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Validation of a RP-LC method for the simultaneous determination of isoniazid, pyrazinamide and rifampicin in a pharmaceutical formulation

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Abstract

A simple and accurate liquid chromatographic method was developed and validated for estimation of isoniazid (ISN), pyrazinamide (PYR) and rifampicin (RIF) in combined dosage forms. Drugs were chromatographed on a reverse phase C18 column using a mobile phase gradient and monitored at the corresponding maximum of each compounds. Peaks were identified with retention time as compared with standards and confirmed with characteristic spectra using diode-array detector. Solution concentrations were measured on a weight basis to avoid the use of an internal standard. The method does not require any specific sample preparation except the use of a guard column. The method is linear ($r^2 > 0.999$), precise (RSD%: 0.50% for ISN, 0.12% for PYR and 0.98% for RIF), accurate (overall average recovery yields: 98.55% for ISN, 98.51 for PYR and 98.56% for RIF) and selective. Due to its simplicity and accuracy the method is suitable for routine quality control analysis of antitubercolosis combination dosage form.

Keywords: Isoniazid; Pyrazinamide; Rifampicin; Validation; High-performance liquid chromatography

1. Introduction

The tuberculosis treatment is an increasing worldwide problem due to the fact that the effectiveness of modern chemotherapy has been blunted by the high incidence of primary drug resistance, especially in developing countries. A 6-months regimen with rifampicin (RIF) and isoniazid (ISN), supplemented in the initial 2-months intensive phase by pyrazinamide (PYR), is highly effective. This modern short-course regimen can be simplified by the use of a fixed combination of the used drugs [1].

There are several fixed combination preparations containing ISN, PYR and RIF available on the world market and both the International Union Against Tuberculosis and Lung Disease (IUATLD) and the World Health Organisation (WHO) recognise the potential benefits of using

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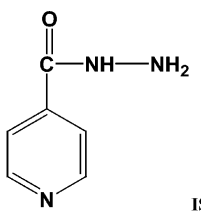
such preparations [2,3]. The structures of ISN, PYR and RIF are reported in Fig. 1.

Many techniques for the determination of ISN (isonicotinic acid hydrazide), PYR (pyrazine carboxylamide) and RIF 3-[(4-methyl-1-piperazinyl) iminomethyl]-rifamycin SV) individually in pharmaceutical formulations and biological samples have been reported [4–11]. The available methods include normal-, reversed-phase high-performance liquid chromatography and spectrophotometry. In the recent past, the contemporary determination of ISN, PYR and RIF from fixed combinations in unit doses by high-performance thin-layer chromatography [12], multivariate spectrophotometric calibration method [13] and colorimetric analysis [14] have been published, however many of these methods suffer from limitations such complex and tedious procedures together with not fully validated studies. The only reported HPLC reverse phase method for the contemporary determination of ISN, PYR and RIF requires the use of an internal standard and tetrabutyl ammonium hydroxide as ion-pairing agent in the mobile phase which shortens column life [15].



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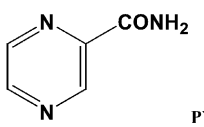

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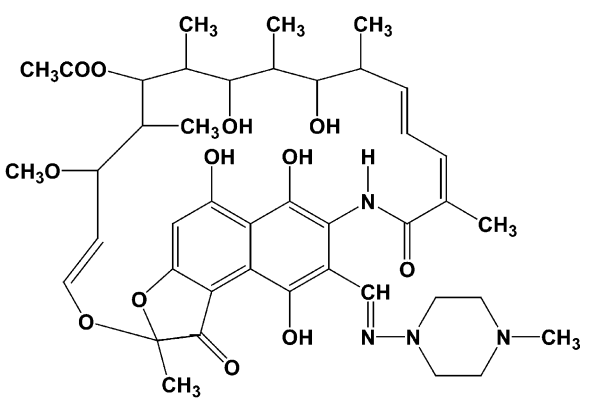

RIFAMPICIN

Fig. 1. Structure of isoniazid, pyrazinamide and rifampicin.

There is therefore a need of a simple HPLC method for systematic control procedures aimed at ensuring the quality of fixed combination preparations, which are made available for clinical use.

A new HPLC method, for the simultaneous determination of RIF, ISN and PYR was developed and validated according ICH guidelines [16,17]. A RP C18 stationary phase was used to separate these active components and a gradient elution profile consisting of phosphate buffer and acetonitrile was used. Data supporting the linearity, precision, specificity, limit of detection (LOD) and recovery are presented. In comparison with previously reported methods [12–15] a stability-indicating study has also been included.

2. Experimental

2.1. Material and reagents

The working standards employed for RIF, ISN and PYR were from Sigma (St. Louis, MO). Tris (hydroxymethyl) methylamine, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were of analytical grade and purchased from Merck (Darmstadt, Germany). Acetonitrile was from Carlo Erba (Milan, Italy).

Water was deionized by passing through a Direct-Q™ system (Millipore, Bedford, MA).

The accuracy of the method was proven by determination of the drugs in laboratory-prepared dosage placebo formulations containing typical excipients (75 mg cross-linked sodium carboxymethylcellulose, 100 mg cellulose microcrystalline, 60 mg sodium starch glycolate, 20 mg Mg stearate, 60 mg talc and 5 mg colloidal silicon dioxide) and spiked with 75 mg ISN, 400 mg PYR and 150 mg RIF.

2.2. Equipment

Chromatographic experiments were performed with a HPLC system consisting of a Hewlett Packard HP 1100 liquid chromatograph (Palo Alto, CA) with a Rheodyne sample valve (20-μl loop) equipped with a Hewlett Packard HP 1100 diode-array detector (DAD) connected to a HPLC ChemStation, (Revision A.04.01).

2.3. HPLC method

The column was a Lichrospher 100 RP18 (250 × 4 mm I.D.) (5 μm) (Merck, Darmstad, Germany) and the guard column was a Lichrospher 100 RP18 (5 μm).

Gradient elution was carried out with a mobile phase of acetonitrile (A) and 50 mM phosphate buffer pH 3.5 (B). The gradient profile was (A:B) 3:97 v/v for 5 min, then a linear gradient to 50:50 v/v at 30 min and return to 3:97 v/v in 10 min. Chromatography was performed at room temperature using a flow rate of 1 ml/min and a run time of 40 min.

The operating UV wavelengths, set at the corresponding maximum for each compound by using a DAD, were 254 nm for RIF, 261 nm for ISN and 265 nm for PYR.

Under the chromatographic conditions described the retention times (minutes) for ISN, PYR and RIF were, respectively, about 4.4, 8.0 and 31.4.

2.4. Procedure

Solutions were prepared on a weight basis and volumetric flasks were used as suitable containers in order to minimize solvent evaporation. Prior to injecting solutions, the column was equilibrated with the mobile phase flowing during the gradient. Quantitation was accomplished using an external standard method.

2.5. Preparation of the solutions

2.5.1. Reference solutions

One hundred and fifty milligram of ISN were taken in a 100 ml volumetric flask, dissolved and diluted to volume with deionized water to a final concentration of 1.5 mg/ml. Four hundred milligram of PYR were taken in a 50 ml volumetric flask dissolved and diluted to volume with deionized water to a final concentration of 8 mg/ml. Hundred and fifty milligram of RIF were taken in a 50 ml volumetric flask dissolved and diluted to volume with methanol to a final concentration of 3 mg/ml.

Reference solutions were kept at 4 °C until before use. One millilitre of each solutions were kept in a 20 ml volumetric flask and diluted to volume with 50 mM phosphate buffer (pH 3.5)/methanol 50:50 (v/v) to a final concentration of 75 μg/ml ISN, 150 μg/ml RIF and 400 μg/ml PYR (working solution).

2.5.2. Calibration solutions

Quantitative assays were performed by means of the external standard procedure. The prepared stock solutions of ISN, PYR and RIF were suitably diluted with 50 mM phosphate buffer (pH 3.5)/methanol 50:50 (v/v).

2.5.3. Sample solutions

Ten tablets were weighed to determine the average tablet weight and finely powdered. An accurately weighed powder sample corresponding to the half of the average weight equivalent to 37.5 mg ISN, 75 mg of RIF and 200 mg of PYR was placed in a 50 ml volumetric flask and diluted to volume with 50 mM phosphate buffer pH 3.5/methanol 50:50 (v/v). The sample was ultrasonicated for 10 min at 40 °C and then for 30 min at temperature of tap water, filtered and 1 ml of the filtrate diluted to 10 ml with the same solvent in a volumetric flask.

2.6. Validation

Once the chromatographic and the experimental conditions were established, method validation was performed following ICH specification.

Linearity was determined by building three calibration curves. For the construction of each calibration curve six calibration standard solutions were prepared at concentrations ranging from 25 to 200% of the working solution (75 μg/ml ISN,

150 μg/ml RIF and 400 μg/ml PYR). Each standard solution was injected once. Calibration curves of standard RIF, ISN and PYR were generated by plotting the analyte peak area vs the concentration of the drugs. Linearity is confirmed if the RDS% values of the slope and the intercept are less then 3%.

To ensure the validity of the analytical procedure, a system suitability test (SST) was established. Relative standard deviation of the area, tailing factor and retention time were the chromatographic parameters selected for the SST; they were calculated from six dilutions (working solutions) of the reference solutions, each injected once. The parameters values should meet the following criteria: RSD% of the three analytes peak areas $< 3\%$, tailing factors for the three analytes (USP *Tailing Factor*) < 2 [18], retention time standard deviation within $\pm$ seconds of the expected retention time.

The limit of quantitation (LOQ) was defined as the lowest concentration that can be determined with acceptable accuracy and precision, which resulted in a peak area of two times the LOD. The LOD was determined by diluting solutions of known concentration until the response was three times the noise.

The system precision (injection repeatability) was determined by performing five repeated analysis of the same working solution while the intermediate precision was assessed by analysing different standard solutions in three different days (*inter-day*) and different sample preparations of a single lot on the same day and on three different days. Recovery was evaluated by comparing the theoretical and measured concentration of a placebo formulation spiked with 75 mg ISN, 400 mg PYR and 150 mg RIF and typical excipients.

2.7. Stability/specificity

To ensure that the method is stabiliy-indicating and to check for degradation products, stress studies to force degradation were performed on the three reference solutions as follows. The samples were analysed against a freshly prepared working solution. The reference solutions of ISN, PYR and RIF were stored at 4 °C far from light, at room temperature far from light, at room temperature and light and analysed after 24 h, 3 days and 1 week after diluting them to the working concentration. The solution is considered stable if in the described storage conditions variation in the concentration is inferior to 2%.

Specificity studies were also carried out by dissolving the analytes in aqueous 0.1 N HCl, 0.3% H_2O_2, 50 mM Tris buffer pH 8 and analysing the samples after 1 month. Peak purity was evaluated using a diode-array UV detector and peak purity software, which compared spectra at the peak upslope, apex and downslope. The spectra were normalized and superimposed for comparison. If identical spectra are found for each point on the peak, then the peak is considered pure.

3. Result and discussion

The main problem in the development of a method for the contemporary analysis of ISN, PYR and RIF was to find a suitable combination of mobile phases to separate the components. Preliminary isocratic studies on a reverse phase C18 column with different mobile phase combination of acetonitrile and phosphate buffer were considered. The optimum pH of the phosphate buffer was found to be 3.5. The large range of polarity of the drugs made difficult the obtainment of a complete resolution of the analytes in a reasonable time. High percentage of organic solvent was needed for the elution of RIF in reasonable time, therefore, it was decided to develop a linear gradient based on the best isocratic conditions. A steep gradient elution (10 min from 3 to 50% acetonitrile) led to a drifting of the baseline and for that reason the 'first choice' gradient was changed to 25 min from 3 to 50% acetonitrile. The analytical profiles obtained by injection of a standard working solution and of an extracted spiked placebo formulation are reported in Fig. 2a and 2b, respectively.

The filtration process of the method was qualified by comparing three separately filtered sample preparation (powder sample containing 37.5 mg ISN, 75 mg RIF, 200 mg PYR and a mixture of exicipient) against a non filtered stan-

Table 1
System suitability results

Compound	RSD% of the area	USP tailing factor	Retention time (min) ± min
ISN	1.6	1.55	4.380 ± 0.84
PYR	0.9	1.73	8.021 ± 0.77
RIF	2.5	1.73	31.366 ± 0.67

System suitability was checked by injecting the working solution six times.

dard solution of the same concentration. The acceptable criteria for the filtration study were that the mean recovery of the filtered relative to the standard solution should be 100.0 ± 1.5% with a RSD < 2.0. The results obtained met the acceptance criteria (overall average recovery yelds: 99.24 ± 1.23% RSD for ISN, 101.25 ± 0.39% RSD for PYR and 99.74 ± 0.37% RSD for RIF).

The SST is an integrated part of the analytical method and it ascertains the suitability and effectiveness of the operating system. The results of the SST are reported in Table 1. The symmetry factors for the analytes peaks were less than 2.0, the RSD% of the peak areas responses less than 2.5% and the migration times within seconds.

The linear regression analysis obtained plotting the peak areas of the three analytes vs concentration showed excellent correlation coefficients (correlation coefficient greater than 0.999) and the linearity data are reported in Table 2.

The system precision (injection repeatability) is a measure of the method variability that can be expected for a given analyst performing the analysis and was determined by performing five repeated analysis of the same working solution. The RSD% for ISN, PYR and RIF response was found to be 0.50, 0.12 and 0.98%, respectively.

The intermediate precision was assessed by analysing three working solutions in three different days (*inter-day*, Table 3) and different sample preparations of the placebo formulation fortified with the drugs on the same day and on three different days (Tables 4 and 5).

The limits of quantitation (LOQ) were 6.0, 7.5 and 4.0 μg/ml for RIF, ISN and PYR, respectively, and the LOD were estimated to be 3.0, 3.5 and 2.0 μg/ml for RIF, ISN and PYR, respectively. In Fig. 3 is reported a chromatogram of a standard solution corresponding to the LOQ of the method.

Recovery data from the study are reported in Table 6. The mean recovery data obtained were within 2%. Overall average recovery yields were 98.55, 98.51 and 98.56% for ISN, PYR and RIF, respectively. Since the results obtained were within the acceptable ± 3% range, the method was deemed to be accurate.

Forced degradation studies provided informations of the stability-indicating properties and specificity of the method. The reference solution of ISN and PYR were stable for 1 week at room temperature and light under the described conditions while the same conditions caused the peak area of RIF to decrease 10.78% suggesting the preparation of the solution every day.

Table 2
Linear regression analysis

Compound	Slope[a] ± RSD%	Intercept[a] ± RSD%
ISN	30.48 ± 1.92	−183.24 ± 2.49
PYR	63.81 ± 1.14	999.73 ± 1.27
RIF	43.69 ± 1.63	−255.36 ± 2.55

[a] Mean ($n = 3$).

Table 3
Intermediate precision: standards

Compound	Amount (μg/ml)	Found[a] (μg/ml)	RSD%
ISN	75	75.25	2.21
PYR	400	394.50	0.45
RIF	150	151.62	2.22

[a] Mean ($n = 9$).

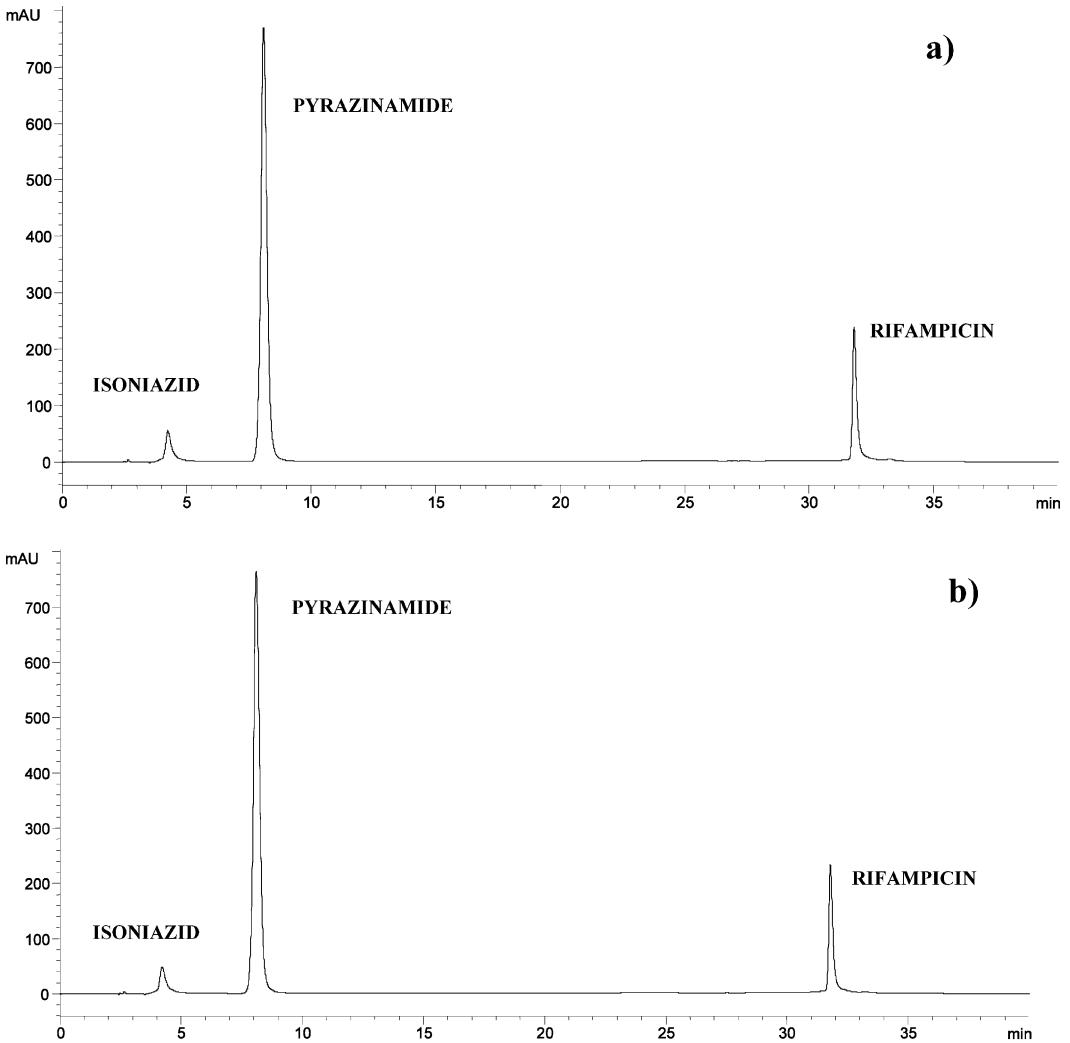

Fig. 2. (a) Chromatogram of a standard solution (75 μg/ml ISN, 150 μg/ml RIF and 400 μg/ml PYR). (b) Analytical profile obtained by injection of an extracted spiked placebo formulation (75 μg/ml ISN, 150 μg/ml RIF and 400 μg/ml PYR).

Table 4
Intermediate precision: assay (*intra-day*)

Compound	% of the label[a]	RSD%
ISN	98.32	0.33
PYR	100.05	0.35
RIF	98.43	0.5

[a] Mean of two extractions (each analysed in duplicate).

Table 5
Intermediate precision: assay (*inter-day*)

Compound	% of the label[a]	RSD%
ISN	97.76	0.50
PYR	100.05	1.69
RIF	99.07	0.61

[a] Mean of two extractions (each analysed in duplicate) in three different days.

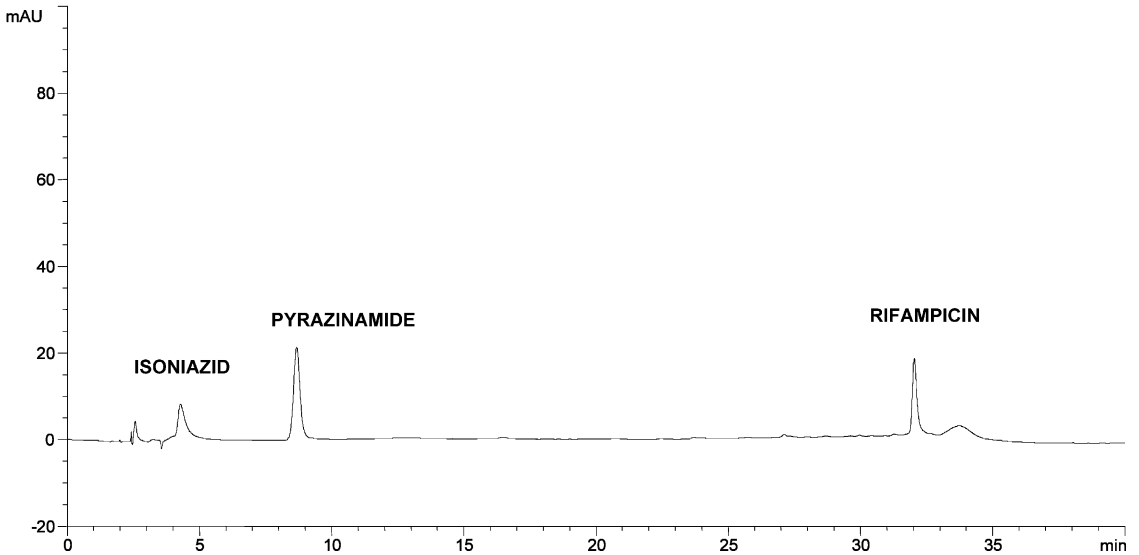

Fig. 3. Chromatogram of a standard solution corresponding to the LOQ (7.5 μg/ml ISN, 6.0 μg/ml RIF and 4.0 μg/ml PYR).

Table 6
Recovery from spiked placebo tablets

Compound	Mean recovery %[a]	±SD	RSD%
ISO	98.55	0.79	0.80
PYR	98.51	0.71	0.72
RIF	98.56	0.94	0.96

[a] Mean of two extractions (each analysed in duplicate).

The analysis of stressed solution stability samples in HCl and H_2O_2 presented degradations peaks resolved from the peaks of the active substances; the peak purity approach revealed that any degradation product coeluting with ISN, PYR and RIF.

The selectivity was demonstrated showing that peaks of analytes were free of interference from degradation products indicating that the proposed method can be used in a stability assay.

4. Conclusion

The developed RP HPLC provides a convenient and efficient method for the separation and determination of ISN, PYR and RIF in combined dosage form and represents a progress in respect to existing procedures for its simplicity and selectivity.

The results of validation showed that the proposed RP-HPLC method is linear, precise, accurate and selective and can be employed for the assay of ISN, PYR and RIF in dosage form and stability studies.

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