	18.07	100.44	99.64
	36	100	36	35.87	99.65	
	36	150	54	53.37	98.84	

Table 7.6: Accuracy Study of FEXI (n = 3)

10.5.2.4 Precision**10.5.2.4.1 Repeatability (n = 6)**

The repeatability study of FEXI have been performed by multiple injections of the samples of the drugs (n = 6). The repeatability data for the FEXI is shown in the table below.

Conc. of FEXI ($\mu\text{g/ml}$)	Area
10	1043.91
	1055.67
	1047.13
	1058.72
	1049.81
	1046.45
Mean	1050.28
SD	5.75
% RSD	0.54

Table 7.7: Repeatability Study of FEXI (n = 6)

10.5.2.4.2 Intraday Precision (n = 3)

The Intraday precision for the FEXI has been performed by taking multiple injections (n = 3) in a same day at different 25, 100, 150 % Levels. The data for the intraday precision is shown in table below.

FEXI		
Conc. (µg/ml)	Mean area ± SD	% RSD
9	255.9 ± 3.3	1.30
36	1040.2 ± 3.6	0.34
54	1558.3 ± 5.4	0.30

Table 7.8: Intraday Precision of FEXI (n = 3)

10.5.2.4.3 Interday Precision (n = 3)

The Interday precision for the FEXI has been performed by taking multiple injections (n = 3) in different day at different 25, 100, 150 % Levels. The data for the intraday precision is shown in table below.

FEXI		
Conc. (µg/ml)	Mean area ± SD	% RSD
9	265.2 ± 3.9	1.49
36	1043.6 ± 4.3	0.41
54	1559.4 ± 7.9	0.51

Table 7.9: Interday Precision of FEXI (n = 3)

10.5.2.5 LOD and LOQ

It has been calculated from the n=5 samples from the calibration curve slope and standard deviation. The LOD & LOQ value are found to be 0.46 & 1.39 ug respectively for FEXI.

10.5.3 Application of the Developed Analytical Method to Formulation

The proposed analytical method been tested in assay analysis % Assay of the Label claim on the WINTHROP™ Each tablet contains FEXI Fexinidazole 600mg. Analytical method successfully applied to the estimation of drugs in marketed pro-duct by comparing with the standard and the sample formulation. The assay result are shown in the table below.

	Serial No	Label claim (mg)	Result (mg)	% Label Claim	Avg % Assay	SD	% RSD
FEXI	1	600	586.94	97.82	97.92	0.15	0.15
	2	600	588.56	98.09			
	3	600	587.05	97.84			

Table 7.10: Assay of Formulation WINTHROP™ (n = 3)

10.5.4 Dissolution Studies

The dissolution profile method is developed of these drugs been performed from the tablet dosage form n=6 and it shows % Drug release and % Cumulative drug release Dissolution. The analytical developed method is applied successfully in the dissolution profile studies. The results of dissolution studies are shown below.

Time Min	Area of Sample	Drug Release Conc. ug/ml	Drug Release in mg as per Label claim	% DR Drug Release	% CDR Cumulative Drug Release
FEXI					
			Label Claim FEXI 600mg		
10	687.78	23.730	284.769	47.461	47.458
20	1211.42	41.812	501.747	83.624	83.885
30	1317.34	45.469	545.637	90.939	91.400
40	1411.19	48.710	584.525	97.420	97.922
50	1421.27	49.058	588.701	98.116	98.654
60	1428.29	49.300	591.610	98.601	99.142

Table 7.11: Dissolution Study of Formulation WINTHROP™

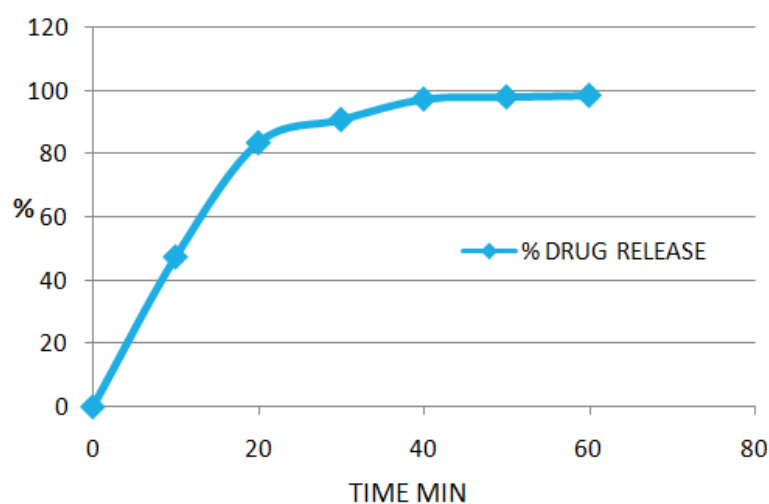


Figure 7.9: % Drug Release for FEXI

10.5.5 Summary of Results

Sr No	Parameters	Results FEXI
1	System Suitability: Theoretical plates- Tailing Factor- Retention time min-	3872 1.34 2.45
2	Precision (%RSD)	0.54
3	Linearity (R^2)	0.9999
4	Accuracy (% Recovery)	99.64
5	LOD (ug/ml)	0.46
6	LOQ (ug/ml)	1.39
7	% Assay	97.91
8	Dissolution % Drug Release at 40min	97.92

10.6 CONCLUSIONS

The Analytical HPLC method for FEXI drug has been successfully developed and validated. The analytical method is optimized in testing, analysis of the drug in individual as well in the in formulation and all validation parameters., are performed in the acceptance criteria as per ICH regulatory guideline. Method that has been developed., is been, optimized to analyse minimum conc. of drugs in-pure form and, in testing-analysing marketed formulation. Dissolution method for the drug have been developed for solid dosage form. The HPLC analytical method is applied in the estimation for dissolution profile studies of the marketed dosage form. Accurate precise method developed., can be used for analysis of FEXI drug as well as individual in as Assay method and dissolution testing procedures in academics, research, analytical laboratories and pharmaceutical industries.