CONTRIBUTION TO THE DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY BY THE UV DETECTION METHOD FOR ISONIAZID AND OMEPRAZOLE DETERMINATION

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The objective of this study was to develop and validate a method for isoniazid and omeprazole determination in human serum. Solid phase extraction techniques have been used for sample preparation. The analytical method was applied on a Thermo Fisher Scientific Surveyor Plus chromatographic system, equipped with an autosampler and UV-VIS with diode array detector. Separation was performed on a C8 chromatographic column Octasilil (Purospher RP8) of the 250 mm x 4.6 mm i.d. type (5 µm). A mixture of 10 mM triethylamine (with a pH value of 10.5): acetonitrile (67:33, v/v) has been used as mobile phase. The obtained retention times were the following: 2.323 min for isoniazid; 3.497 min for 2-pyridylamine (used as an internal standard); 4.013 min for omeprazole and 6.837 min, respectively, for lansoprazole (used as internal standard). Detection was in UV at 260 nm for isoniazid and 2-pyridylamine, and at 300 nm, respectively, for omeprazole and lansoprazole. The method is linear, selective, accurate and precise in the 50-5000 ng/mL concentration range.

Keywords: HPLC-UV, validation, isoniazid, omeprazole

INTRODUCTION

Tuberculosis, a contagious disease caused by infection with *Mycobacterium tuberculosis* (Koch bacillus, BK), is usually localized in the lung, however extra-pulmonary tuberculosis can affect other organs, as well.[1] In Romania, tuberculosis is a public health problem with an incidence of new cases expressed as 105.9 per 100000 inhabitants reported in 2008, a statistics which classifies our country in the top of the European countries.[2]

In the Clinical Hospital of Pulmonary Diseases of Iasi, 9647 patients (out of which 7281 represented new cases) were hospitalized and treated for tuberculosis between January 2003-December 2010. The highest incidence of new cases and relapses was registered in 2005 (151.3 cases per 100000 inhabitants). Isoniazid, rifampicine and pyrazinamide are first-line anti-tuberculosis drugs administered in such cases. It was observed that numerous patients diagnosed with tuberculosis developed a gastrointestinal disease after the administration of anti-tuberculosis drugs. In this case, to avoid such secondary effects, medication for pulmonary tuberculosis based on isoniazid was associated with omeprazole.

The literature of the field makes mention of several analysis methods for isoniazid and omeprazole by HPLC, but only a few studies discuss their simultaneous determination. On the other hand, the proposed method has validation parameters superior or comparable to those obtained by other methods.[3-7]

The scope of the present paper was to develop and validate a method for isoniazid and

omeprazole determination in biological fluids by high performance liquid chromatography with UV detection. Its validation was performed in accordance with ICH guidelines (International Council of Harmonization), and some methodologies recommended in literature.[8-16]

EXPERIMENTAL

Standards and reagents

Isoniazid (reference substance) and 2-pyridylamine (internal standard-IS) were purchased from Sigma – Aldrich (Germany), omeprazole (reference substance) and lansoprazole (IS) were purchased from Molekula BioChimica (Germany). Triethylamine (R), formic acid (R), 25% ammonia aqueous solution were of high purity, gradient-grade acetonitrile was provided by Merck (Germany) and gradient-grade methanol by Sigma – Aldrich (Germany). All analyses were performed with high-purity water produced by a Millipore system. Fisher Scientific Hyper SEP Retain PEP were purchased from Thermo Fisher Scientific (SUA).

Sample preparation

All samples were thawed and homogenized before extraction. A volume of 1 mL blood serum was spiked with 0.05 mL internal standard (10000 ng/mL 2-pyridylamine or 5000 ng/mL lansoprazole). The samples were centrifuged for 10 min at 5000 rpm. Prior to the application of the samples on the SPE cartridge, the Fisher Scientific Hyper SEP Retain PEP were washed with 2 mL methanol and 2 mL deionized water. After sample loading at a pressure of 5 psi, each cartridge was rinsed with 3 x 1 mL of 5% methanol in deionized water, with a flow rate of 1-2 mL/min. The sorbent bed was dried thoroughly under a 10 inch positive pressure (10 min) and by centrifugation (10 min, 5000 rpm). The SPE cartridge was eluted with 0.75 mL acetonitrile constituted in basic media (10% ammonia) and 0.75 mL acetonitrile constituted in acidic media (10% formic acid). The final eluate was concentrated under a gentle air stream at 40 °C until dryness and resolubilised in 1 mL mobile phase. Being a complex matrix, blood serum can produce serious problems in chromatographic analysis. In this case, SPE is recommended as a simple and rapid technique, which also reduces the effects of the matrix.

Instrumentation and chromatographic conditions

Analyses were carried out using a Thermo-Fischer Scientific Surveyor HPLC Plus System with a pumping system, equipped with autosampler and UV-VIS diode array detector. An Octasilil C8 (Purospher Star-RP 8) 250 mm x 4.6 mm i.d. (5µm) column was used as stationary phase.

The solvents constituting the mobile phase were 10 mM triethylamine (pH = 10.5)/acetonitrile (67/33, v/v). An isocratic elution was applied and the flow rate was maintained at 1.0 mL/min during the whole run of the samples, at a column temperature of 25 °C. The PDA Plus detector was set to scan in the 190-330 nm range, at a 10 Hz frequency. Two wavelengths were employed: 260 nm for isoniazid and 2-pyridylamine; 300 nm for omeprazole and lansoprazole, respectively. The injected volume was of 5 µL.

For HPLC method development and validation, the same software – Chrom Quest – was used for data processing. The software permitted to determine the purity of the chromatographic signals for omeprazole and lansoprazole (internal standard), as well as for isoniazid and 2-pyridylamine (internal standard). The following validation parameters were studied: specificity/selectivity, linearity of the method, precision, accuracy/average recovery, detection and quantification limits (LOD and LOQ, respectively).

RESULTS AND DISCUSSION

Specificity and selectivity: under the above-mentioned conditions, the method is specific for the simultaneous determination of isoniazid and omeprazole in serum samples. Each time, the peaks specific to the compounds of interest (isoniazid and omepazole), as well as to the internal standards (2-pyridylamine and lansoprazole), were obtained at a well-defined retention time (Figs. 1 and 2). Peak purity was studied, the results obtained evidencing pure peaks. Moreover, the analysis of a blank solution at 260 nm and 300 nm evidenced no peaks at the retention times corresponding to the studied substances, which means that there were no interferences with blood serum. On the other hand, the absorption spectra showed possible interferences with some substances, such as pyrazinamide and rifampicin. Ethambutol and streptomycin do not interfere.

Quantification and quality assurance: multi-level calibration curves were used for quantification, a good linearity being achieved for the tested intervals, including the whole concentration range found in the samples.

To study method linearity, nine sets of solutions for each of the two reference standards, with a concentration between 50-5000 ng/mL, have been prepared and injected. 10 mg reference standards (isoniazid or omepazole, respectively) were dissolved in 10 mL solvent (water or methanol) and 1 mL was diluted to 100 mL with the same solvent. A volume of 10 mL of this solution was diluted to 20 mL with blood serum, a 10000 ng/mL concentration being thus obtained. No further dilution was performed with blood

serum. For quantification, the external standard method was used. Three successive determinations have been made for each solution, and the peak area was measured (Table 1).

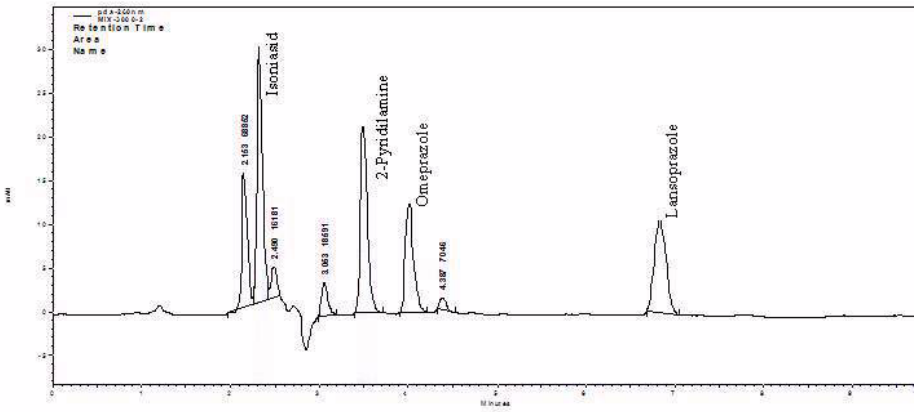

Figure 1: Chromatogram of isoniazid and omepazole and of internal standards (2-pyridylamine and lansoprasole), recorded at λ = 260 nm

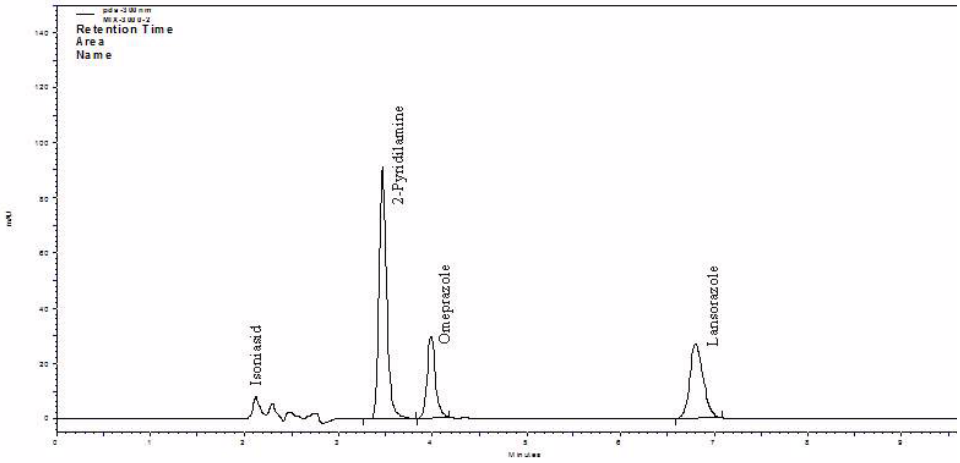

Figure 2: Chromatogram of isoniazid and omepazole and of internal standards (2-pyridylamine and lansoprazole), recorded at λ = 300 nm

Table 1
Peak area for isoniazid and omeprasole linearity study

Concentration	Isoniazid peak area				Omeprazole peak area			
(ng/mL)	1	2	3	Average	1	2	3	Average
50	1978.8	1982.4	1942.4	1967.9	2393	2563	2565	2507
100	4947	4956	4856	4919.7	4927	4856	4999	4927.3
200	10940	10856	10564	10786.7	11025	11625	11325	11325.0
500	25075	25656	25412	25381.0	26736	26856	27025	26872.3
1000	48971	48565	48763	48766.3	56848	56545	56235	56542.7
2000	87245	87555	88025	87608.3	107062	107569	106999	107210.0
3000	126335	127566	126589	126830.0	149652	148958	149632	149414.0
4000	169096	169856	168756	169236.0	206399	206986	207865	207083.3
5000	218891	219658	218564	219037.7	260604	260526	260665	260598.3

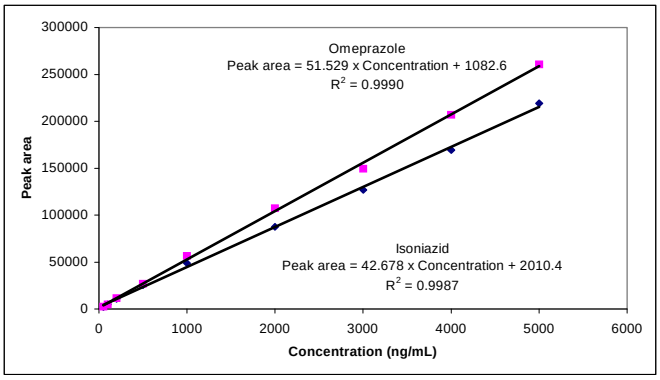

Figure 3: Calibration curves obtained for isoniazid and omeprazole

Table 2
Precision of isoniazid and omeprazole determination

No.	Day	Peak area			
		Isoniazid	Omeprazole	2-pyridilamine	Lansoprazole
1		149652	206399	281797	293190
2		148958	206986	282658	295653
3	1	149632	207865	284256	294566
4		148658	207566	286167	282858
5		147896	205965	286588	285658
6		148236	206253	284566	286545
7		149563	206856	275559	281797
8	2	147856	207456	275986	282658
9		147236	207996	278956	284256
10		147655	207409	286687	286687
Mean		148534.2	207075.1	282733.4	286847
SD		889.6003	698.473	4207.465	287337.7
RSD (%)		0.5989	0.3373	1.4881	1.2625

The average peak area was plotted as a function of concentration (Fig. 3), and the regression equation and regression coefficient were calculated. The resulted mean calibration curves revealed a direct proportionality relationship between the area of the analytical signal and sample concentration over the studied range, thus demonstrating the linearity of the method.

For isoniazid and omeprazole, respectively, the method was linear in the 50-5000 ng/mL range, the regression coefficient being of 0.9987 and 0.9990, respectively. To demonstrate the precision of the method, repeatability has been assessed on a series of 10 determinations for 5000 ng/mL isoniazid and 5000 ng/mL omeprazole, as well as for the two internal standards used (5000 ng/mL lansoprazole and 10000 ng/mL 2-pyridilamine), under the same chromatographic conditions and in two different days. Relative standard deviation for the peak area was evaluated. The maximum values obtained for relative standard deviation were below 2% (Table 2).

Data in Table 2 show a relative standard deviation (RSD) below 2%, which testifies the precision of the method.

To demonstrate the accuracy of the method, three samples containing 300, 500 and 700 ng/mL isoniazid and omeprazole were analysed under the same conditions. Using the equation of calibration, the practical concentrations and the recovery values were calculated (Table 3).[17]

For isoniazid, mean recovery is of 100.0% in the 95.8-104.4% range, while for omeprazole, mean recovery is of 93.7% in the 90.6-97.7% range, which demonstrates the accuracy of the method.

As the concentration of interest is less than 1000 ng/mL, the detection limit (LOD) and the quantification limit (LOQ) were calculated for the 50-1000 ng/mL range. For this concentration

range, the standard error of linear regression is of 765.58 for isoniazid and 679.92 for omeprazole, respectively.

So, LOD and LOQ were calculated as 3.3 × standard error of regression/slope and 10 × standard error of regression/slope. For this concentration range, the following results were obtained: LOD = 51.6 ng/mL, LOQ = 156.5 ng/mL for isoniazid, and LOD = 39.6 ng/mL, LOQ = 119.9 ng/mL, respectively, for omeprazole.

Table 3
Accuracy of isoniazid and omeprazole determination

No.	Isoniazid concentration			Omeprazole concentration		
	Theoretical	Practical	Recovery (%)	Theoretical	Practical	Recovery (%)
1		313.3	104.4		271.7	90.6
2	300	299.2	99.7	300	276.7	92.2
3		287	95.8		278.4	92.8
4		500.5	100.1		467.3	93.5
5	500	494.8	99.0	500	463.8	92.8
6		504.7	100.9		471.8	94.4
7		709.6	101.4		684.0	97.7
8	700	697.2	99.6	700	655.4	93.6
9		693.3	99.0		668.0	95.4
	Mean		100.0	Mean		93.7
	Min		95.8	Min		90.6
	Max		104.4	Max		97.7

CONCLUSION

The UV-HPLC method for isoniazid and omeprazole determination (after optimization of the method parameters) was validated according to ICH guidelines Q2A and ICH Q2B. The results indicate that, over the 50-5000 ng/mL concentration range, the method has good linearity, precision (RSD = 0.5989% for isoniazide and RSD = 0.3373% for omeprazole, respectively) and accuracy (mean recovery = 100.0 over the 95.8-104.4% range for isoniazid, and 93.7%, respectively, for the 90.6-97.7% range for omeprazole, respectively). The concentration–response relationship from the present method indicates a good linearity for isoniazid (r^2 = 0.9987) and omeprazole (r^2 = 0.9990) over the studied concentration range. The limits of detection (LOD) and the limits of quantification (LOQ) were established for isoniazid and omeprazole under the conditions established for the methods. The obtained values – LOD = 51.6 ng/mL and LOQ = 156.5 ng/mL for isoniazid; LOD = 39.6 ng/mL and LOQ = 119.9 ng/mL, respectively, for omeprazole – are superior to those obtained by other authors (a better quantification limit LOQ = 600 ng/mL[3] or 200 ng/mL[4] for isoniazid or 1.52 μg/mL[5] for omeprazole, a better precision (RSD = 1.6-4.2% for isoniazid[6] or RSD = 0.4-8.5% for omeprazole[7]).

A simultaneous extraction procedure was carried out for both reference substances, isoniazid and omeprazole, using SPE techniques with polymeric sorbents, permitting to separate the polar and nonpolar compounds (acids, bases, amphoteric substances). Due to the short time necessary for the detection of the compounds considered, the here applied method can be very well adapted for pharmacokinetic studies.

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