RESEARCH ARTICLE

Simultaneous Determination of Pyridoxine HCl, Isoniazid, Trimethoprim and Sulfamethoxazole by Double Divisor Ratio Spectra Derivative Spectroscopic Method

Kush Prashant Kansara, Hiral Narendra Dave*

Department of Pharmaceutical Quality Assurance, Parul Institute of Pharmacy, Parul University, Vadodara, Gujarat, India.

Received: 26th November, 2023; Revised: 28th February, 2024; Accepted: 15th April, 2024; Available Online: 25th June, 2024

ABSTRACT

Tuberculosis could be regarded as a serious and enduring menace to global health. A significant hurdle in tuberculosis treatment lies in drug resistance, including the emergence of multiple drug resistance. A regulatory agency has recently endorsed the fixed-dose combination of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole as a robust therapy for cases of tuberculosis resistant to multiple drugs. Therefore, a successful attempt was made to develop and validate a novel spectroscopic method, i.e., double deviation ratio spectra derivative spectroscopic method, for the simultaneous determination of pyridoxine HCl, Isoniazid, trimethoprim, and sulfamethoxazole in their pure form. The optimized divisor concentration in the developed method was found to be a mixture of 6 µg/mL of sulfamethoxazole and 16 µg/mL of pyridoxine HCl for the determination of isoniazid and trimethoprim in the same quaternary mixture. Similarly, the optimized divisor concentration for the determination of sulfamethoxazole and pyridoxine HCl was found to be a mixture of isoniazid and trimethoprim with concentrations of 10 and 12 µg/mL, respectively. With the developed method, the linearity for pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole was found to be in the range of 8 to 40 µg/mL, 10 to 30 µg/mL, 12 to 36 µg/mL, and 4 to 12 µg/mL, respectively. The results of the accuracy study, in terms of percentage recovery, were found to be 99.92 ± 0.978, 100.04 ± 1.731, 99.87 ± 1.811, and 98.83 ± 0.922 for the simultaneous determination of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole, respectively, using the developed method. The developed method has been successfully validated as per the ICH guidelines.

Keywords: Double divisor ratio spectra derivative spectroscopy, Isoniazid, Multiple drug-resistant tuberculosis, Pyridoxine HCl, Sulfamethoxazole, Trimethoprim.

International Journal of Pharmaceutical Quality Assurance (2024); DOI: 10.25258/ijpqa.15.2.38

How to cite this article: Kansara KP, Dave HN. Simultaneous Determination of Pyridoxine HCl, Isoniazid, Trimethoprim and Sulfamethoxazole by Double Divisor Ratio Spectra Derivative Spectroscopic Method. International Journal of Pharmaceutical Quality Assurance. 2024;15(2):790-795.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Mycobacterium tuberculosis stands as the causative agent behind the onset of tuberculosis (TB) pathogenesis. Regular and close interaction with TB patients notably elevates the risk of contracting the illness. An individual receiving treatment for active tuberculosis possesses the capacity to transmit the disease so frequently, maintaining a relatively high frequency of transmission. For the treatment of latent TB, medical practitioners utilize isoniazid, rifampin, or a combination of isoniazid and rifampicin.[1] The duration of treatment varies from three to 9 months, contingent on the medications administered. A 2 month prescription of rifampicin, ethambutol, pyrazinamide, and isoniazid in combination is followed by a subsequent 4 month regimen of rifampicin and isoniazid alone.[2] This has been the established treatment regimen for drug-susceptible pulmonary TB since 2010.[3] During the concluding four-month period, ethambutol might be employed as a substitute for isoniazid in patients displaying strong tolerance. In cases where multiple drug-resistant tuberculosis (MDR-TB) is detected, treatment spanning 18 to 24 months necessitates the inclusion of at least four antibacterial medications. As discussed previously, combination therapy is one of the choices for the smooth treatment of MDR-TB. A recently endorsed fixed-dose combination of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole, with the respective ratios of 1:12:6.4:32, is recommended widely for the treatment of MDR-TB.[4]

The UV-visible spectrophotometric method stands out as the most frequently employed and straightforward approach for determining the concentrations of various active pharmaceutical

Author for Correspondence: hiral.dave16194@paruluniversity.ac.in

ingredients and products.[5-8] Advancement of UV spectroscopy, i.e., double divisor ratio spectra derivative method, can be considered as one of the rapid and easy techniques for determining multiple components simultaneously, utilizing an advanced form of derivative spectroscopy.[9] Various methods have been reported for the determination of pyridoxine HCl, isoniazid, trimethoprim and, sulfamethoxazole individually or in combination with other active pharmaceutical ingredients. One bioanalytical method has been reported for the determination of pyrazinamide, rifampicin and isoniazid by reverse-phase high-performance liquid chromatography (RP-HPLC).[10] Simultaneous determination of quaternary mixture of ethambutol hydrochloride, isoniazid, pyrazinamide and rifampicin has been reported previously.[11] Similarly, pyridoxine hydrochloride, isoniazid, pyrazinamide, and rifampicin combination has been reported in their pharmaceutical formulations by RP-HPLC method.[12] Therefore, there is a significant research gap in the simultaneous determination of the recently approved combination of sulfamethoxazole, isoniazid, trimethoprim, and pyridoxine HCl, despite the abundance of literature on various tertiary and quaternary mixtures of anti-TB drugs or drugs used in the treatment of MDR-TB, especially with the use of advanced derivative spectroscopy. The current research article provides a novel, rapid and validated spectroscopic method for the simultaneous determination of sulfamethoxazole, isoniazid, trimethoprim, and pyridoxine HCl.

MATERIALS AND METHODS

Materials

Pure drug samples of isoniazid, sulfamethoxazole and trimethoprim were obtained from Balaji Drugs Dealers: API and Pharmaceutical Polymer, Surat, Gujarat. The pure sample of Pyridoxine HCl was obtained from Benz Chem Enterprises, Vadodara, Gujarat. Throughout the process of developing and validating the method, a wide variety of chemicals were utilized to ensure the thorough and effective development and validation of the newly created approach. They were obtained from the local authorized chemical suppliers.

Methods

Equipment

A Shimadzu - 1900 double beam UV-visible spectrophotometer with UV Probe software, version 2.34, was used during the overall process of method development and validation as a core instrument for overall research. Apart from this, a variety of equipment such as UV-visible spectrophotometer (Shimadzu 1800), digital weighing balance (Mettler Toledo, Switzerland), FTIR (Bruker, Germany), and melting point apparatus (Veego, India) were used as per the requirement throughout the research.

Selection of solvent and selection of wavelength

Sulfamethoxazole and trimethoprim exhibited high solubility in methanol and ACN. Isoniazid and pyridoxine HCl were also freely soluble in water and soluble in methanol. To assess the stability of working solutions in their respective solvents, four distinct solutions containing 10 µg/mL of each drug were scanned using a UV spectrometer using methanol as a solvent. The standard solution of isoniazid (10 µg/mL), sulfamethoxazole (10 µg/mL), trimethoprim (10 µg/mL), and pyridoxine HCl (10 µg/mL) were scanned separately in the range of 200 to 400 nm.

Preparation of standard stock solution

Stock solutions were prepared for pyridoxine HCl, isoniazid, trimethoprim and sulfamethoxazole, respectively, using methanol as a solvent with the final concentration of 1000 µg/mL for each drug separately, which were labeled as stock solution I-IV respectively. The mixture of pyridoxine HCL, isoniazid, trimethoprim, and sulfamethoxazole, with the respective ratios of 1:12:6.4:32 was prepared to get the final concentration of stock solution as 100 µg/mL of pyridoxine HCl, 1200 µg/mL of isoniazid, 640 µg/mL of trimethoprim and 3200 µg/mL of sulfamethoxazole, respectively. The solution mixture was labeled as stock V.

Preparation of working standard solutions of each isoniazid, sulfamethoxazole, trimethoprim, and pyridoxine HCl

From the stock solutions I-IV, various dilutions/ working standard solutions were made for individual drugs in the range of 8 to 40 µg/mL, 10 to 30 µg/mL, 4 to 12 µg/mL and 12 to 36 µg/mL of pyridoxine HCl, isoniazid, trimethoprim and sulfamethoxazole, respectively using methanol as a solvent.

Preparation of working standard solutions of mixture of isoniazid, sulfamethoxazole, trimethoprim, and pyridoxine HCl

Working standard solution of a mixture of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole was prepared from stock solution V with the respective ratios of 1:12:6.4:32 for further application of double divisor ratio spectra derivative method.

Optimization of divisor concentration for simultaneous determination of isoniazid, sulfamethoxazole, trimethoprim, and pyridoxine HCl using double divisor ratio spectra derivative spectroscopy

The optimized divisor concentration in the developed method was found to be a mixture of 6 µg/mL of sulfamethoxazole and 16 µg/mL of pyridoxine HCl for determination of isoniazid and trimethoprim in the same quaternary mixture. Once the ratio spectra were obtained, the first derivative amplitude was measured to get respective zero crossing points for isoniazid and trimethoprim. Similarly, the optimized divisor concentration for determination of sulfamethoxazole and pyridoxine HCl was found to be a mixture of isoniazid and trimethoprim with a concentration of 10 and 12 µg/mL, respectively. Further, the ratio spectra were obtained and converted to first derivative spectra to get zero crossing points of sulfamethoxazole and pyridoxine HCl. Thus, after the application of the double divisor ratio spectra derivative method, the analytical wavelength was found to be 269, 287, 262, and 291 nm for pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole, respectively.

Validation of the developed method

- *Linearity range*

From the stock solutions, various dilutions and working standard solutions were prepared for individual drugs in the following ranges: 8 to 40 µg/mL for pyridoxine HCl, 10 to 30 µg/mL for isoniazid, 12 to 36 µg/mL for trimethoprim, and 4 to 12 µg/mL for sulfamethoxazole, respectively, using methanol as a solvent. Linearity was assessed for all four drugs by applying the optimized parameters of the developed method, along with their corresponding analytical wavelengths: 269 nm for pyridoxine HCl, 287 nm for isoniazid, 262 nm for trimethoprim, and 291 nm for sulfamethoxazole.

- *Accuracy*

By employing the standard addition method, the accuracy of the method was assessed through the calculation of the percentage recovery of standards. In the standard laboratory mixture of all four drugs, each individual drug was spiked with a specific concentration. The standard laboratory mixture of pyridoxine HCl, isoniazid, trimethoprim and sulfamethoxazole was diluted in such a way to get the final concentration of the respective drugs as 8, 10, 12 and 4 µg/mL. The respective standard solution of the standard laboratory mixture was determined and calculated to obtain the percentage of each standard simultaneously. Each individual standard was spiked at 50, 100, and 150% levels. At these levels, the spiking concentrations of pyridoxine HCl were 4, 8, and 12 µg/mL in the standard laboratory mixture of all four drugs. Similarly, the spiking concentrations for isoniazid were 5, 10, and 15 µg/mL. The spiking concentrations for trimethoprim were 6, 12, and 18 µg/mL. Additionally, the spiking concentrations of sulfamethoxazole were 2, 4, and 6 µg/mL. Subsequently, the percentage of standard recoveries was determined for each drug as part of the recovery study after applying the optimized conditions of the newly developed spectroscopic method.

- *Precision*

The method's precision was assessed through repeatability, as well as intraday and interday precision. The repeatability of the method was verified by repeatedly taking (n = 6) standard solutions of 24, 20, 24, and 8 µg/mL of pyridoxine HCl, isoniazid, trimethoprim and sulfamethoxazole, respectively. For intraday precision, three replicates of three concentrations of the standard solution (16, 24, and 32 µg/mL) of pyridoxine HCl, (15, 20, and 25 µg/mL) of isoniazid, (18, 24, and 30 µg/mL) of trimethoprim, and (6, 8, and 10 µg/mL) of sulfamethoxazole were analyzed on the same day. For interday precision, three replicates of three concentrations of the standard solution (16, 24, and 32 µg/mL) of pyridoxine HCl, (15, 20, and 25 µg/mL) of isoniazid, (18, 24, and 30 µg/mL) of trimethoprim, and (6, 8, and 10 µg/mL) of sulfamethoxazole were analyzed over 3 days. %RSD was calculated for repeatability, intraday precision and interday precision.

- *Reproducibility*

The reproducibility of the developed method was determined using different UV-visible spectrophotometers, namely Shimadzu 1800 and Shimadzu 1700. Three concentrations of the standard solution (16, 24, and 32 µg/mL) of pyridoxine HCl, (15, 20, and 25 µg/mL) of isoniazid, (18, 24, and 30 µg/mL) of trimethoprim, and (6, 8, and 10 µg/mL) of sulfamethoxazole were analyzed in triplicate. After the application of the developed method, the %RSD was calculated.

- *Robustness*

The robustness of the method was tested with minor modifications in the detection wavelength (± 1 nm) for all four drugs simultaneously. Three random concentrations were selected from the standard range of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole. The %RSD was calculated.

Limit of detection and limit of quantification

The LoD and LoQ were estimated using the following equations.

LoD = 3.3 × (Standard deviation/Mean Slope of regression line or Calibration curve)

LoQ = 10 × (Standard deviation/Mean Slope of regression line or Calibration curve)

Experimental verification was conducted to confirm the final result of LoD and LoQ.

RESULTS AND DISCUSSION

Solubility Study

Isoniazid, sulfamethoxazole, trimethoprim and pyridoxine HCl were stable at room temperature for 24 hours and at freezing conditions for 48 hours using methanol as a common solvent. The mixture of all four drugs was also stable at room temperature for 24 hours when methanol was used as a solvent. Therefore, methanol was chosen as the common solvent for the subsequent analytical method development and validation for the simultaneous determination of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole.

Optimization of Divisor Concentration for Simultaneous Determination of Isoniazid, Sulfamethoxazole, Trimethoprim, and Pyridoxine HCl using Double Divisor Ratio Spectra Derivative Spectroscopy

Utilizing UV-visible spectroscopy effectively for the analysis of multiple components presents a formidable task when done without the prerequisite steps of separating and purifying individual substances. However, the potential for success is now enhanced through the utilization of software advancements, especially in the case of spectroscopy. The zero-order spectra of isoniazid, sulfamethoxazole, trimethoprim, and pyridoxine HCl, as represented in Figure 1, exhibited complete overlap, highlighting the need to employ advanced derivative spectroscopy, specifically by generating ratio spectra from the combined drug mixtures.

One of the biggest challenges to opting for the application of ratio spectra was to optimize divisor concentrations of respective drugs to get accurate results in the form of concentrations of individual drugs from the mixture without the aid of prior separation of individual drugs. A significant

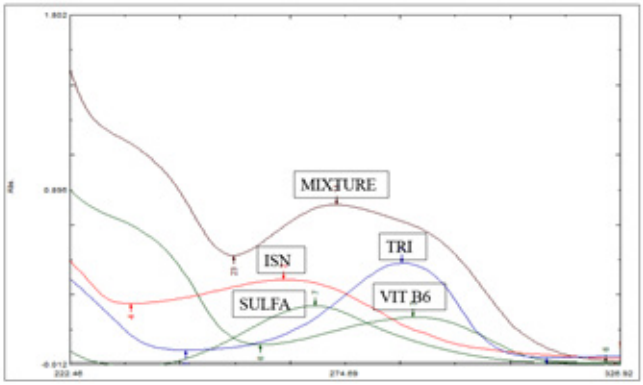

Figure 1: The overlay zero order spectra of pyridoxine HCl (Vit B6), isoniazid (ISN), trimethoprim (TRI) and sulfamethoxazole (SULFA) along with a standard laboratory mixture of all four drugs

hurdle in adopting the utilization of ratio spectra was the optimization of the divisor concentrations for each respective drug. This optimization aimed to achieve precise outcomes in the form of individual drug concentrations within the mixture, all without relying on the prior separation of the individual components. Due to the complexity of the mixture being quaternary in nature, the feasibility of employing the ratio spectra derivative method was precluded. To address this issue, the solution came in the form of employing the double divisor ratio spectra derivative method. This approach enabled the simultaneous determination of isoniazid, sulfamethoxazole, trimethoprim, and pyridoxine HCl without necessitating the individual separation of these drugs. The fundamental principle behind the utilization of the double divisor ratio spectra derivative method for analyzing a quaternary mixture was to optimize the divisor composition by creating a blend of two drugs. This blended divisor was then divided by each individual drug component and a quaternary mixture of drugs as well. Later, first-order derivative ratio spectra were obtained to identify the zero-crossing points of drugs and determine the analytical wavelengths for each drug.

Based on the preceding discussion and its application, the optimized divisor concentrations within the developed method were established as a mixture of 6 µg/mL of sulfamethoxazole and 16 µg/mL of pyridoxine HCl. These concentrations, in the form of a mixture, were employed for the determination of isoniazid and trimethoprim in the given quaternary mixture. After acquiring the ratio spectra, the amplitude of the first derivative was measured to determine the respective zero-crossing points for isoniazid and trimethoprim. Similarly, for the determination of sulfamethoxazole and pyridoxine HCl, the optimized divisor concentration was discovered to be a mixture of isoniazid and trimethoprim at concentrations of 10 and 12 µg/mL, respectively. Subsequently, the ratio spectra were obtained and transformed into first derivative spectra to pinpoint the zero-crossing points for sulfamethoxazole and pyridoxine HCl. Consequently, following the implementation of the double divisor ratio spectra derivative method, the analytical wavelengths were determined as 269 nm for pyridoxine HCl, 287 nm for isoniazid, 262 nm for trimethoprim, and 291 nm for sulfamethoxazole.

Validation of Developed Method for Simultaneous Determination of Pyridoxine HCl, Isoniazid, Trimethoprim and Sulfamethoxazole

The developed double divisor ratio method was successfully validated successfully for the simultaneous determination of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole, encompassing all validation parameters as per the ICH guidelines.

The accuracy study was carried out in the form of %recovery of spiked samples of individual drugs in a mixture. The results of the accuracy study are represented in Table 1. The results of validation parameters as per ICH guidelines of analytical method validation were shown in Table 2.

Table 1: Results of accuracy study for simultaneous determination of pyridoxine HCl, isoniazid, trimethoprim and sulfamethoxazole

Drug	*%Level (Spiking)*	*Amount of sample mixture (µg/mL)*	*Amount of Standard spiked (µg/mL)*	*Total sample (µg/mL)*	*Recovered amount (µg/mL)*	*%Recovery**	*Mean Recovery*	*S.D.*
Pyridoxine HCl	50	8	4	12	11.98	99.83		
	100	8	8	16	15.84	99.0		
	150	8	12	20	20.19	100.95	99.92	0.978
Isoniazid	50	10	5	15	14.87	100.87		
	100	10	10	20	19.72	98.05		
	150	10	15	25	25.31	101.2	100.04	1.731
Trimethoprim	50	12	6	18	17.89	99.88		
	100	12	12	24	23.74	98.15		
	150	12	18	30	29.54	98.46	98.83	0.922
Sulfamethoxazole	50	4	2	6	5.97	99.05		
	100	4	4	8	7.89	98.62		
	150	4	6	10	10.19	101.95	99.87	1.811

*n=3 concentration/3 replicates, S.D.= Standard deviation

Table 2: Results of validation parameters for simultaneous determination of pyridoxine HCl, isoniazid, trimethoprim and sulfamethoxazole

Parameters	*Double divisor ratio spectra derivative spectroscopic method*			
	Pyridoxine HCl	*Isoniazid*	*Trimethoprim*	*Sulfamethoxazole*
Analytical wavelength (nm)	269	287	262	291
Range (µg/mL)	8–40	10–30	12–36	4–12
Linearity	0.9994	0.9991	0.9998	0.9991
Intercept	0.0017	0.0021	0.0013	-0.0031
Slope	0.0029	-0.0032	0.00014	-0.0081
Accuracy	99.92 ± 0.978	100.04 ± 1.731	99.83 ± 0.922	98.87 ± 1.811
Intraday precision* (%RSD)	0.890	0.916	1.63	1.35
Interday precision* (%RSD)	1.16	1.07	1.53	1.19
Reproducibility* (%RSD)	0.823	1.14	1.38	1.12
LoD (µg/mL)	1.93	2.23	3.06	1.3
LoQ (µg/mL)	5.8	6.77	9.21	3.82
Robustness With different wavelength (%RSD)	1.523	1.493	1.493	1.306
Repeatability** (%RSD)	1.81	0.884	0.529	1.392

*n= Mean value of three determinations
**n=Mean value of six determinations

CONCLUSION

An effective, specific, and precise analytical technique using the double divisor ratio spectra derivative concept was successfully developed and validated. This method enabled the simultaneous determination of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole without the necessity of prior individual drug separation. This method was proficiently utilized for the conventional assessment of pyridoxine HCl, isoniazid, trimethoprim, and sulfamethoxazole within blended mixtures, yielding outcomes that aligned with established benchmarks. Hence, the outcomes of this study hold significant advantages for both researchers and industry professionals alike.

ACKNOWLEDGMENTS

The authors express their gratitude to Balaji Drugs Dealers (API and Pharmaceutical Polymer), located in Surat, Gujarat, for supplying the pure samples of isoniazid, trimethoprim, and sulfamethoxazole. Additionally, the authors extend their appreciation to Benz Chem Enterprises, based in Vadodara, Gujarat, for providing a pure sample of Pyridoxine HCl.

REFERENCES

1. Menzies D, Adjobimey M, Ruslami R, Trajman A, Sow O, Kim H, Obeng Baah J, Marks GB, Long R, Hoeppner V, Elwood K. Four months of rifampin or nine months of isoniazid for latent tuberculosis in adults. New England Journal of Medicine. 2018; 379(5):440-53. DOI: 10.1056/nejmoa1714283
2. Blomberg B, Fourie B. Fixed-dose combination drugs for tuberculosis: application in standardised treatment regimens. Drugs. 2003; 63:535-53. DOI: 10.2165/00003495-200363060-00002
3. Lobue P, Menzies D. Treatment of latent tuberculosis infection: an update. Respirology. 2010; 15(4):603-22. DOI: 10.1111/j.1440-1843.2010.01751.x
4. WHOPAR Part 6. Isoniazid, Pyridoxine hydrochloride, Sulfamethoxazole, Trimethoprim – A fix dose combination. World Health Organization Report. 2017: 1. https://extranet.who.int/pqweb/sites/default/files/HA639part6v1.pdf
5. Nayif AA, Al-Da,amy MA, Kadhim SH. Spectrophotometric Determination of Cu(II) Using (DMIPNI) as a New Reagent Derived from 4,5-Diphenyl Imidazole. International Journal of Drug Delivery Technology. 2022;12(2):522-527. DOI: 10.25258/ijddt.12.2.10
6. Ahmed AM, Anwar SM, Hattab AH. Spectrophotometric determination of phenylephrine hydrochloride in pharmaceutical preparations by oxidative coupling reaction. Int. J. Drug Delivery Technol. 2020; 10:323-7. DOI: 10.25258/ijddt.12.2.10
7. Patel V, Patel M, Velhal N, Paramar K, Patel J. Analytical Method Development and Validation for the Spectrophotometric Estimation of Hipuuric Acid Prodrug(Methenamine Hippurate). International Journal of Pharmaceutical Quality Assurance. 2023;14(1):76-80. DOI: 10.25258/ijpqa.14.1.13
8. Saddam NS, Kadhim KH. Spectrophotometric determination of drug clonazepam in pure form and pharmaceutical tablets by oxidative coupling reaction with chlorpromazine hydrochloride. Int. J. Pharm. Qual. Assu. 2019;10(02):342-8. doi: 10.25258/ijpqa.10.2.22
9. Dave HN, Mashru RC, Thakkar AR. Simultaneous determination of salbutamol sulphate, bromhexine hydrochloride and etofylline in pharmaceutical formulations with the use of four rapid derivative spectrophotometric methods. Analytica Chimica Acta. 2007 Jul 30;597(1):113-20. DOI: 10.1016/j.aca.2007.06.035
10. Prasanthi B, Ratna JV, Phani RSC. Development and validation of RP-HPLC method for simultaneous estimation of rifampicin, isoniazid and pyrazinamide in human plasma. J Anal Chem. 2015; 70(8):1015–22. DOI:10.1134/S1061934815080146
11. Suárez-González J, Cáceres-Pérez AR, Oliva A, Santoveña-

Estévez A, Fariña JB. Development and Optimization of a New UPLC-UV/MS Method through DoE and MLR for Detecting Substandard Drug Products to Treat Tuberculosis. Molecules. 2022; 27(20):7141. DOI: 10.3390/molecules27207141

12. Dhal SK, Sharma R. Development and validation of RP-HPLC method for simultaneous determination of pyridoxine hydrochloride, isoniazid, pyrazinamide and rifampicin in pharmaceutical formulation. Chemia Analityczna. 2009; 54(6):1487-1500. http://beta.chem.uw.edu.pl/chemanal/PDFs/2009/CHAN2009V54P01487.pdf.