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Development and validation of a spectrofluorimetric method for the estimation of pyrazinamide in bulk and formulation

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Abstract
A simple, accurate, precise, sensitive and cost-effective spectrofluorimetric method was developed and validated for the estimation of pyrazinamide in bulk and formulation. The relative fluorescence intensity of pyrazinamide was measured in Distilled Water: Methanol (40:60%v/v) at an excitation wavelength of 365 nm and an emission wavelength of 510nm. Proposed method was found to be linear over the range of 50 to 1000 ng/ml with correlation coefficient 0.9999. Proposed method was validated using different analytical method validation parameters viz. Accuracy, precision, LOD, LOQ, robustness and ruggedness using QC standards as per the ICH guidelines**.** The percentage recovery was found to be 100.79 ± 0.69 % and percentage RSD values were found to be less than 2 for accuracy and precision studies. The detection and quantification limits for the proposed method were found to be 4.1259ng/ml and 9.6369ng/ml, respectively. A simple, accurate, precise, sensitive yet cost-effective spectrofluorimetric method was developed for the estimation of pyrazinamide in bulk and formulation. The said spectrofluorimetric method was found to be economic as it comprises water as a solvent.

Keywords: Spectrofluorimetry, pyrazinamide, validation, excitation, emission

Introduction

Pyrazinamide is an essential first-line antitubercular drug that plays a crucial role in the treatment of tuberculosis (TB). As a key component of the standard TB therapy regimen, accurate and reliable quantification of pyrazinamide is of paramount importance for ensuring its quality and efficacy in both bulk and formulation forms. Consequently, the development and validation of a robust analytical method to estimate pyrazinamide content is of great significance.

Several analytical techniques, including high-performance liquid chromatography (HPLC), gas chromatography (GC), and UV-visible spectroscopy, have been employed for the determination of pyrazinamide. However, these methods suffer from various limitations such as time-consuming sample preparation, high cost, complexity, and low sensitivity. In recent years, spectrofluorimetry has emerged as a promising alternative due to its enhanced sensitivity, selectivity, simplicity, and cost-effectiveness.

The spectrofluorimetric method utilizes the principles of fluorescence spectroscopy to estimate the concentration of a specific analyte. It involves the measurement of the emitted fluorescence intensity of a compound upon excitation with a specific wavelength of light. Pyrazinamide possesses intrinsic fluorescence properties, making it amenable to spectrofluorimetric analysis. By exploiting the unique spectral characteristics of pyrazinamide, a spectrofluorimetric method can be developed for its accurate determination.

The present study aims to develop and validate a spectrofluorimetric method for the estimation of pyrazinamide in bulk and formulation samples. The method will be designed to offer several advantages, including simplicity, rapidity, high sensitivity, low cost, and excellent precision. Validation parameters such as linearity, specificity, accuracy, precision, robustness, and system suitability will be evaluated to ensure the reliability and suitability of the developed method.

The validated spectrofluorimetric method will be applied to analyze commercially available pyrazinamide formulations, enabling the determination of their pyrazinamide content. This will facilitate quality control and monitoring of pyrazinamide formulations, ensuring their compliance with regulatory standards and ultimately enhancing the effectiveness of TB treatment.

In conclusion, the development and validation of a spectrofluorimetric method for the estimation of pyrazinamide in bulk and formulation samples hold significant importance in the pharmaceutical field. This research endeavor aims to contribute to the existing knowledge by offering a reliable and cost-effective analytical approach for the quantification of pyrazinamide. The outcomes of this study will aid in improving the quality control measures and therapeutic outcomes of pyrazinamide-based TB treatments, benefiting patients worldwide. [4-7]

Pyrazinamide, chemically known as pyrazine carboxamide,is an important drug primarily used in the treatment of tuberculosis (TB). It exhibits a unique chemical structure and possesses various physico-chemical properties that make it amenable to analysis using spectrofluorimetry.

IUPAC Name: 5-(Pyrazin-2-yl) pyrazine-2-carboxamide
Molecular Formula: C5H5N3O
Molecular Weight: 123.11 g/mol

Pyrazinamide is commercially available under different brand names, and its pharmaceutical properties contribute to its effectiveness in treating TB. The accurate estimation of pyrazinamide content in its bulk form and formulations is crucial for ensuring its quality and therapeutic efficacy.

The physico-chemical properties of pyrazinamide include a partition coefficient of 1.19 at pH 7.0 in water. This

coefficient indicates the distribution of pyrazinamide between two immiscible phases, providing insight into its solubility and lipophilicity.

Pyrazinamide also exhibits specific acid-base properties. The pKa value, which represents the strength of an acid or base, can provide information about its ionization behavior in different pH environments. The pKa value of pyrazinamide as the strongest acidic site is not readily available in the information provided.

Considering the unique physico-chemical properties and pharmaceutical significance of pyrazinamide, the development of an accurate, precise, and cost-effective spectrofluorimetric method for its estimation is warranted. This method aims to utilize the inherent fluorescence properties of pyrazinamide to enable its quantitative analysis.

The developed and validated spectrofluorimetric method can be effectively applied to estimate the pyrazinamide content in marketed formulations, ensuring their compliance with regulatory standards and enhancing the quality control measures in the pharmaceutical industry.

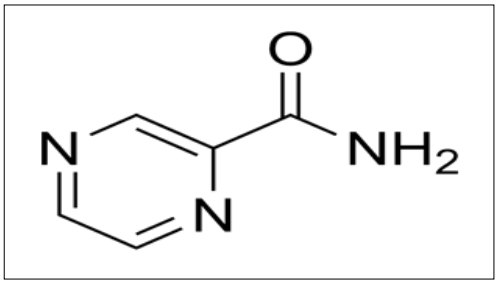

Fig 1: Chemical structure of Pyrazinamide

Materials and Method

Material

Pyrazinamide has been purchased from TCI Chemicals (India) Pvt. Ltd, Chennai. Methanol was purchased from Merck. All the chemicals of analytical grade were used for the proposed study.

Instruments used

The spectrofluorimetric study was carried out with a Shimadzu RF-5301 fluorimeter to determine levels of fluorescence in the Pyrazinamide. A Xenon 150w lamp was used as a light source. Quartz cells having 48mm height, 10mm path length with 0.5mm slit width were used for fluorescence measurement. Weighing balance (Vibra HT, Essae) with internal calibration mode was used for the weighing purpose.

Preliminary analysis

A preliminary analysis was carried out to determine the excitation and emission wavelength of Pyrazinamide. Various solvents like distilled water, methanol, acetonitrile and their combinations were used to determine appropriate media for Pyrazinamide. Pyrazinamide showed maximum fluorescence intensity in Methanol: Water (40:60%v/v) as a media. Initially, Pyrazinamide solution of 100ng/ml strength was prepared in methanol. Prepared solution was scanned spectrofluorimetrically to obtain the excitation and emission wavelengths. The scanning was performed over 220 nm to 600 nm range and excitation and emission wavelength were found to be 365nm and 510nm (figure.2) respectively.

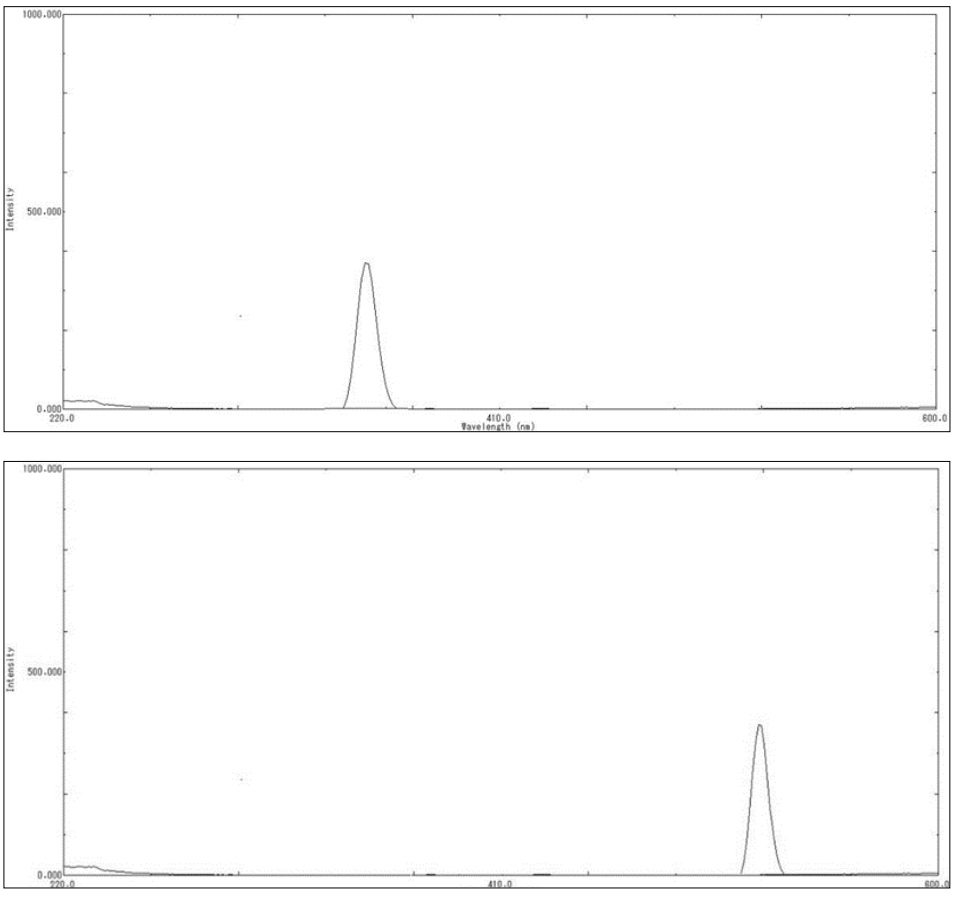

Fig 2: Excitation and Emission Spectra of Pyrazinamide

Preparation of Standard Stock Solution

Accurately weighed 5 mg of Pyrazinamide was transferred into the calibrated volumetric flask and dissolved in 10 ml water to achieve a stock solution of 1000 µg/ml (stock-I). Stock- I solution was suitably diluted with water to achieved further calibration standards.

Construction of Calibration Curve

Calibration curve was prepared by diluting the stock-I (1000 µg/ml) solution to achieve the seven different calibration standards representing CAL STD 1 (50ng/ml), CAL STD 2 (100ng/ml), CAL STD 3 (200ng/ml), CAL STD 4 (300ng/ml), CAL STD 5 (600ng/ml), CAL STD 6 (900ng/ml),

CAL STD 7 (1000ng/ml) strength. The fluorescence intensity was measured at pre-defined excitation and emission wavelengths of 365 and 510 nm respectively. The calibration curve representing concentration vs. Fluorescence intensity was plotted using excel program of Microsoft office 2013. Above mentioned procedure was repeated three times, so that reproducible results can be obtained.

Spectrofluorimetric Method Validation

Validation is the process which provides a high degree of assurance, so as to produce a desired result and meeting its predetermined specifications and quality characteristics. Developed fluorimetry method for the estimation of Pyrazinamide was validated as per the ICH guidelines. Different validation parameters like linearity and range, accuracy, precision, robustness, ruggedness, limit of detection (LOD) and limit of quantitation (LOQ) were calculated using predefined calibration standards and or quality control standards as described below [11-12].

Linearity and Range

Linearity of the proposed spectrofluorimetric method was calculated by using seven different calibration standards. After analysis of calibration standards, calibration curves in terms of Concentration vs. Fluorescence intensity plots were developed and subjected to linear least square regression analysis. R^2 value was considered to be important factor for determining the linearity of the proposed method.

Accuracy

To determine the accuracy of the method, different quality control solutions were prepared independently from stock-I i.e., LQC: 60ng/ml, MQC: 450ng/ml and HQC: 950ng/ml and analyzed at level of 80%, 100% and 120% of its predefined concentrations to the predefined concentrations, different amounts of Pyrazinamide were added (standard addition method) and the accuracy was calculated on the basis of percent recovery.

Precision

The precision of the method was checked by preparing different quality control solutions independently from stock-I i.e., LQC: 60ng/ml, MQC: 450ng/ml and HQC: 950ng/ml at three different time intervals in a day. Same procedure was followed on three different consecutive days so as to obtain inter-day variation. The fluorescence intensities for Pyrazinamide were recorded and the results were expressed as % Relative Standard Deviation (%RSD).

Robustness

Robustness of the proposed spectrofluorimetric method was established by changing composition of the ethanol by ± 1.0 %. MQC samples of Pyrazinamide were prepared in methanol with and analyzed at 365nm and 510nm (excitation-emission wavelength of Pyrazinamide). The results were calculated in terms of % RSD.

Ruggedness

Ruggedness of the proposed method was studied by analyst variation. MQC samples of Pyrazinamide were prepared by three different analysts of the laboratory and were analyzed at 365nm and 510nm. The results were calculated in terms of % RSD.

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

LOD and the LOQ of the drug were calculated by using the following equations as per ICH guidelines.

$LOD = 3.3 \times SD/S$

$LOQ = 10 \times SD/S$

Where, SD= standard deviation of lower most concentration of calibration curve

Where, SD= standard deviation of lower most concentration of calibration curve

S= Slope of calibration curve.

Estimation of Pyrazinamide in Bulk and Marketed Formulation

The Pyrazinamide content in its marketed formulation (Pyzina – Pyrazinamide Tablets IP 500mg.) was estimated using pre-validated UV-Visible spectrophotometric method. Tablet formulation contents (labeled strength: 500 mg/tablet) were dissolved in 1 ml of co-solvent system to achieve a stock solution of 1500 ng/ml (n=5). Said solution was suitably diluted with co-solvent system and analyzed for the Pyrazinamide content using proposed spectrofluorimetric method.

Results and Discussion

Construction of Calibration Curve

Quantification of Pyrazinamide samples by any instrumental method of analysis needs a reproducible calibration curve and a mathematical equation stating correlation between concentration and the response. As compare to graphical method, above stated method is widely accepted and reproducible in nature. To establish linearity of the proposed method, seven different calibration standards were prepared from the stock solution and analyzed at excitation wavelength 365nm and emission wavelength 510nm by spectrofluorimeter. Least square linear regression analysis

was performed for the obtained spectrofluorimetric data using MS-excel 2013. Calibration curve was repeated five times for reproducibility. Various concentrations and their fluorescence intensities with mean ± standard deviation was reported (Table 1).

Table 1: Calibration standard data for Pyrazinamide.

S. No.	Concentration (ng/ml)	Fluorescence intensity
1	50	52.112 ± 0.6123
2	100	99.231 ± 0.1345
3	200	196.754 ± 1.0342
4	300	297.685 ± 0.5873
5	600	599.874 ± 0.9561
6	900	885.356 ± 0.4125
7	1000	997.259 ± 0.2867

Spectrofluorimetric Method Validation

Linearity and range

Linearity and range are the important parameters of analytical method that demonstrates the limit within which the intended method is to be used for its optimum performance. Considering the prime importance of linearity and the range, seven-point calibration curve of Pyrazinamide was plotted covering a range of 50-1000 ng/ml. Different concentrations and the respective mean fluorescence intensities values are depicted in table 1. Calibration curve when subjected to least square regression analysis yielded an equation; y = 0.9916x + 0.6835 with correlation coefficient 0.9999 (figure 3). From the linearity study, it was revealed that, developed method was linear over the concentration range of 50 to 1000ng/ml.

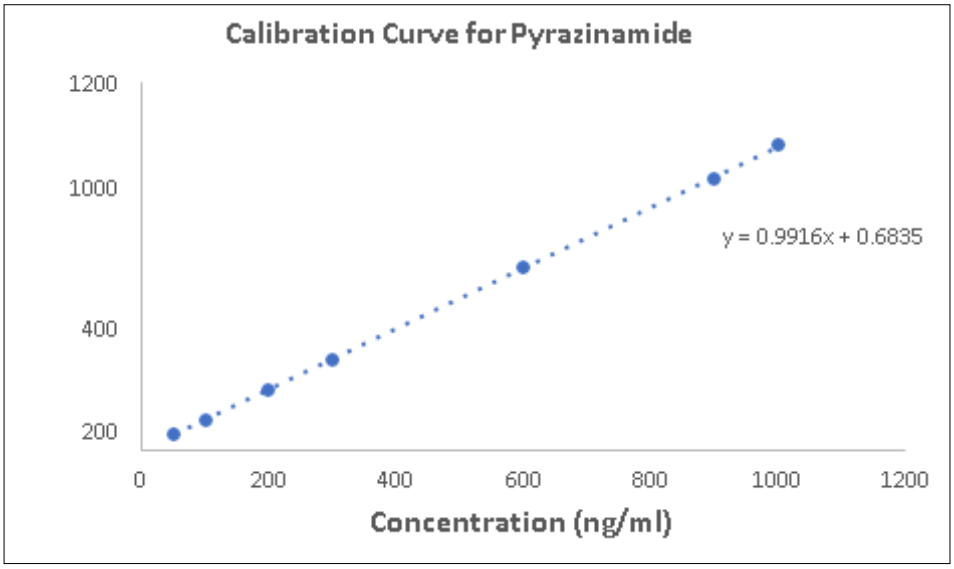

Fig 3: Calibration Curve for Pyrazinamide

Accuracy

Accuracy is the closeness of test results to the true value obtained by method. The accuracy of an analytical method should be established over its calibration range so that at any point of determination, results obtained would be accurate. For Pyrazinamide, accuracy was determined using recovery studies. At 80%, 100% and 120% standard addition, mean recovery of Pyrazinamide was found in between 99.47% to 100.18%. The relative standard deviation (% RSD) was found to be less than 2 as shown in table 2. From the results of accuracy studies, it was predicted that developed method is highly accurate.

Table 2: Accuracy data of Spectrofluorimetric method for Pyrazinamide

Sr. no.	Concentration (%)	Origin level (ng/ml)	Amount added (ng/ml)	% recovery	Mean % recovery	% RSD
1	80	60	48	98.89	99.47	1. 076
2	80	60	48	100.32		
3	80	60	48	99.68		
4	100	450	450	100.12	100.18	0.134
5	100	450	450	99.81		
6	100	450	450	100.54		
7	120	950	1140	98.47	99.67	0.884
8	120	950	1140	100.98		
9	120	950	1140	99.92		

Precision

Precision is defined as closeness of agreement among the individual test result when the method is applied repeatedly to multiple sampling of homogeneous sample. Precise analytical method leads to accurate results. Intra-day and inter-day precision of spectrofluorimetric method was established at LQC: 60ng/ml, MQC: 500ng/ml and HQC: 950ng/ml levels of Pyrazinamide. The results expressed in terms of mean fluorescence intensity values, % assay and % RSD for the intra- day and inter-day precision study are demonstrated in table 3 and table 4 respectively. Percent RSD values of intra-day precision study were found to be in between 0.121 and 0.875, whereas those of inter-day precision study were in between 0.419 and 1.689 overall; % RSD values of less than 2 demonstrated that developed spectrofluorimetric method is precise and reproducible.

Table 3: Intra-day precision data of Spectrofluorimetric method for Pyrazinamide

		Morning			Afternoon			Evening		
S. No	**Concentration Range (ng/ml)**	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**
1	60	60.104	100.26	0.384	60.372	100.82	0.875	60.651	101.26	0.549
2	450	449.254	99.87	0.162	450.407	99.64	0.121	449.288	99.89	0.232
3	1000	950.467	100.59	0.151	950.258	100.55	0.209	950.936	100.53	0.136

Table 4: Inter-day precision data of Spectrofluorimetric method for Pyrazinamide

		Day 1			Day 2			Day 3		
S. No	**Concentration Range (ng/ml)**	**Amount (Mean)**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**	**Mean**	**% Assay**	**% RSD**
1	60	60.663	99.79	0.615	60.979	100.89	1.431	60.266	101.02	1.689
2	450	450.126	100.72	0.944	449.262	100.36	0.885	450.051	100.13	0.736
3	1000	950.439	99.98	1.012	950.422	99.55	0.419	950.209	99.75	0.590

Robustness

Robustness of an analytical method is the measure of its capacity to remain unaffected by small but deliberate change in method parameters. It is an important parameter of analytical method as a small, un-intentional change in method parameters like solvent composition, buffer strength, pH etc. May occur during routine use and may hamper the performance of said method. It is expected that such change should not alter the performance of the method. Therefore, robust analytical method is preferred. Robustness of proposed spectrofluorimetric method was performed by changing the pH of water. After analysis, it was found that change in pH of water did not affect the performance of method. % RSD values were found to be in between 0.75 and 1.46 (table 5). Percent RSD values below 2 depicted that proposed spectrofluorimetric method is robust in nature.

Table 5: Robustness data of Spectrofluorimetric method for Pyrazinamide

S. No.	**Concentration (ng/ml)**	**Mobile phase composition (% v/v)**	**Amount**	**% RSD**
1	450	39:61	425.449	0.89
2	450	40:60	425.721	0.75
3	450	41:59	426.563	1.46

Ruggedness

Ruggedness of analytical method is the degree of reproducibility of test results obtained by analysis of the same samples under a variety of conditions, such as different laboratories, different analyst. In order to determine the ruggedness of proposed spectrofluorimetric method, Pyrazinamide solutions were prepared and analyzed by different analysts. Sample analysis and data processing resulted into % RSD values between 0.229 and 0.621. Results of ruggedness studies revealed that proposed spectrofluorimetric method was rugged as it showed % RSD values less than 2 (table 6).

Table 6: Ruggedness data of Spectrofluorimetric method for Pyrazinamide

S. No.	**Concentration (ng/ml)**	**Analyst**	**Amount**	**% RSD**
1	450	I	425.972	0.621
2	450	II	425.547	0.341
3	450	III	425.369	0.229

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

Limit of quantification (LOQ) represents the lowermost concentration that can be analyzed with acceptable accuracy and precision. Limit of detection (LOD) of an individual analytical procedure is the lowest amount of an analyte in a sample which can be detected but not necessarily quantitated as an exact value. From the standard deviation of lower most concentration and the slope of the calibration curve, LOD and LOQ of proposed spectrofluorimetric method was found to be 4.1259 ng/ml and 9.6369 ng/ml respectively (table 7). Lower LOQ value indicated that proposed method is sensitive enough to quantify the Pyrazinamide content of samples at its lower level.

Table 7: LOD & LOQ data for Spectrofluorimetric method for Pyrazinamide:

1	LOD	4.1259 ng/ml
2	LOQ	9.6369 ng/ml

Estimation

The developed spectrofluorimetric method was successfully applied for estimation of Pyrazinamide in marketed formulation. By proposed method, Pyrazinamide content in the Pyzina

– Pyrazinamide Tablets IP 500mg was found to be 100.79 ± 0.69 % respectively.

Conclusion

A simple, accurate, sensitive and precise spectrofluorimetric method for the estimation of Pyrazinamide was developed and validated. The proposed method was found to be robust and rugged in nature with high accuracy and precision. Proposed method was successfully used for the estimation of Pyrazinamide in its marketed formulation.

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References

1. Bhusari S, Karnik H, Wakte P. Development and validation of a spectrofluorimetric method for the estimation of Tenofovir in bulk and formulation. World J Adv Eng Technol Sci,2023:8(01):231–238.
2. European Medicines Agency. Note for guidance on validation of analytical procedures: text and methodology, 1995, 1-15.
3. ICH Harmonized Tripartite Guideline. Validation of analytical procedures: text and methodology Q2 (R1), 1994.
4. Boots AW, Haenen GR, Bast A. Health effects of quercetin: from antioxidant to nutraceutical. Eur J Pharmacol,2008:585(2–3):325–37.
5. Craig CR, Stitzel RE. Modern pharmacology with clinical applications. 6th ed. Lippincott Williams & Wilkins, 2004: p. 588.
6. Zhou HY, Chen XH, Li W, Guan SY. Mass analysis of tenofovir disoproxil fumarate and determination of fumarate combination ratio by NMR. Chin J Pharm Anal,2012:32(12):2180–2183.
7. Kalpana J, Himaja M, Anbarasu C. Rapid stability indicating RP-HPLC method for simultaneous quantification of related impurities of antiretroviral drugs. Asian J Chem,2015:27(7):2393–2395.
8. Abdel Hay MH, Gazy AA, Shaalan RA, Ashour HK. Selective RP-HPLC DAD method for determination of tenofovir fumarate and emtricitabine in bulk powder and in tablets. Acta Chromatogr,2015:27(1):41–54.
9. Srinath A, Sneha B, Alladi A, Ahmed R, Kulkarni R. Method development and validation for simultaneous estimation of lamivudine, tenofovir and efavirenz in combined tablet dosage form by RP-HPLC and UV-spectroscopic method. Int J Pharm Sci Res,2014:5(12):5491–5497.
10. Bhusari S, Karnik H, Wakte P. Development and validation of a spectrofluorimetric method for the estimation of Tenofovir in bulk and formulation. World J Adv Eng Technol Sci,2023:8(01):231–238. DOI: https://doi.org/10.30574/wjaets.2023.8.1.0033.
11. Bhusari S, Karnik H, Wakte P. Development and validation of UV-spectrophotometric method for estimation of pterostilbene in Pterocarpus marsupium. World J Adv Res Rev,2023:17(01):1123–1131.
12. Jullien V, Treluyer J, Pons G, Rey E. Determination of Tenofovir in human plasma by high-performance liquid chromatography with spectrofluorimetric detection. J Chromatogr B Anal Technol Biomed Life Sci,2003:785(2):377–381.
13. Sunitha PG, Kaliappan I. Validated first-order derivative spectrophotometry for simultaneous determination of emtricitabine and Tenofovir disoproxil fumarate in pharmaceutical dosage form. J Drug Deliv Ther,2014:4(2):9–11.
14. International Conference on Harmonisation (ICH). Topic Q2 (R1): Validation of analytical procedures text and methodology, 2005.
15. Bhusari S, Karnik H, Wakte P. Development and evaluation of matrix-type sustained release tablet of metformin HCl by using natural polymer. MOENIA J,2023:10(3):81-112. DOI: 10.37896/MOENIA10.3/311.
16. The United States Pharmacopoeia (USP 38). Chromatography, 2015, 621.
17. Center for Drug Evaluation and Research of the Food and Drug Administration (FDA). Reviewer Guidance Validation of Chromatographic Methods, 1994.