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RESEARCH ARTICLE

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF FEXINIDAZOLE IN BULK FORM

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Abstract: An RP-HPLC technique with high sensitivity and precision has been devised to accurately determine the concentration of Fexinidazole in its bulk formulation. The maximum wavelength (λmax) of Fexinidazole was determined to be 264 nm in Methanol: Phosphate Buffer 7.4 solution at a ratio of 75:25 % v/v. The approach demonstrates a high level of sensitivity, with a linear range of 2 to 10 µg/ml. The regression equation for this range is y = 203134x + 9713.5, with a r2 value of 0.9997. This approach is validated and tested in accordance with the criteria specified in the ICH guidelines and USP. The detection limit and quantitation limit were determined to be 0.008 µg/ml - 0.02 µg/ml, respectively. The results indicated that the technique is precise, specific, and repeatable, with a relative standard deviation (RSD) of less than 2%. Additionally, the procedure is straightforward, cost-effective, time-efficient, and suitable for determining Fexinidazole in bulk forms.

Keywords: Fexinidazole, HPLC method, Quantitative Analysis, AMD, Method development.

INTRODUCTION

Fexinidazole, a prodrug and a nitroimidazole with 2 substitutions in its position 5, is orally bioavailable in the treatment of both early and advanced human African trypanosomiasis (HAT) caused by Trypanosoma brucei gambiense. Known as sleeping sickness, it can result from the bite of an inflected tsetse fly, resulting in a biphasic illness.

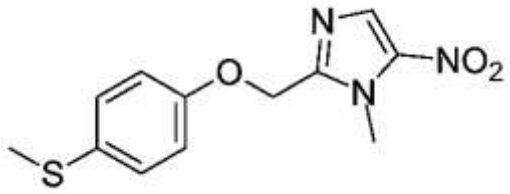

Fig 1: Structure of Fexinidazole

Fexinidazole is a compound that has great potential for pharmaceutical use in the treatment. Its atomic mass is 279 Daltons (Da), and its molar mass is 31 grams per mole. Before fexinidazole came, the treatment options available for second-stage human African trypanosomiasis (MSHAT) and meningoencephalitis HAT were the antimonial drugs, Melarsoprol and others such as Eflornithine, plus the combined treatment known as NECT - Nifurtimox-Eflorn. Despite their effectiveness, these treatments were not without challenges. First and foremost, as each of these therapeutic regimens requires quite a long period of intensive intravenous infusion, it becomes rather cumbersome to be implemented on a large scale, particularly in low-resource settings where the health care infrastructure is limited. Besides, treatment with Melarsoprol has a high degree of toxicity upon administration; therefore, it is less favoured in treating patients.

The current study makes use of a chromatographic technique to highlight the enhancement of analytical powers for the quantification of Fexinidazole. The prime objective is to develop and validate a relatively simple but superbly selective HPLC method. The method should be capable of determining Fexinidazole in different molecular compositions and hence contribute to the development of innovative analytical techniques in pharmaceutical research.

MATERIAL AND METHODS

Instruments:

Chromatographic partitioning was done on a Jasco MD-2010 Plus small fluid chromatographic setup. This instrument is highly advanced, developed to assist the researcher with accurate chromatographic analysis. It was fitted with a programmable UV detector at different frequencies that would allow flexibility and accuracy in the detection of different

substances. This system was further supplied with a fixed 20µl loop Rheodyne injector that delivered consistent volumes of samples. A C18 column was used as the stationary phase. The Cosmosil C18 column has a dimension of 4.6mm x 250mm and the size of the particle is 5µm.
Moreover, a UV-2012 spectrophotometer with twin shafts was used for the various spectrophotometric measurements and associated measurement activities. It contributed significantly to the assessment of absorbance values and other spectrophotometric attributes of samples under study. To obtain the weight of the reagents and samples with the required high accuracy, a Wenser High Precision Balance, model PGB 100, was used. This electric balance gives associated accurate measurements, impacting directly on the goodness of the results for the experiment.

Reagents and chemicals

The pure drug in the form of tablets of fexinidazole was procured from a reputed chemical supplier, Yarrow Chem, Mumbai. The pure drug procured in the form described above was used as a reference standard for the present study. HPLC-grade methanol and water were obtained from Merck Specialties Private Limited, a well-known supplier of Mumbai. The chromatographic and spectrophotometric analyses carried out during the research needed methanol and water of high purity in order to be fairly reliable and reproducible.

Optimization of Detection Wavelength

The selection of wavelengths for quantitative analysis requires them to yield the best response for the substances being detected, and optimization of wavelengths was conducted using various UV detectors to achieve satisfactory results. In the current investigation, standard medication solutions of 10µg/ml of each of the Fexinidazole was prepared in methanol. Having learned UV spectra of that medication to contain wavelength 264 nm of the future study was selected.

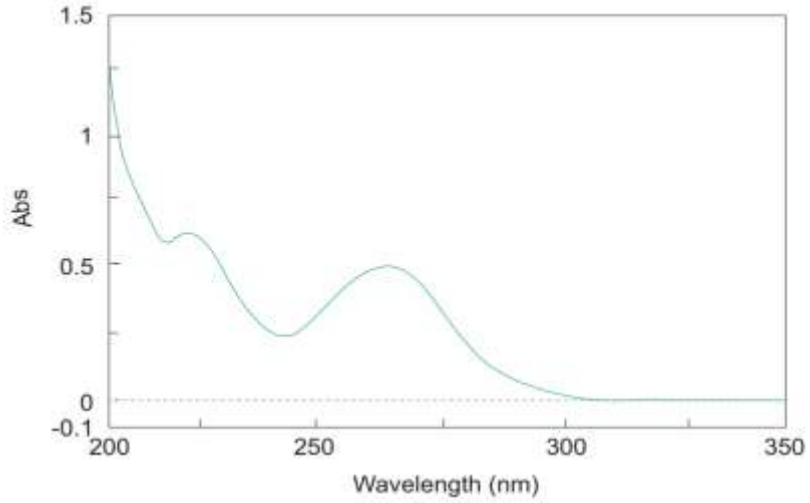

Fig. 3: UV Spectra of Fexinidazole Showing λmax 264 nm

Chromatographic conditions

The study used Cosmosil C18 (with a measurement of 4. 6mm x 250mm, along with the particle size: EPC X [6% acetonitrile mobile phase, 5µm] for separation of chromatography at a discovery frequency of 264 nm, and methanol: phosphate buffer 7.4 (75:25) for elution. Sample infusion was used to guage the elution, with a stream rate that was particularly adjusted to 1.0 ml/min. The standard and sample arrangement were also used.

Preparation of Standard solutions

A similar dissolvable was used to make up the volume after accurately weighing and transferring 10 mg of Fexinidazole to 10 ml volumetric flasks. This made it possible to make an essential stock arrangement of the drug at a concentration of 1000µg/ml.

Optimisation of RP-HPLC method

The HPLC approach was simplified to assess Fexinidazole, optimizing the technique with different mobile phases. The Methanol: Phosphate Buffer 7. 4 (75:25) column was used, with appropriate retention periods, hypothetical plates, and

high resolution. A chromatogram of Fexinidazole was also shown, providing a summary of chromatography parameters at 5µm.

Table 2: Optimized parameter

Parameters	Conditions
Column	Cosmosil C18 (4.6mm x 250mm, Particle size: 5µm)
Mobile Phase	Methanol: Phosphate Buffer 7.4 (75:25)
Flow Rate	1.0 ml/min
Wavelength	264 nm
Injection Volume	20 µl
Detector	UV-3000-M
Run Time	5 min
Retention Time	Approx. 4.8 min

Validation of RP-HPLC Method

This optimization of the HPLC method was performed based on ICH Q2 (R) guidelines.

Linearity

To assess linearity a 1000 µg/ml stock solution was diluted with sample solutions and aliquots placed in volumetric flasks, where the mobile phase was adjusted so fexinidazole concentrations ranged from 2 to 10 µg/ml. Each of these solutions was pipetted into system three times and data plotted as standard curves. Peak areas were found and regression factors calculated. Calibration curves for fexinidazole sample are illustrated in Figure 2; linearity characteristics are detailed Table 3.

Accuracy

For accuracy and reliability of recovery study, this method "% recovery" or standard addition was performed. To do this pure fexinidazole was added to pre analyzed sample in a specific quantity whereby the process was repeated optimized again. The optimized process gave percentage recovery illustrated in Table 5.

Precision

Intra-day precision and accuracy of the proposed method were confirmed via repeatability study done using three independent injections computed % RSD from standard sample containing 100% fexinidazole (6 µg/ml). These results are summarized in Tables 7 and 8.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

From the slope(s) of calibration curve and standard deviation (SD) of peak areas, LOD and LOQ were calculated. Therefore, LOD = 3.3 (SD/slope) while LOQ = 10 (SD/slope).

Robustness

To evaluate method robustness, several chromatographic parameters were altered, including mobile phase, detection wavelength and flow rate; variations in % coefficient were also determined. Minor modifications were made under optimal conditions to assess stability. A fluctuation of ±2 nm was applied on the detection wavelength. The adsorbent used was the YMC-Triart C18 column having a flow rate of 1 ml/min. The solutions at the test concentration of 100% was injected three times into the system with specified changes made as indicated. Results for % RSD are listed in table 9.

Ruggedness

Ruggedness was evaluated through examining how different external factors affect processing. As stated, before some factors were purposely changed to study method's robustness which involved changing conditions inside equipment, different analysts across regions or change in atmospheric conditions.

The analysis of marketed formulations

Twenties of Fexizole commercial formulation tablets were obtained, each weighing 600mg of Fexinidazole, they were ground into fine powder. Approximately 10mg of tablet sample was weighed accurately and into a volume flask of 100ml. The diluent filled up to the mark for making volume. The solution was sonicated for about 10 minutes while spinning intermittently. From that, a 100ug/ml stock solution was prepared after passing through 0.45um membrane filter.

System Suitability

The evaluation of system suitability was important to ascertain whether the method would give reliable and precise results or not. The system suitability test involved injecting 6ul/ml of fexinidazole in the chromatographic system three times. The tailing factor (T) and the number of theoretical plates (N) were established as illustrated in table 13.

RESULT AND DISCUSSION

Linearity*:*
Linearity study resulted in sufficient data indicating that the method bears strong linearity characteristics within the tested concentration range. In this way, peak area measurements correlating with the Fexinidazole concentration are plotted to construct the calibration curve, as illustrated in Figure 1. The results indicated that the method was linear throughout the tested concentration range, thus proving the ruggedness and accuracy of the analytical procedure.
A linear equation, following regression analysis of the data, was worked out: y = 203134x + 9713.5. The goodness-of-fit, represented by r^2, was found to be 0.9997, very near to 1.
This result proves instrumental and method reliability to produce accurate results that are reproducible for the same application in the quantification of Fexinidazole in different kinds of samples.

Table 3: Summary of results of Linearity

Conc. (µg/ml)	Peak Area
2	415236
4	812365
6	1245947
8	1632548
10	2036481

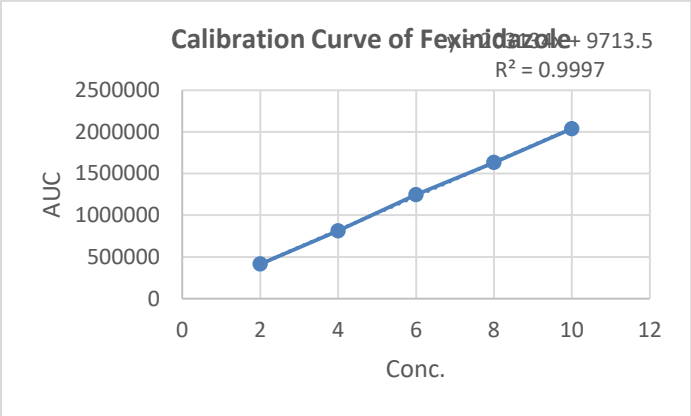

Figure 4: Calibration curve of Fexinidazole

Accuracy

The degree of accuracy can also be used to define how close the values got by that procedure are to the actual values. With regards to the accuracy testing results, one got to know that the aforementioned approach was found to be accurate as it was falling under the levels of tolerance which are acceptable. Furthermore, the % RSD is computed for the Fexinidazole and all the analyzed values are the % RSD which falls within the limits. From the Table 5, the acceptable degree of rounding was within the range but not more than 2.

Table 5: summary of Results of Accuracy

Level of addition	Standard added (µg/ml)	conc. (µg/ml)	Total conc. (µg/ml)	Area obtained*	Std Area	Drug recovered (µg/ml)	% Recovery
50%	2	4	6	1245568	1245947	6.00	99.97
	2	4	6	1243024		5.99	99.77
	2	4	6	1248876		6.01	100.24
100%	4	4	8	1645856	1632548	8.07	100.82
	4	4	8	1653264		8.10	101.27
	4	4	8	1632028		8.00	99.97
150%	6	4	10	2031255	2036481	9.97	99.74
	6	4	10	2036587		10.00	100.01
	6	4	10	2035654		10.00	99.96

Table 6: % recovery data

Level of addition	% Mean recovery*	SD	% RSD
50%	99.99	0.24	0.24
100%	100.7	0.66	0.66
150%	99.9	0.14	0.14

Precision

The “repeatability of measurements in which samples are drawn from a homogeneous population under set conditions” and is presented in relative standard deviation can be defined as precision. The result has been achieved within the specified limits. Moreover, the RSD has been calculated for the results that have been mentioned here. Both accuracy and precision were established at less than 2 percent as expected in table 7 and 8.

Table 7: Intraday precision summarised

Sr. No.	Conc. (µg/mL)	Area	Mean	SD	%RSD
1	2	415356	415794.67	501.25	0.12
2	2	415687			
3	2	416341			
4	6	1243265	1247771.33	21021.71	1.68
5	6	1259784			
6	6	1240265			
7	10	2031248	2030564.00	6702.23	0.33
8	10	2036898			
9	10	2023546			

Table 8: summary of Interday Precision

Sr. No.	Conc. (µg/mL)	Area	Mean	SD	%RSD
1	2	415632	415869.33	739.15	0.18
2	2	416698			
3	2	415278			
4	6	1240526	1243146.00	4825.70	0.39
5	6	1248715			
6	6	1240197			
7	10	2030125	2035778.33	4899.58	0.24
8	10	2038416			
9	10	2038794			

LOD and LOQ

The LOD and LOQ were determined by the following equations: LOD = (3. 3× std. Deviation /slope); LOQ = (10× std. Slope adjusted from linear and deviation is made from accurate.

Consideration of the stated equations, which are the LOD and LOQ values for Fexinidazole that were found are 0.08 & 0.26 µg/mL

Robustness

Reliability of the findings establish that even if slight modifications are incorporated in to the method, the approach would still remain robust enough. When the flow rate is taken into consideration, along with the wavelength, they were somewhat brought down to some other lower figure or higher one than the actual figure to check whether the peak area and the retention time has fallen within or over the limit or not. Summary of results obtained when varying parameters on 15µg/mL solution is presented in Table 9 below.

Table 9: Data of robustness study

Sr. No	Parameter	Condition	Area	Mean	SD	%RSD
1	Change in Flow rate (ml/min)	0.9	1245678	1243948	3112.04	0.25
2		1	1245810			
3		1.1	1240355			
1	Change in Wavelength (nm)	262	1245163	1242110	5305.57	0.43
2		264	1235984			
3		266	1245184			

Ruggedness

Ruggedness is an experiment which is conducted in order to establish the impact of external factors on the process. To determine stability of the created approach, parameter was changed intentionally. Such parameter includes; variation of system, any analyst, different atmospheric conditions. Analysts did a quite extensive research of ruggedness. The table beneath discussed the results that have been conducted.

Table 10: Data of ruggedness study

Sr. No	Analyst	Conc. (µg/ml)	Area	Mean area*	SD	% RSD
1	Analyst-I	6	1245655	1243666.33	6355.79	0.51
			1248790			
			1236554			
2	Analyst-II	6	1245689	1247515.00	7933.21	0.64
			1256202			
			1240654			

Specificity

Since it was learnt that the impurities as well as excipients doesn't Bind to the standard medication, then the method now determines that it is particular or specific. The specificity outcome is presented in below table.

Table 11: Data of specificity

Drug conc. (µg/ml)	Excipients (µg/ml)	Total conc. (µg/ml)	Area	Mean	SD	%RSD
2	4	6	415356	415455	203.45	0.05
2	4	6	415689			
2	4	6	415320			
4	4	8	813254	812451.667	694.86	0.09
4	4	8	812045			
4	4	8	812056			
6	4	10	1241005	1241079	4571.45	0.37
6	4	10	1236545			
6	4	10	1245687			

% Assay of Marketed formulation

Examination of the marketed formulation %>Assay of Market formulation
The % Assay of the marketed formulation of Fexizole-600mg Healing Pharma was determined and presented in the following table.

Table 12: Data of % Assay of marketed formulation

Sr. No.	Formulation	Area of Standard	Area of Marketed Formulation	% Assay
1	Fexizole-600mg (Healing Pharma)	1245947	1224125	98.25

System Suitability Parameters:

The study of system appropriateness involved making three replication injections of 1. 0% test concentration; the observed values of the number of total theoretical plates as well as factor of asymmetry are adequate. The chromatographs clearly support the presence of a positive detection of Fexinidazole at 4. For the other students who are sitting in front for 8 minutes, the simply allow them to be deprived of any possible interruption.

Table 13: System suitability parameter

Sr. No.	conc. (µg/ml)	Retention Time (min)	Theoretical plates	Asymmetry Factor
1	6	4.812	8646	1.23
2	6	4.816	8234	1.21
3	6	4.863	7982	1.23
4	6	4.875	8103	1.24
5	6	4.803	8956	1.25
6	6	4.835	8256	1.24
Mean		4.83	8362.83	1.23
SD		0.03	366.77	0.01
%RSD		0.61	4.39	1.11

CONCLUSION

The approach clarified for assessing pure and pharmaceutical forms of Fexinidazole is highly effective. Its easy use, acceptable degree of accuracy and precision, speediness in execution, and capacity for selectivity characterize it. Also, a straightforward-to-prepare mobile phase that predominantly costs less contributes to its practicality for routine use. Furthermore, its robustness is observable from the excellent rates of recovery thereby yielding reliable results under different testing conditions.

This technique is also very well-suited for evaluating Stability Fexinidazole in bulk and dosage forms. This means that it can be used as one of the important tools for quality control in pharmaceutical laboratories where there are strict standards on the purity and stability of a certain drug product (Zhang et al., 2017). Besides that, its overall usefulness lies in this method being time-efficient and resourceful while maintaining consistent performance throughout. Therefore, based on these features, this method offers holistic solutions to exact estimation of Fexinidazole; ensuring the applicator meets desirable threshold values when it comes to purity level or stability level within different formulations. For instance, both routine quality verification and studying drug stability may employ it.

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