Reversed-Phase High Performance Liquid Chromatographic Analysis of Three First Line Anti-Tuberculosis Drugs

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Active pharmaceutical ingredients such as isoniazid, pyrazinamide and rifampicin are among the most important first-line anti-tuberculosis drugs. A simple, rapid and sensitive reversed phase-high performance liquid chromatographic assay method for the simultaneous determination of isoniazid, pyrazinamide and rifampicin has been developed. Separation of the interest compounds was achieved in a 10 min chromatographic run in gradient elution mode on a Zorbax SB-C18 stainless steel column (150 × 4 mm, 5 µm) using a guard column containing the same stationary phase. The gradient elution was carried out with a mobile phase of 10% CH_3CN aqueous solution for channel A and 50% CH_3CN in pH = 6.8 phosphate buffer (20 mM), to which 1.5 mL triethylamine were added for channel B. Quantification of the analyzed substances was carried out spectrophotometrically at 269 nm. Detection limits of 0.48 mg/L for isoniazid, 0.52 mg/L for pyrazinamide and 0.48 mg/L for rifampicin were established for the developed assay method. The present work showed that the proposed analysis method was advantageous for simple and rapid analysis of the active pharmaceutical ingredients in pharmaceuticals and biological fluids.

Keywords: isoniazid, pyrazinamide, rifampicin, first-line anti-tuberculosis drugs, HPLC

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Pulmonary tuberculosis, the chronic infectious disease caused by Mycobacterium tuberculosis, represents a global health emergency causing an increasing number of deaths in developing countries [1, 2]. A resurgence of tuberculosis has been noticed in central and Eastern Europe in recent years and the increase in poverty, poor living conditions, malnutrition and lack of medication are among the main causes [3]. Currently, more than one third of the world's population is infected with Mycobacterium tuberculosis, with approximately 8 million new cases and 2 million deaths reported each year [4]. Presently, control of tuberculosis is still a challenge and it is estimated that between 2002 and 2020 nearly 1 billion people will have been affected by the disease if proper control measure will not be instated [5].

Isoniazid (I), pyrazinamide (P), rifampicin (R) and ethambutol (EB) are active pharmaceutical ingredients (APIs) used as first-line anti-tuberculosis drugs. The use of fixed-dose combination tablets of the four active APIs for tuberculosis treatment is preferred [6].

Various techniques have already been reported for the analysis of anti-tuberculosis APIs in various pharmaceutical formulations and biological samples [7,-9]. Among them, reversed phase-high performance liquid chromatography (RP-HPLC) is a sensitive enough and suitable method to quantify those pharmaceutical substances included in the study, as well as many others [10-23].

The present work aimed to develop a simple, rapid and sensitive reversed phase-high performance liquid chromatographic assay method for simultaneous quantification of isoniazid, pyrazinamide and rifampicin in various drug products.

Experimental part

Pure active isoniazid, pyrazinamide and rifampicin were purchased from the Control Department of Pharmaceutical and Biological Products, Antibiotics Company, Iasi, Romania. APIs tablets were purchased from the same company. Acetonitrile (CH_3CN) of HPLC reagent grade was obtained from Sigma Aldrich, and ultrapure water (18.2 MΩ×cm) was obtained with UltraPure Option Q Lab equipment. Chromatographic analysis has been performed with an Agilent 1100 Series HPLC system with UV-Vis detection by performing manual injections with a 20 µL sample loop. Separation of the interest compounds was achieved under gradient elution mode on a Zorbax SB-C18 (150×4 mm, 5 µm) stainless steel column with a guard column containing the same stationary phase. The gradient elution was carried out with a mobile phase of 10% aqueous solution CH_3CN for channel A and 50% CH_3CN in *p*H = 6.8 phosphate buffer (20 mM), (plus 1.5 mL triethylamine) for channel B. The analytes can be analyzed in a chromatographic run of 10 min at a flow rate of 1 mL/min. The gradient profile (A:B) was 100:0 (v/v) for 4 min, then 0:100 for 0.5 min and then it was kept constant at 0:100 (v/v) for 5.5 min.

Results and discussions

In order to determine the optimum wavelength for detection, absorption spectra of all analytes were registered in the 200-600 nm range using a SPECORD 210 UV-Vis spectrophotometer. As figure 1 shows, detection of isoniazid, pyrazinamide and rifampicin was best done at 269 nm.

Stock standard solutions were prepared by dissolving accurately weighed reference isoniazid (99.65%), pyrazinamide (99.48%) and rifampicin (99.86%) in mobile phase B in order to achieve 1000 mg/L concentration levels. For the determination of the specific retention time (tR) single component standards were injected into the HPLC system under the chromatographic conditions described previously. Mixed standards were prepared by appropriate dilution and by mixing mobile phase B with each stock

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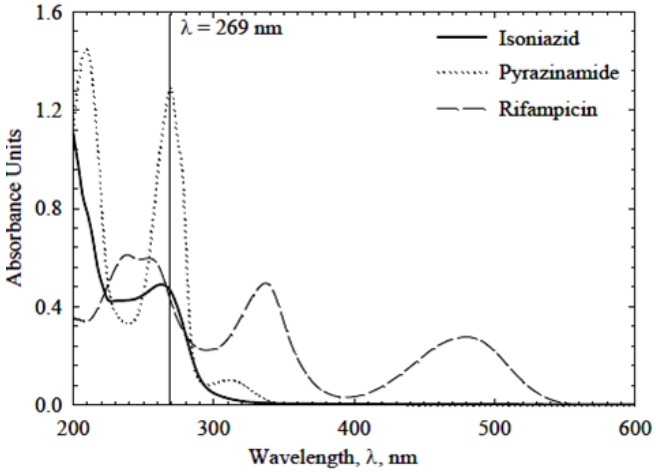

Fig. 1. UV-Vis absorption spectra of isoniazid, pyrazinamide and rifampicin

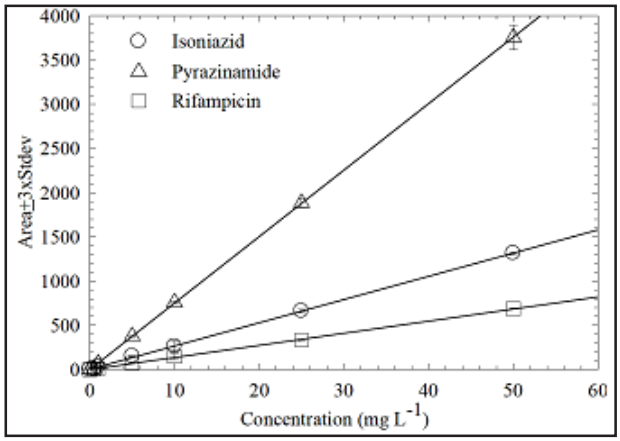

Fig. 3. Calibration curves for isoniazid, pyrazinamide and rifampicin

solution in a 10 mL volumetric flask to achieve selected final concentrations in the calibration range. Figure 2 shows a separation chromatogram of all three APIs within 10 minutes, including the detection at 269 nm. The elution order was $tR_{isoniazid} < tR_{pyrazinamide} < tR_{rifampicin}$ in agreement with the polarity of the interest APIs expressed as log P: -0.64 for isoniazid [20], -1.884 for pyrazinamide [22] and 3.719 for rifampicin [23].

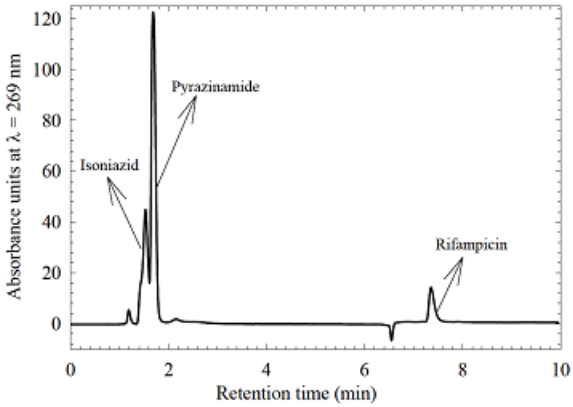

Fig. 2. Chromatogram of isoniazid, pyrazinamide and rifampicin

The calibration curves for mixtures containing standards of isoniazid, pyrazinamide and rifampicin were investigated in the 0.5–50 mg/L concentration range. The calibration curves of standard isoniazid, pyrazinamide and rifampicin were generated by plotting each analyte peak area against concentration. Linear regression analysis has been applied to plots of the interest APIs peak area versus concentration. Calibration curves for isoniazid, pyrazinamide, and rifampicin are presented in figure 3. Very good correlation coefficients were obtained and the linearity data are reported in table 1.

The limit of detection (LoD) has been estimated based on the ratio between three times the standard deviation, estimated from 10 successive injections of the lowest concentration standard, and the sensitivity of the method for each one of the APIs.

The quantification of the interest APIs in pharmaceutical samples was performed by means of external standard procedure. Three batches of isoniazid, pyrazinamide and rifampicin tablets were analyzed using the described method. Five tablets for each of the APIs were weighed to determine the average tablet weight. After homogeneous grounding to a fine powder, a certain amount of each APIs was accurately weighted and quantitatively transferred into a 100 mL volumetric flask by adding mobile phase B.

The samples were ultrasonicated to achieve better dissolution and then filtered through a 0.45 µm Millipore filter to obtain clear solutions before injecting. For each sample, three replicate injections were made to evaluate the reproducibility of the preparation of the sample, of injecting and that of tablet content. Neither of the investigated tablets contained compound that interfered with the determination of either of the APIs. The averaged concentration of APIs in each sample and the associated RSDs showed good reproducibility of the method and of the injection. The amounts of each API have been compared to the labeled value (table 2).

The admissibility criteria from Pharmacopoeia specifies that there should not be less than 99.0% and no more than 101% of isoniazid, 99.0% and 100.5% of pyrazinamide, and 97.0% and 102.0% of rifampicin in the drug product. The data in table 2 showed that the analyzed tablets met the criteria [24].

Table 1

CALIBRATION RESULTS AND LOD VALUES FOR ISONIAZID, PYRAZINAMIDE AND RIFAMPICIN

APIs	Formula	M (g/mol)	Points	R^2	Slope	Offset	LoD (mg/L)
Isoniazid	$C_6H_7N_3O$	137	6	0.9997	26.239	4.098	0.472
Pyrazinamide	$C_5H_5N_3O$	123	6	0.9997	75.424	-8.395	0.542
Rifampicin	$C_{43}H_{58}N_4O_{12}$	823	6	0.9995	13.687	-0.377	0.456

Table 2
QUANTIFIED AMOUNT OF API PER TABLET/CAPSULE

Batch N°	API	Labelled amount (mg)	Quantified amount (mg)	STDEV×2	% API
1	Isoniazid	300	303.2	6.66	101.07
	Pyrazinamide	500	498.18	9.64	99.64
	Rifampicin	150	149.43	1.15	99.62
2	Isoniazid	300	299.96	5.78	99.99
	Pyrazinamide	500	501.10	4.90	100.22
	Rifampicin	150	150.64	5.49	100.43
3	Isoniazid	100	100.03	6.80	100.03
	Pyrazinamide	500	501.34	9.46	100.27
	Rifampicin	150	150.55	9.76	100.37

Conclusions

The performed work shows that a new and accurate HPLC method can be used to assay isoniazid, pyrazinamide and rifampicin used as active pharmaceutical ingredients in drug tablets used for the treatment of tuberculosis. The method saves time (10 minutes chromatographic run for the analysis of all three APIs) as well as organic solvents. The proposed method showed low detection limits (isoniazid 0.472 mg/L, pyrazinamide 0.542 mg/L, rifampicin 0.456 mg/L) and very good linearity. The present method is simple and fast. Moreover, the analytical equipment used is an integral part of the infrastructure presently existent in almost any analytical laboratory. The proposed method can be used for the simultaneous analysis of isoniazid, pyrazinamide and rifampicin in pharmaceutical formulations used for the treatment of tuberculosis.

Acknowledgements: The authors gratefully acknowledge the POSCCE-O 2.2.1, SMIS-CSNR 13984-901, Project No. 257/28.09.2010, CERNESIM, for the infrastructure used.

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Manuscript received: 17.08.2018