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RP-HPLC Analytical Method Development and Validation For Estimation of the Drug Sparfloxacin using Tinidazole as Internal Standard in Bulk and Pharmaceutical Dosage Forms

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ABSTRACT
A rapid and reproducible HPLC method has been developed and validated for the estimation of the drug Sparfloxacin using Tinidazole as internal standard in bulk drug and pharmaceutical dosage forms. The estimation was carried out using C18 column (150mm x 4.6mm, 5µm); mobile phase consisting of buffer at pH 4.5 and Acetonitrile; the flow rate of 1.0mL/min and ultraviolet detection at 300 nm. The retention time of Sparfloxacin was reported to be 2 min, the lowest Retention Time ever reported. The method was validated with respect to Precision, Linearity, Accuracy, Ruggedness and Robustness as per ICH Guidelines. The LOD and LOQ have also been established and found to be 75µg/mL and 250 µg/mL respectively. The validated method was successfully applied to the commercially available pharmaceutical dosage forms, yielding very good and reproducible result.

KEYWORDS
Sparfloxacin • Tinidazole • HPLC • LC Method development and validation.

INTRODUCTION
Since last couple of years, our continuous efforts are to contribute to our Pharmaceutical society with a very user friendly as well as fast eluting HPLC analytical method for various Pharmaceutical products [1-3] . As we are aware that faster estimation of the product plays a very important role in the productivity of the organization and this is the need of the day, we have tried to develop a faster method for the estimation of drugs. In continuation to our interest, we have selected the anti-bacterial drug Sparfloxacin in the present work. This time we have tried to use Tinidazole as an internal standard, which will help the analyst in providing the error free analytical results.

Sparfloxacin is a fluoroquinolone antibiotic which is used in the treatment of bacterial infections. It is an antibacterial drug. Quinolones inhibit the bacterial DNA gyrase or the topoisomerase IV enzyme, thereby inhibiting DNA replication and transcription. Quinolones can enter cells easily and therefore are often used to treat intracellular pathogens such as Legionella pneumophila and Mycoplasma pneumoniae. The IUPAC name of Sparfloxacin is 5-amino-1-cyclopropyl-7-[(3*R*,5*S*)3,5-dimethylpiperazin-1-yl]-6,8-difluoro-4-oxo-quinoline-3-carboxylic acid and the structure of the same is represented in Fig.1.

A few methods are available in the literature for the estimation of Sparfloxacin [4-9]. In the current work, sincere efforts are put to develop a faster method for the determination of Sparfloxacin using Tinidazole as an internal standard. Moreover, during validation, LOD and LOQ have also been established so that this method can be adopted and utilized by others for a clinical pharmacokinetic study.

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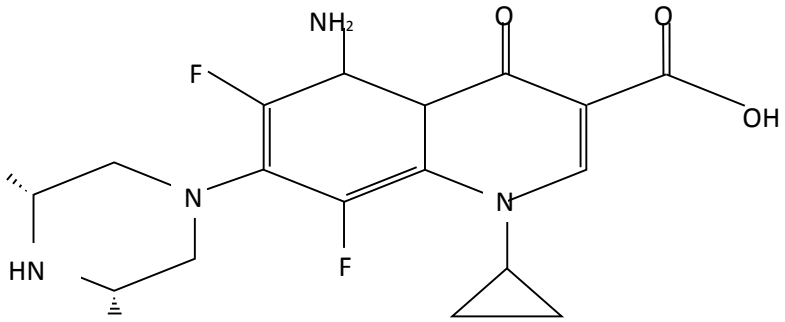


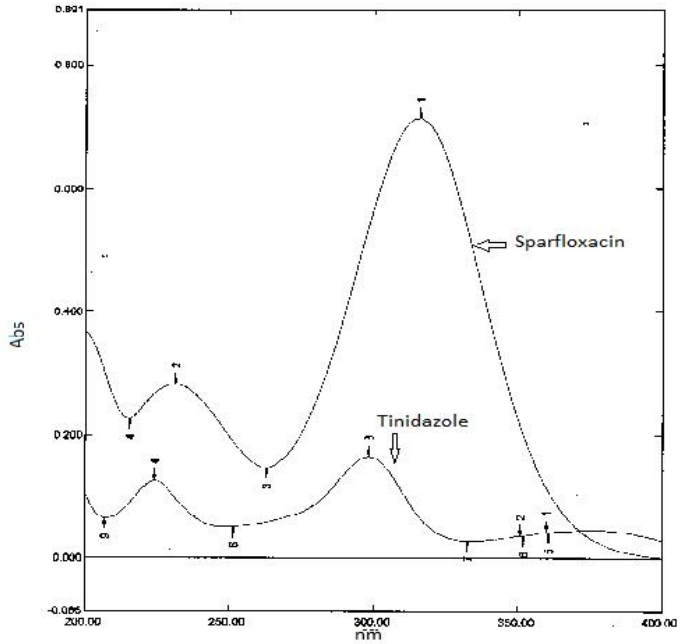
SPARFLOXACIN

Fig. 1: Chemical structure of Sparfloxacin

MATERIALS AND METHOD

Method Development

Both the drugs, Sparfloxacin and Tinidazole were scanned by UV, individually, in a wavelength range of 200-400 nm and maxima for each drug was measured. The maxima for Sparfloxacin was found to be 359.80nm, 350.80nm, whereas, for Tinidazole maxima were found at 316.20nm and 231.20nm. The corresponding UV spectrum graphs of Sparfloxacin and Tinidazole are as shown in Fig.2. To optimise the UV maxima, various HPLC experiments were performed at various wavelengths starting from 230nm to 350nm. The best response has been observed at 300nm therefore, it was selected for carrying out the experimental work.



Wave length(nm)	1	2	3	4	5	6	7	8	9
Sparfloxacin	359.8	350.8	298.2	224.2	360.6	351.8	332.6	251.4	206.8
Tinidazole	316.2	231.2	262.6	215.4					

Fig. 2: UV spectrum of Sparfloxacin and Tinidazole

Chromatographic conditions:

Various columns as well as mobile phase combinations have been tried to develop the desired method. Finally we have been able to work out an isocratic method which can be adopted very easily by any analyst. The chromatographic conditions which has been optimised was on stationary phase of C18 column with dimension of 150mm L x 4.6mm D, 5µm. Mobile phase was prepared with Buffer and Acetonitrile (50:50). Buffer was prepared by dissolving 6.8g of Potassium dihydrogen orthophosphate in 1000 ml of water. The pH was adjusted to 4.5 with Orthophosphoric acid. Injection volume was 20µL. The pump flow rate was 1.0 mL/min. The column temperature was maintained at room temperature (~ 27ºC). The eluent was detected at 300 nm.

Materials, Reagents and Chemicals:

Sparfloxacin and Tinidazole Standards were obtained from Startech Labs. Tablets used for the experiment, Sparkind was manufactured by Discovery mankind, Sparta was manufactured by Alembic pharmaceuticals and Zospar was manufactured by FDC Limited. HPLC grade Acetonitrile, Potassium dihydrogen Orthophosphate and Orthophosphoric acid were obtained from Merck, Darmstadt, Germany.

Equipments:

UV Visible spectrophotometer used was Shimadzu, model UV-2450. The HPLC instrument used was Schimadzu make, Model LC 2010 CHT. Class VP Software was used for data acquisition.

Preparation of Standard solution:

Stock solution of Sparfloxacin was prepared by taking 25 mg of Sparfloxacin in 25mL volumetric flask and diluted upto the mark with mobile phase. Standard solution of 0.1 mg/mL of Sparfloxacin (treat this as 100 % for various experimental purpose) was prepared by taking 1mL of Sparfloxacin stock solution in 10mL volumetric flask and dilute upto the mark with mobile phase. Stock solution of Tinidazole was prepared by taking 25mg of Tinidazole in 25mL volumetric flask and dilute upto the mark with mobile phase. Standard solution of 0.1 mg/mL of Tinidazole (treat this as 100 % for various experimental purpose) was prepared by taking 1mL of Tinidazole stock solution in 10mL volumetric flask and diluted upto the mark with mobile phase.

Preparation of Linearity solutions:

For Linearity 150%, 125%, 100%, 75%, 50% & 25% solutions were prepared.150% Solution was prepared by dissolving 15 mL of each of Sparfloxacin and Tinidazole stock solution in 100mL volumetric flask and diluted upto the mark with mobile phase. 20.83 mL of 150% solution was taken in 25 mL volumetric flask and diluted upto the mark with mobile phase for preparing 125% solution. 16.7mL of 150% solution was taken in 25 mL volumetric flask and diluted upto the mark with mobile phase for preparing 100% solution. 12.5mL of 150% solution was taken in 25 mL volumetric flask and diluted upto the mark with mobile phase for preparing 75% solution. 8.3mL of 150% solution was taken in 25 mL volumetric flask and diluted upto the mark with mobile phase for preparing 50% solution. 4.2mL of 150% solution was taken in 25 mL volumetric flask and diluted upto the mark with mobile phase for preparing 25% solution.

Preparation of Accuracy Solution:

Solution was prepared by taking 25mg of Sparfloxacin standard in 25mL volumetric flask and diluted upto the mark with mobile phase. Further 1mL of the above solution and 1mL Tinidazole standard stock solution was taken in 25mL volumetric flask and diluted upto the mark with 50%, 100% & 150 % linearity solutions for getting the respective spiked solution.

Preparation of Sample solution for Batch Analysis:

The method was applied to the analysis of three commercial samples available in the market, Sparkind, Sparta and Zospar. The average weight of ten tablets was calculated and found to be402.2mg, 370.6mg and 297.8mg of Sparkind, Sparta and Zospar respectively. The tablet was crushed to a homogeneous mixture and 50.25mg, 45.23mg and 34.22mg of the tablets Sparkind, Sparta and Zospar respectively were dissolved in 25mL of the mobile phase. The solutions were sonicated for 5 minutes followed by cyclomixing for 5 minutes to extract

the drug in solution. The resulting solutions were filtered by using Millipore syringe filter (0.42µ). The resulting clear solutions were injected in HPLC in duplicate as per the developed method.

Analytical Method Validation

Specificity of the method:

The parameter, specificity of the method was performed to know the Retention Time of Sparfloxacin in the presence of internal standard Tinidazole, in standard and in the sample.

System Precision:

System Precision (System Suitability) test is used to verify if the resolution and reproducibility of the chromatographic systems are adequate for the analysis to be done. The tests are based on the fact that the equipment, electronics, samples to be analyzed constitutes an integral system that can be evaluated as such. The limits for system suitability were set for Theoretical plates, Resolution, Asymmetry.

Linearity:

To measure linearity of the method, concentrations of the standard mixture, 25%, 50%, 75%, 100%, 125% and 150% were prepared and injected and chromatogram was recorded. A graph was plotted for the concentration of the corresponding drug versus Area. The Correlation coefficient(r) for Sparfloxacin and internal standard, Tinidazole was calculated.

Accuracy:

It is very important that the developed method is accurate, which is evaluated by the recovery of the drugs. To determine the recovery in sample preparation method of standard additions was made for measuring the recovery of the drugs. To the known standard concentrations (0.1mg/ml) of the drug ,50%, 100% and 150% of Sparfloxacin was added. Three different solutions were prepared as mentioned above under sample preparation for Accuracy. The accuracy was expressed as the percentage of Sparfloxacin recovery.

LOD and LOQ:

The determination was done with various concentrations of the drug to find out the LOD and LOQ level of the drug. Precision at LOQ level has been performed to evaluate the effectiveness of the method. For this purpose the analysis of the drug was done at the LOQ level. Six injections of the drug were done and the respective chromatograms were recorded. The repeatability of retention times and peak area of standard drug was measured. This study has been performed so that the method can be effectively applied and extended to Clinical Pharmacokinetic studies, if required by somebody.

Robustness:

Robustness of the method has been checked by obtaining chromatograms with slight changes in the method. Changes were made in the Flow rate, pH, mobile phase composition, and the analysis was done. The flow rate as per the developed method is 1mL/min. This has been purposely changed to 0.8mL/min and 1.2mL/min and the chromatogram was obtained. The pH as per the developed method is 4.5. Variation of ±1 to the pH was done purposely and the chromatograms were obtained. The mobile phase composition as per the developed method is Buffer: Acetonitrile (50:50). This was changed to Buffer : Acetonitrile (45:55) and Buffer : Acetonitrile (55:45).

Ruggedness:

In order to demonstrate the Ruggedness, analysis was performed on different days and different chemists and the chromatograms were obtained and checked for any variation in the results. The percentage RSD for the retention time and area was calculated.

Performance of the method on Commercial Samples:

As a final verification of the method, the method was applied to the analysis of drug in commercial tablets. For this purpose, performance test of the method has been conducted on the market samples, Sparkind, Batch

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No.C6CGL001, manufactured by Discovery mankind, Sparta, Batch No. 11262003A, manufactured by Alembic pharmaceuticals and Zospar, Batch No. SPT0091, manufactured by FDC Limited.

RESULTS AND DISCUSSION

The present work involves estimation of Sparfloxacin in bulk and dosage forms using Reverse Phase HPLC. The method was developed and optimised in such a way that the product can be analysed rapidly using a simple methodology , which results in saving sophisticated instruments and chemist's valuable time. This is the reason that currently UFLC (Ultra-Fast Liquid Chromatography) technique [10,11] is gaining a lot of importance due to its high speed of analysis, though most of the Pharmacopeia still have the HPLC methods in their monographs. This time, we have tried to use Tinidazole as an internal standard. The benefit of using internal standard in the analytical method is that it helps the analyst in attaining the consistency in the end result. The current analytical method is rapid and encouraging. The developed method has been validated as per the ICH guidelines. While validating the method we have also established the levels of LOD and LOQ with the intention of utilizing this analytical method by somebody for their clinical pharmacokinetic study, which is not in the scope of the current work. Findings of Validation parameters are expressed in following lines.

Specificity of the method:

The Retention times of the drug Sparfloxacin and the internal standard Tinidazole were measured individually and found to be 2.058 min and 1.508 min, respectively. The retention time of a mix solution of Sparfloxacin and internal standard Tinidazole was measured and found to be 2.058 min and 1.508 min, respectively. The retention time of Sparfloxacin and Tinidazole was found to be 2.058min and 1.517 min, respectively for the sample solution (market sample in the form of Tablet spiked with the internal standard). This shows that there is no specific change in the retention time of the drug individually or in the presence of an internal standard in bulk and dosage forms. This indicates that there is no any interference of the internal standard or the excepient and hence the method can be used for the determination of Sparfloxacin in the presence of an internal standard; both in bulk and pharmaceutical dosage forms. Respective HPLC chromatograms are represented in the Fig. 3, 4 and 5.

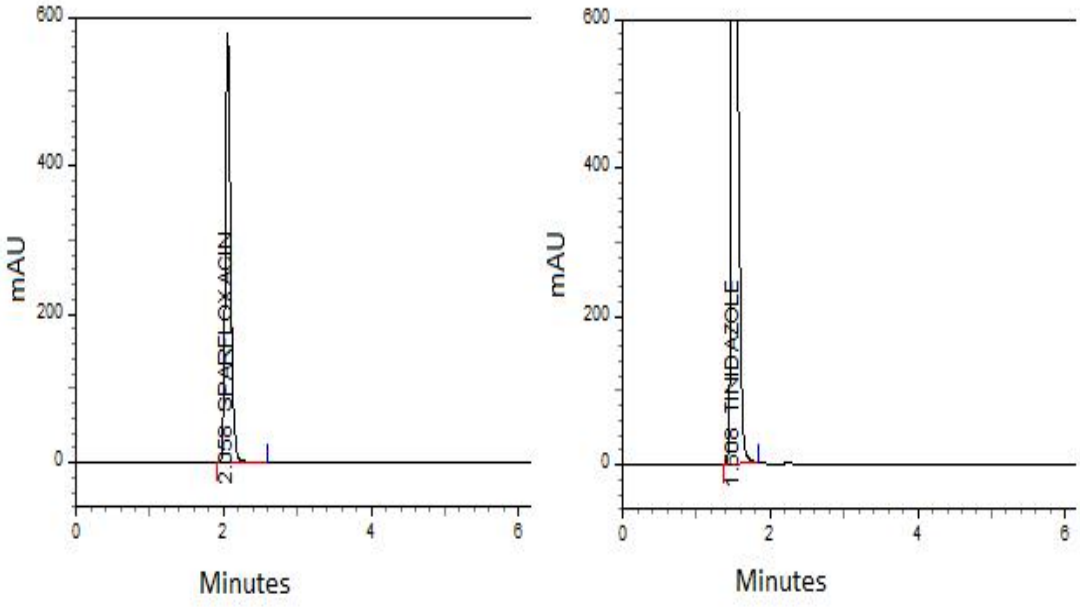

Fig. 3: Chromatograms of Sparfloxacin and Tinidazole individually

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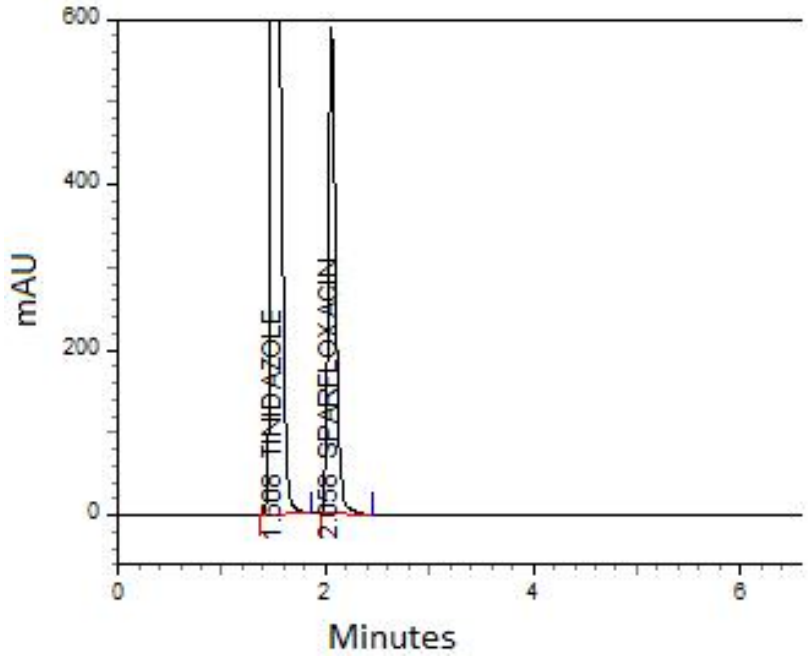

Fig. 4: Chromatogram of Standard Sparfloxacin solution containing Tinidazole as an internal standard

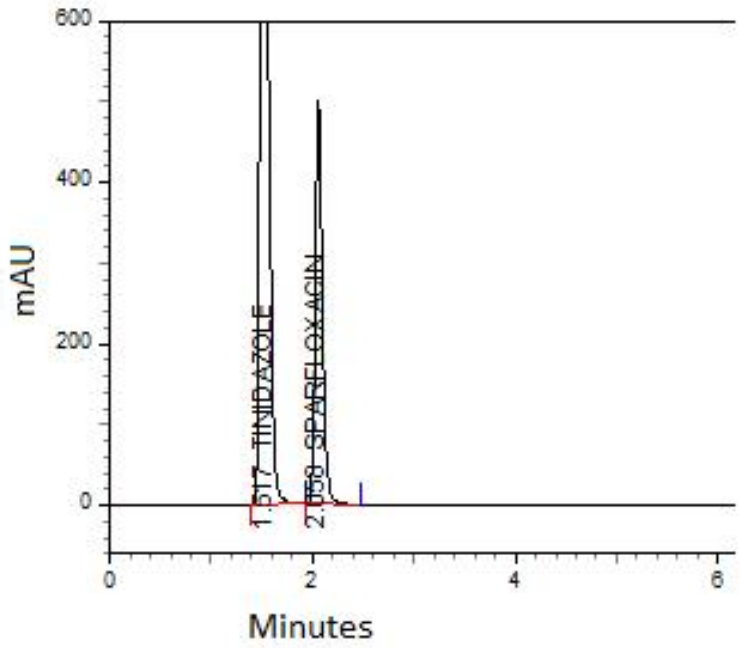

Fig. 5: Chromatogram of market Sample of Sparfloxacin spiked with Tinidazole as an internal standard

Precision:

For measuring system precision, five injections of the standard mix were injected and the respective chromatograms were obtained. The Retention time, Areas, Resolution, Theoretical plates and peak Asymmetry were calculated. Percentage RSD value was calculated. All the values have been found to be well within the acceptable range. The results obtained are given in following Table 1.

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Table 1: System Precision Results

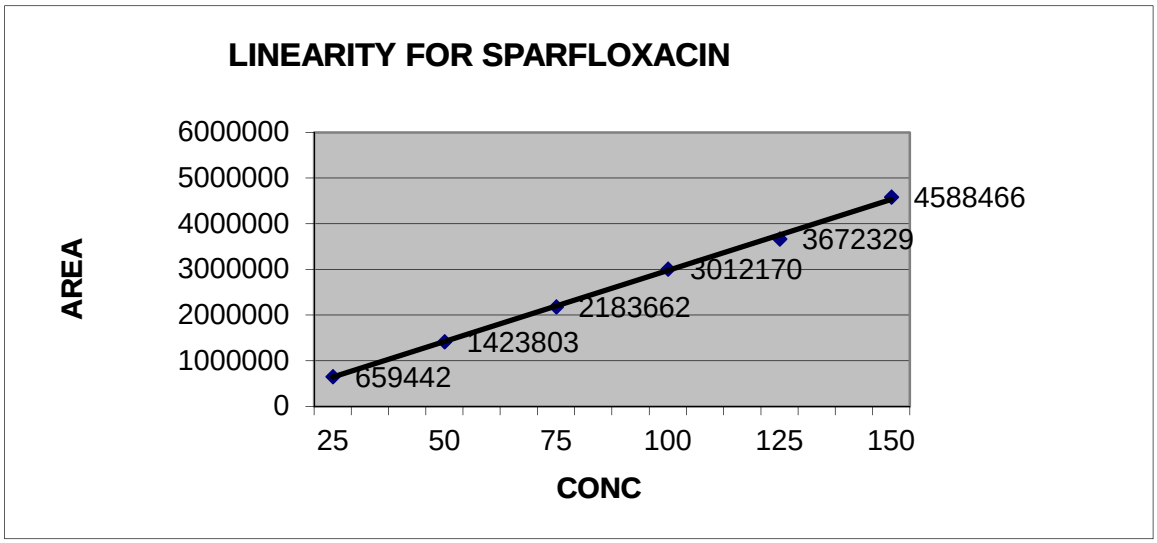
		Average	SD	%RSD
Sparfloxacin	Retention Time	2.06	0.003	0.17
	Area	3009379	2022.86	0.07
	Theoretical plates	3368.35	63.66	1.89
	Resolution	3.804	0.02	0.44
	Asymmetry	1.256	0.005	0.44
Tinidazole	Retention Time	1.508	0	0
	Area	8447374	11183.4	0.13
	Theoretical plates	1695.31	26.42	1.56
	Resolution	0	0	0
	Asymmetry	1.26	0.02	1.53

Linearity:

Linearity of an analytical method is the ability of the method to elicit test results that are directly proportional to the concentration of the analyte. The concentration of both the analytes were found to be proportional to the area and the response of the detector. The correlation coefficient (r) obtained was calculated and it was found to be greater than 0.99 for Sparfloxacin and Tinidazole, which is well within the acceptance range. The response was found to be linear over the range of 0.025mg/mL to 0.15mg/mL for Sparfloxacin and Tinidazole. The results are shown in Table 2 and respective linearity graphs are represented in Fig.6.

Table 2: Linearity results

	Linearity Range	Correlation Coefficient
Sparfloxacin	0.025-0.15mg/mL	0.9995
Tinidazole	0.025-0.15mg/mL	0.9918



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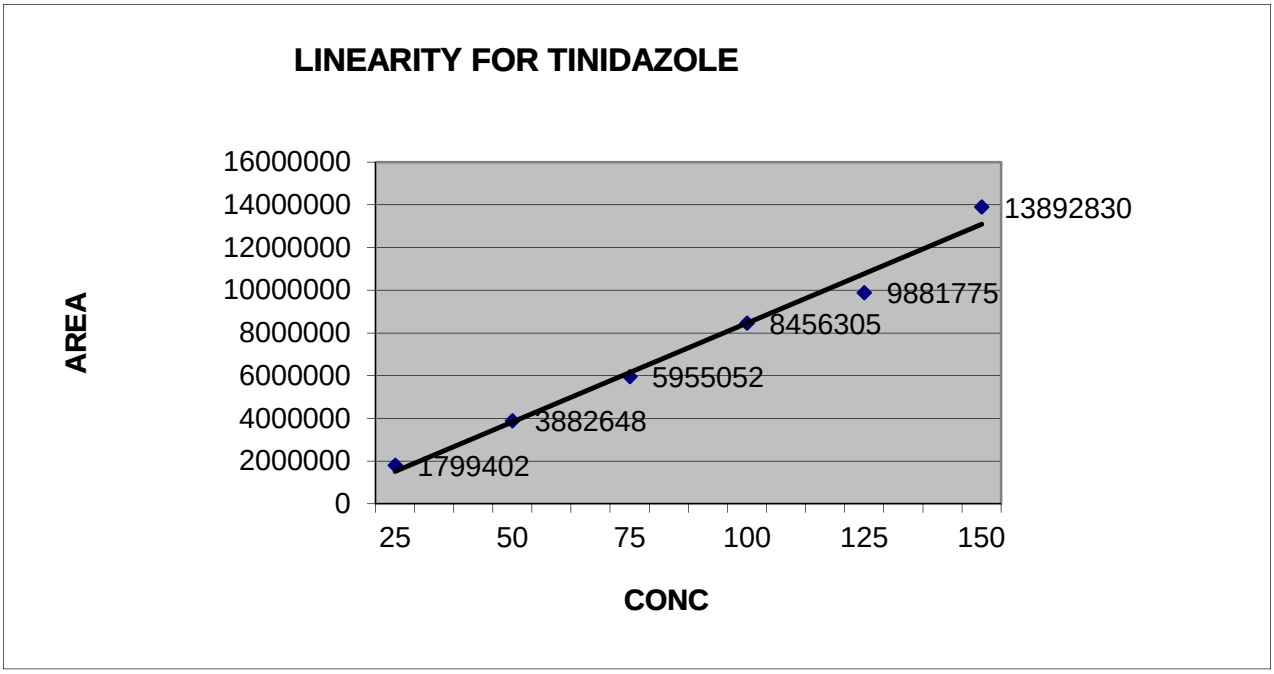

Fig. 6: Graphs Showing Linearity of Sparfloxacin and Tinidazole

Accuracy:

For measuring the accuracy of the method, 3 spiked solutions have been prepared as mentioned in Materials and method section under the heading Preparation for accuracy solution. Chromatograms were obtained for all the three spiked solutions and recoveries have been calculated. As per the ICH guidelines, the acceptable range of recovery should be between 80-120%. The results obtained were in the range of 97.16 and 103.82, which indicates that the recoveries are well within the acceptance range. Therefore, the method is accurate and it can be used for the estimation of Sparfloxacin. Details are presented in Table 3.

Table 3: Results for Accuracy of the method

Accuracy at---→	50 %	100 %	150 %
Area (Known Concn + spiked)(a)	4828726	6363804	7890810
Area Sparfloxacin Known Conc (b)	3173405	3173405	3173405
Area Sparfloxacin (a-b)	1655321	2990399	4717405
Sparfloxacin Area (expected)	1592856	3120355	4855320
% Recovery (a-b)x100/c	103.92	95.84	97.16

LOD and LOQ:

The LOD of sparfloxacin using the S/N ratio was found to be 75µg/mL and the LOQ of Sparfloxacin using the developed method was found to be 250µg/mL. Precision at LOQ level has also been performed and the percentage RSD values for Area and Retention Time were calculated. Results obtained has been shown in Table 4. % RSD obtained for RT and Area well within the acceptable limit.

Table 4: Results of Precision at LOQ Level

	Sparfloxacin		Tinidazole	
	Retention Time	**Area**	**Retention Time**	**Area**
1	2.058	5093	1.508	63171
2	2.027	4968	1.516	62850
3	2.064	5127	1.52	64562
4	2.049	5326	1.523	60284
5	2.062	5471	1.519	72356
6	2.108	5267	1.534	68954
Average	2.06	5208.67	1.52	65362.83
SD	0.026	181.08	0.009	4457.7
%RSD	1.29	3.48	0.56	6.81

Robustness:

Due to deliberate change in the flow rate, pH and mobile phase composition of the method, excellent performance of the method was observed. This indicates that the method is Robust. The results obtained are shown in Table 5.

Table 5: Robustness results

		Retention time%RSD	**Area %RSD**
Different Flow Rates			
0.8mL/min	Sparfloxacin	0.17	0.1
	Tinidazole	0	0.09
1.2mL/min	Sparfloxacin	0	0.02
	Tinidazole	0.32	0.1
Different pH values			
4.0	Sparfloxacin	1.04	0.24
	Tinidazole	1.16	0.05
5.0	Sparfloxacin	0.84	0.06
	Tinidazole	1.86	0.04
Different Mobile phase composition			
Buffer:Acetonitrile (45:55)	Sparfloxacin	0.29	0.79
	Tinidazole	0.85	0.25
Buffer:Acetonitrile (55:45)	Sparfloxacin	0.42	0.58
	Tinidazole	0.38	0.17

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Ruggedness:

Analysis performed on different days and by different analysts . Data evaluated and % RSD of Area and Retention time were calculated for various trials and data obtained are tabulated in Table 6. Based on the data it is evident that the method is Rugged.

Table 6: % RSD on different days and different Analysts

DAY 1	**Standard %RSD**	
	Retention Time	**Area**
Sparfloxacin	0.24	0.17
Tinidazole	0.41	0.28
DAY 2	**Standard %RSD**	
	Retention Time	**Area**
Sparfloxacin	12.65	0.01
Tinidazole	6.13	0.03
ANALYST 1	**Standard %RSD**	
	Retention Time	**Area**
Sparfloxacin	0.62	0.06
Tinidazole	0.48	0.04
ANALYST 2	**Standard %RSD**	
	Retention Time	**Area**
Sparfloxacin	0.42	2.38
Tinidazole	0.66	0.84

Performance of the Method on Commercial Formulations:

For evaluating the performance of the method, three market samples (Tablets) have been taken namely Sparkind, Sparta and Zospar . As per the label claim, all three commercial tablets contain 200mg of Sparfloxacin. The quantification of the drug was done in all the three formulations and the results have been found to be in the range of 99.8 to 100.3 %. This indicates that the method developed by us can be used for the estimation of Sparfloxacin in any of the pharmaceutical dosage forms. The results obtained are shown in Table 7.

Table 7: Estimation of Sparfloxacin in commercial samples

	Label claim	**Acquired data**	**% Recovery**
Sparkind	200mg/tab	205.67mg/tab	102.84%
Sparta	200mg/tab	198.67mg/tab	99.34%
Zospar	200mg/tab	199.19mg/tab	99.60%

CONCLUSION

A rapid, precise, user friendly and reproducible HPLC Method for estimation of Sparfloxacin using Tinidazole as an internal standard in Pharmaceutical dosage forms was developed and validated as per ICH Guidelines. The LOD and LOQ measurements were also established for the further scope of utilizing this method. This method can be used by the industries and academic institutions for the estimation of Sparfloxacin.

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