ORIGINAL ARTICLE

SIMULTANEOUS DETERMINATION OF ISONIAZID AND RIFAMPICIN BY UV SPECTROPHOTOMETRY

MARIANA TILINCA[1], GABRIEL HANCU[2]*, ELEONORA MIRCIA[3], DIANA IRIMINESCU[2], AURA RUSU[2], ROBERT ALEXANDRU VLAD[2], ENIKŐ BARABÁS[4]

[1]*Cell and Molecular Biology, Faculty of Medicine, University of Medicine and Pharmacy Târgu Mureş, Romania*
[2]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy Târgu Mureş, Romania*
[3]*Department of Organic Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy Târgu Mureş, Romania*
[4]*Department of Botany and Cell Biology, Faculty of Pharmacy, University of Medicine and Pharmacy Târgu Mureş, Romania*

**corresponding author: gabriel.hancu@umftgm.ro*

Manuscript received: September 2016

Abstract

One of the most effective antituberculosis treatments is the combination between isoniazid and rifampicin. Two alternative UV spectrophotometric methods were developed for the simultaneous determination of isoniazid and rifampicin in mixture, by employing simultaneous equation method and first-derivative spectrophotometry respectively. The determinations were performed in methanol. In the simultaneous equation method, the absorbtion maxima of the two drugs, 263 nm and 338 nm, were selected for estimation of isoniazid and rifampicin respectively. The second method was based on a first-derivative spectrophotometric method involving the determination of the analytes at their respective zero crossing point, the determinations were made at 263 nm for rifampicin and 290 nm for isoniazid respectively. Both substances obeyed Lambert-Beer's law in a concentration range between 5 - 50 µg/mL. The analytical performances of the proposed methods were verified regarding their accuracy, linearity, precision, selectivity and sensitivity according to ICH guidelines. The proposed methods can be successfully applied in routine laboratory analysis for the determination of isoniazid and rifampicin in combined dosage forms.

Rezumat

Unul dintre cele mai eficiente tratamente antituberculoase este reprezentat de combinația dintre izoniazidă și rifampicină. Au fost dezvoltate două metode alternative de spectrofotometrie în UV pentru determinarea simultană a izoniazidei și rifampicinei în amestec, aplicând metoda ecuațiilor simultane și spectrofotometria derivată de ordinul I. Determinările s-au efectuat utilizând ca solvent metanolul. În metoda ecuațiilor simultane, au fost selecte maximele de absorbție ale celor doi analiți, 263 nm și 338 nm, pentru determinarea izoniazidei respectiv a rifampicinei. A doua metodă s-a bazat pe o metodă spectrofotometrică derivată de ordinul I care a implicat determinarea analiților prin tehnica „*zero-crossing*", determinările efectuându-se la 263 nm pentru rifampicină, respectiv 290 nm pentru izoniazidă. Ambele substanțe respectă prevederile legii Lambert-Beer în intervalul de concentrații 5 - 50 µg/mL. Performanțele analitice ale metodei au fost verificate în ceea ce privește acuratețea, linearitatea, precizia, selectivitatea și sensibilitatea conform prevederilor ICH. Metodele dezvoltate pot fi aplicate cu succes în analizele de laborator de rutină pentru determinarea izoniazidei și rifampicinei din amestecuri.

Keywords: isoniazid, rifampicin, UV spectrophotometry, simultaneous equation method, derivative spectrophotometry

Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis*; the bacteria usually attack the lungs, but can also damage other parts of the body. Most people who are exposed to TB never develop symptoms because the bacteria can live in an inactive form in the body; but if the immune system weakens, TB bacteria can become active [1, 2].

INH (isonicotinic acid hydrazide) is an anti-mycobacterial agent which is bactericidal for both extracellular and intracellular organisms. It is the primary drug for the treatment of TB when the disease is caused by isoniazid-sensitive strains of the *Mycobacterium tuberculosis*. INH is a colourless, odourless, white crystalline powder slowly affected by exposure to air and to light; soluble in water, methanol and alcohol, slightly soluble in chloroform, very slightly soluble in ether [3, 4].

RIF (3-[[(4-methyl-1-piperazinyl)-imino]-methyl)]-rifamycin) is a semisynthetic derivative of rifamycin antibiotics which are produced by the fermentation of the strain *Streptomyces mediterranei*. The primary indications for RIF are for treatment of TB (pulmonary and extra-pulmonary lesions) and for leprosy. It is also useful for elimination of *Neisseria meningococci* in carriers and for Gram

positive (*Staphylococcus aureus* and *S. epidermidis*, *Streptococcus pyogenes*, *S. viridans* and *S. pneumoniae*) and Gram negative bacteria (*Haemophilus influenzae* type B). RIF is a red odourless powder; very slightly soluble in water, acetone, alcohol, ether, soluble in methanol and ethyl acetate, freely soluble in chloroform [3, 4].

Isoniazid (INH) and Rifampicin (RIF) are probably the most efficient antitubercular agents available in modern therapy; they are usually administered in combination, because of the high drug resistance shown by *Mycobacterium tuberculosis*, being a component of all combined antituberculosis chemotherapy regimens recommended by WHO [5].

The combination of these drugs has high therapeutic advantages as it increases the treatment adherence and reduces the risk of resistance or relapses, treatment costs and errors in drug administration; however, the combination of drugs brings new challenges to the pharmaceutical industry with respect to the development of new analytical methods for their simultaneous determination.

Literature survey revealed high performance liquid chromatography (HPLC) [6, 7, 8], capillary electrophoresis (CE) [9, 10], voltammetric [11], spectrophotometric analysis combined with multivariate regression [12, 13], derivative spectrophotometric [14] and visible spectrophotometric [14, 15] methods for the simultaneous determination of INH and RIF.

The spectrophotometric methods for multicomponent sample analysis are based on the properties that the absorbance of a solution is the sum of absorbances of individual components and the measured absorbance is the difference between total absorbance of the solution and that of the blank solution. Various spectrophotometric methods can be used for estimation of drugs in combined dosage form including here simultaneous equation method and derivative spectrophotometry [16, 17].

The aim of present work was to develop new simple, sensitive and rapid spectrophotometric methods and their validation, for the simultaneous determination of INH and RIF in combinations.

Materials and Methods

Chemicals and Reagents

Pharmaceutical grade Isoniazid (Merck, Germany) and Rifampicin (Antibiotice Iaşi, Romania) were used in the experiment. All reagents were of analytical grade quality: clorhidric acid, sodium hydroxide (Chimopar Bucharest, Romania), methanol (Merck, Germany). Ultrapure water was produced using a Milli-Q system (Millipore, USA). Commercial pharmaceutical preparation Rifinah® (Sanofi-Aventis, France) containing 150 mg INH and 300 mg RIF was purchased from a local pharmacy.

Apparatus

A Specord 210 UV-VIS (Analytik Jena, Germany) spectrophotometer using matched 1 cm quartz cells and WinAspect 7.01 (Analytik Jena, Germany) software were used in the determinations.

Preparation of standard solutions

100 mg of INH and RIF were dissolved separately in different solvents (water, methanol, 0.1N HCl, 0.1 N NaOH), the solution were sonicated for 5 minutes, diluted to a concentration of 25 µg/mL and scanned separately between 200 - 400 nm against a blank, in order to determine the maximum absorbtion wavelength of both drugs.

Preparation of samples from pharmaceutical forms

Twenty tablets of Rifinah® from the same batch were weighed accurately, average weight was calculated, the tablets were finely powdered in a mortar into a homogenous powder; an amount of powder equivalent to the weight of one tablet was dissolved in 100 mL methanol by sonication for 5 minutes with intermittent shaking. The solution was filtered and the aliquot portion of filtrate was further diluted to get a final concentration of 12.5 µg/mL INH and 25 µg/mL RIF. The content of INH and RIF in tablet dosage form was calculated using two framed simultaneous equations and derivative method.

Results and Discussion

Preliminary analysis

Spectra of INH and RIF were recorded in different solvents: water, methanol, 0.1N HCl, 0.1N NaOH. Very similar absorption maxima were obtained in the four solvents (Table I); methanol was selected as solvent for developing the spectrophotometric methods; the selection has been made after assessing the solubility and absorption of both drugs in the studied solvents.

Table I

INH and RIF UV absorbtion maxima (λ) in different solvents

Analytes	UV absorbtion maxima λ (nm)			
	Water	Methanol	0.1 N HCl	0.1 N NaOH
INH	264 nm	263 nm	268 nm	266 nm
RIF	242 nm	242 nm	245 nm	245 nm
	258 nm	259 nm	256 nm	256 nm
	335 nm	338 nm	342 nm	333 nm

RIF can be determined both by UV and visible spectrophotometry but INH can be determined directly only in UV, but two of the absorption maxima of INH in UV (242, 259 nm) are very close to one of RIF (263 nm). The absorption spectra of INH and RIF in methanol are presented in Figure 1.

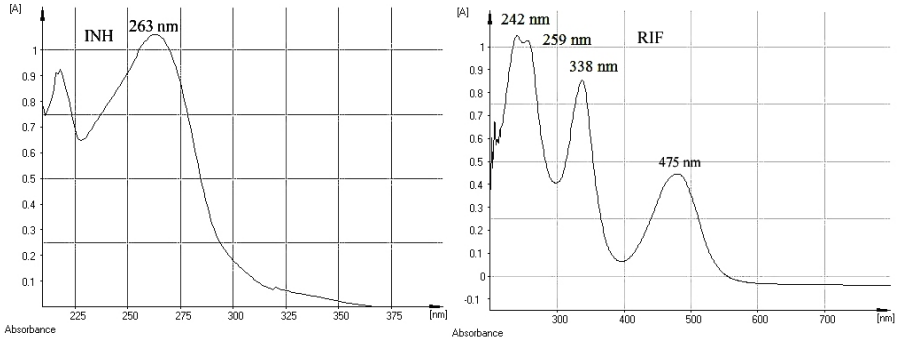

Figure 1.
Absorbtion spectra of INH and RIF respectively in methanol (25 µg/mL)

Simultaneous equation method (Method I)

The overlaid spectra of INH and RIF are presented in Figure 2. From the overlay of the two spectra, two wavelengths 263 nm (λ_{max} for INH) and 338 nm (λ_{max} for RIF) were selected for the estimation of the two analytes by simultaneous equation method.

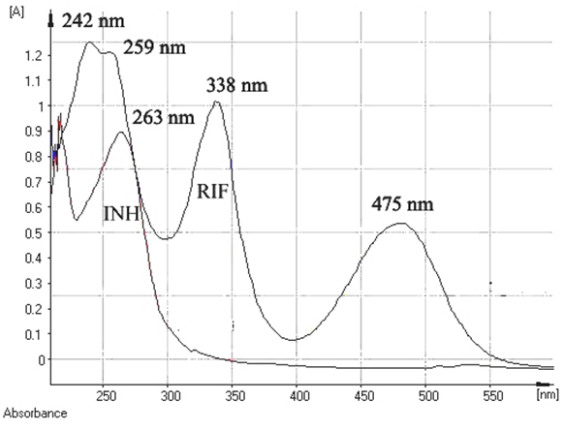

Figure 2.
Overlaid spectra of INH and RIF in methanol (25 µg/mL)

A mixture of INH:RIF 1:1 was measured and it was observed that absorbtivities at 263 nm of the two substances are additive, as the absorbance of the mixture solution is the sum of absorbances of the individual components (Figure 3).

Both substances showed linearity in a concentration range between 5 - 50 µg/mL at their respective wavelength maxima (263 nm for INH and 338 nm for RIF). For simultaneous determination of INH and RIF, a series of solutions (n = 6) of different concentrations from the linearity range were prepared by diluting the stock solutions; absorbance of these series of linearity solutions were recorded at 263 and 338 nm respectively.

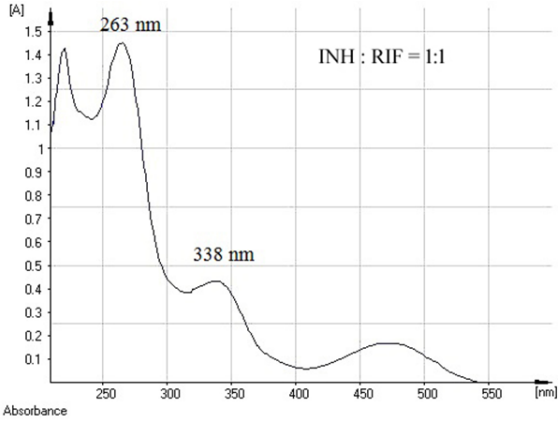

Figure 3.
Spectra of a mixture 1:1 INH and RIF in methanol (25 µg/mL)

The solutions of each drug in triplicate were read against methanol as blank at the selected wavelengths and specific absorbance values were calculated using the below formula:

Specific absorbance, $A^{1\%}_{1\,cm}$ = Absorbance at selected wavelengths/Concentration in g/100 mL.

The $A^{1\%}_{1\,cm}$ was determined at both wavelengths selected for each drug.

Simultaneous equation method was used for the determination using the following formulas:

$$C_x = A_2\, a_{y1} - A_1\, a_{y2} \,/\, a_{x2}\, a_{y1} - a_{x1}\, a_{y2}$$

$$C_y = A_1\, a_{x2} - A2\, a_{x1} \,/\, a_{x2}\, a_{y1} - a_{x1}\, a_{y2}$$

where:

C_x - concentration of INH;

C_y - concentration of RIF;

a_{x1} - $A^{1\%}_{1\,cm}$ of INH at 263 nm;

a_{x2} - $A^{1\%}_{1\,cm}$ of INH at 338 nm;

a_{y1} - $A^{1\%}_{1\,cm}$ of RIF at 263 nm,

a_{y2} - $A^{1\%}_{1\,cm}$ of RIF at 338 nm

A_1 – absorbance of the 1:1 INH:RIF mixture at 263 nm;

A_2 - absorbance of the 1:1 INH:RIF mixture at 338 nm.

First derivative spectrophotometry (Method II)

The zero order spectra were processed to obtain first-derivative spectra using the "zero crossing" technique. The two first derivative spectra were overlaid which shows that INH showed zero crossing at 263 nm, while RIF showed zero crossing at 290 nm (Figure 4). The determinations were made at 263 nm for RIF and 290 nm for INH. The same concentration range was used as for the simultaneous equation method.

Analytical performance

Both methods were validated according to ICH guidelines for validation of analytical procedures regarding precision, linearity, sensitivity and accuracy. Precision, linearity and sensitivity data were determined using a INH:RIF mixture (1:1) obtained from standard solutions.

Precision was studied by measuring intra and inter-day variations in the test method of INH and RIF. Calibration curves were run in triplicates on the same day and for three days at three different concentration levels and % RSD (relative standard deviation) was calculated (Table II).

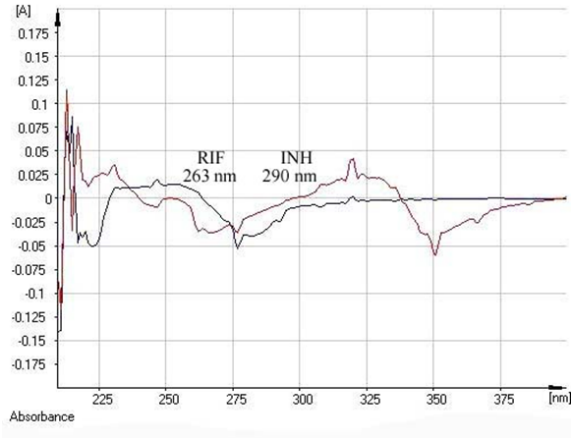

Figure 4.
The overlay of first derivative spectra of INH and RIF in methanol (25 µg/mL)

Table II
Intra- and inter-day precision for the simultaneous determination of INH and RIF

Analyte (µg/mL)	RSD (%)			
	Method I		Method II	
	INH	RIF	INH	RIF
Intra-day precision (n = 6)				
10	0.19	0.21	0.22	0.34
25	0.20	0.23	0.25	0.26
50	0.23	0.27	0.28	0.30
Inter-day precision (n = 18)				
10	0.57	0.55	0.66	0.62
25	0.62	0.64	0.68	0.66
50	0.70	0.76	0.74	0.74

Linear relationship was found in the concentration range of 5 - 50 µg/mL for both INH and RIF and results are shown in Table III. Calibration curve was constructed by plotting absorbance *versus* concentration.

Table III
Linearity data for the simultaneous determination of INH and RIF

Analytes	Method I		Method 2	
	Regression equation	Correlation coefficient	Regression equation	Correlation coefficient
INH	y = 0.041x - 0.121	0.991	y = 0.0418x - 0.065	0.998
RIF	y = 0.041x - 0.082	0.992	y = 0.0416x - 0.044	0.996

The approach based on the standard deviation of the response and the slope of the calibration plots was used to determine detection (LOD) and quantification limits (LOQ). LOD and LOQ values were estimated as [(standard deviation of the response)/(slope of the regression equation)] by multiplying with 3.3 and 10 respectively. The obtained values are given in Table IV.

Table IV
Sensitivity data for the simultaneous determination of INH and RIF

Analytes	Method I		Method 2	
	LOD (µg/mL)	**LOQ** (µg/mL)	**LOD** (µg/mL)	**LOQ** (µg/mL)
INH	2.60	8.58	1.30	4.30
RIF	3.50	11.70	2.30	7.80

To ascertain the accuracy of the developed method and to study the interference of formulation excipients, analytical recovery experiments were carried out by using standard addition method at three different levels (80%, 100% and 120%), by adding the standards to a solution prepared from an appropriate amount of Rifinah® powder; the resulting mixtures were analysed in triplicate and the obtained results were compared with the expected results (Table V).

The % recovery by proposed method was calculated using the below formula.

$$\text{Recovery} = (A - B)/C$$

where: A = total amount of drug estimated (mg), B = amount of drug found on pre-analysed basis (mg) and C = amount of bulk drug added (mg).

Table V

Results of accuracy for the simultaneous determination of INH and RIF

Analytes	Label claim (mg)	Excces drug added (%)	Method I		Method II	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
INH	150	80	99.50	0.67	98.90	0.73
		100	99.15	1.07	99.32	0.88
		120	99.25	0.72	99.24	1.04
RIF	300	80	98.25	0.21	98.80	0.30
		100	98.32	0.65	99.05	0.45
		120	98.12	0.47	99.15	0.38

The optimized method was successfully applied for the simultaneous determination of INH and RIF in the co-formulated original tablet, Rifinah®, containing 150 mg INH and 300 mg RIF. Six samples were assayed. Satisfactory results were obtained for each compound as the found amounts were in good agreement with the label claims (Table VI).

Table VI

Assay results of INH and RIF determination in pharmaceutical formulations (n = 6)

Method	Declared amount (mg)		Found amount (mg)		RSD (%)	
	INH	RIF	INH	RIF	INH	RIF
Method I	150	300	148.84	297.68	0.87	0.92
Method II	150	300	149.14	299.02	0.56	0.64

The method proved to be specific for the determination of INH and RIF from tablets, without excipient interference in drug analysis by both optimized spectroscopic methods. Specificity of the method was also checked by estimating the drug in the presence of excipients such as lactose, starch and magnesium stearate which are mostly available in tablet formulation.

Conclusions

If a sample contains of two absorbing drugs (in our case a combination between INH and RIF) and each of them absorbs at a maximum wavelength different from the other, it may be possible to determine both drugs by the technique of simultaneous equations, provided certain criteria apply. The overlaid spectra of INH and RIF showed that there was interference in the quantitation of the individual drug at their absorption maxima due to absorption of another drug at that particular wavelength, consequently the simultaneous equation method can be used for the simultaneous estimation of the two analytes.

Another option for the simultaneous determination of INH and RIF, which avoids interference due to other drugs in combination or interference due to excipients, is the first derivative spectroscopic method. Derivative spectrophotometry offers an useful means for improving the resolution of mixtures, because it enhances the detection of minor spectral features. It tends to emphasize subtle spectral features by presenting them in a new and visually more accessible way, allowing the resolution of multi-component elements and reducing the effect of spectral background interferences.

The developed methods can be characterized by their simplicity and rapidity requiring only low amounts of analytes and solvent. The described methods give accurate and precise results for the simultaneous determination of INH and RIF from combined pharmaceuticals forms. The methods can be employed for routine analysis in quality control analysis.

References

1. Daniel T., The history of tuberculosis. *Resp Med.*, 2006; 100: 1862-1870.
2. Soroceanu V., Rais C., Ștefănescu E., Brumărel M., Safta V., Adauji S., Priscu V., Taerel AE., Epidemiological and economic aspects of tubercolosis in children. A comparative analysis: Romania *vs.* The Republic of Moldova. *Farmacia*, 2016; 64(1): 152-158.
3. European Pharmacopoeia, 8th ed. Strasbourg: Council of Europe, 2014.

4. Martindale: The Complete Drug Reference, 38th ed. London: Pharmaceutical Press, 2014.
5. Rais C., Taerel A.E., Stefanescu E., Brumărel M., Safta V., Adauji S., Priscu V., Soroceanu V., Epidemiological aspects of tuberculosis in adults in Romania *versus* the Republic of Moldova. *Farmacia*, 2016; 64(4): 643-650.
6. Smith P.J., van Dyk J., Fredericks A., Determination of rifampicin, isoniazid and pyrazinamide by high performance liquid chromatography after their simultaneous extraction from plasma. *Int J. Tuberc. Lung. Dis.*, 1999; 3: S325-328.
7. Fang P.H., Cai H.L., Li H.D., Zhu R.H., Tan Q.Y., Gao W., Xu P., Liu Y.P., Zhang W.Y., Chen Y.C., Zhang F., Simultaneous determination of isoniazid, rifampicin, levofloxacin in mouse tissues and plasma by high performance liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.*, 2010; 878(24): 2286-2291.
8. Zhou Z., Chen L., Liu P., Shen M., Zou F., Simultaneous determination of isoniazid, pyrazinamide, rifampicin and acetylisoniazid in human plasma by high-performance liquid chromatography. *Anal. Sci.*, 2010; 26(11): 1133-1138.
9. Acedo-Valenzuela M.I., Espinosa-Mansilla A., Munoz de la Pena A., Canada-Canada F., Determination of antitubercular drugs by micellar electrokinetic capillary chromatography. *Anal. Bioanal Chem.*, 2002; 374: 432-436.
10. Faria A.F., de Souza M.V.N., Bruns R.E., de Oliveira M.A.L., Simultaneous determination of first-line anti-tuberculosis drugs by capillary zone electrophoresis using direct UV detection. *Talanta*, 2010; 82: 333-339.
11. Hammam E., Beltagi A.M., Ghoneim M.M., Voltammetric assay of rifampicin and isoniazid drugs, separately and combined in bulk, pharmaceutical formulations and human serum at a carbon paste electrode. *Microchem. J.*, 2004; 77: 53-62.
12. Goicoechea H.C., Olivieri A.C., Simultaneous determination of rifampicin, isoniazid and pyrazinamide in tablet preparations by multivariate spectrophotometric calibration. *J. Pharm. Biomed. Anal.*, 1999; 20: 681-686.
13. Li B., He Y., Lv J., Zhang Z., Simultaneous determination of rifampicin and isoniazid by continuous-flow chemiluminescence with artificial neural network calibration. *Anal. Bioanal. Chem.*, 2005; 383: 817-824.
14. Bennetton S.A., Kedor-Hackmann E.R.M., Santoro M.I.R.M., Borges V.M., Visible spectrophotometric and first-derivative UV spectrophotometric determination of rifampicin and isoniazid in pharmaceutical preparations. *Talanta*., 1998; 47: 639-647.
15. Stets S., Tavares T.M., Peralta-Zamora P.G., Pessoa C.A., Nagata N., Simultaneous determination of rifampicin and isoniazid in urine and pharmaceutical formulations by multivariate visible spectrophotometry. *J. Braz. Chem Soc.*, 2013; 7: 1199-1205.
16. Chaudhary J., Jain A., Saini V., Simultaneous estimation of multicomponent formulations by UV-visible spectroscopy: an overview. *Int. Res. J. Pharm.*, 2011; 2: 81-83.
17. Bosch Ojeda C., Sanchez Rojas F., Recent developments in derivative ultraviolet/visible absorbtion spectrophotometry. *Anal. Chim. Acta.*, 2004; 518: 1-24.