Original Research

SIMPLE AND FAST HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF RIFAMPICIN IN THE PLASMA

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ABSTRACT

Aims and Objectives: To develop and validate high-performance liquid chromatographic (HPLC) method for determination of rifampicin (RIF) in the plasma.

Method: To 100 µL aliquot of plasma, 100 µL of acetonitrile containing 4 mg/L of acetanilide as the internal standard (IS) was added. After centrifugation, a 20 µL aliquot of the supernatant was injected into HPLC system. HPLC analysis was accomplished by reversed phase C18 column. The mobile phase was delivered as gradient elution started as 30% acetonitrile and 70% of 20 mM ammonium acetate. The flow rate was set at 1mL/minute.

Results: Calibration curves were linear ($R^2 > 0.997$) in the range of 0.75 to 40 mg/L. Accuracy was good since the percentage of relative error (RE %) was < 7% for all the quality control samples. The relative standard deviation (RSD, %) for both intra- and inter- day precision was less than 8.1%. Mean recovery of RIF and IS from the plasma was 118.8%, and 99.6% respectively. Plasma from 25 patients with tuberculosis was analyzed by this method. The maximum concentration (C_{max}) of rifampicin was in the range of 1.3 - 20.8 mg/L, (mean ± SD = 7.29 ± 4.22 mg/L). Rifampicin concentration was less than 8 mg/L in 80 % of the patients.

Conclusion: a selective, precise and accurate HPLC method for determination of RIF in the plasma was developed and validated. This method is very simple, and fast, which makes it suitable for therapeutic drug monitoring of RIF.

KEY WORDS: HPLC, Rifampicin, Plasma.

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1. INTRODUCTION

Rifampicin is a fundamental drug in the treatment of tuberculosis. It possesses both early bactericidal activity (EBA) and sterilizing activity, which makes it an essential drug to cure the disease, prevent its relapse, and prevent the development of drug resistance strains[1, 2]. Animal studies, and *in vitro* studies demonstrate a relationship between the bactericidal activity of rifampicin and its concentration[3, 4]. In some clinical studies, unresponsiveness of tuberculosis patients to well-supervised regimens was attributed to low concentrations of rifampicin [5-7]. Rifampicin is well absorbed after oral administration. It reaches its maximum concentration (C_{max}) after 2 to 4 hours when taken on an empty stomach. The presence of food in the stomach decreases and delays the absorption of rifampicin[8]. The C_{max} of rifampicin after a dose of 600 mg or 10 mg/kg body weight is expected to be in the range of 8 - 24 mg/L, which is considered by some authors as the normal and target concentration of rifampicin[9]. Some authors suggest increasing the rifampicin dose when its C_{max} is below 4 mg/L[9]. Several HPLC methods for determination of rifampicin alone or with other drugs in biological samples have been published[10-14]. However, some of these methods are liquid chromatography-tandem mass spectrometric methods (LC-MS), in which mass spectrometry techniques are used for compound detection[10, 11]. LC-MS possesses higher sensitivity and selectivity comparing to Ultraviolet (UV) detection; however, this technique is still very expensive and not usually available, especially in developing countries. Some other methods involve solid phase extraction or liquid-liquid extraction [12-14]. Protein precipitation by organic solvent, acids, or metal ions, is a simpler and faster way for plasma clean up[15]. The present work describes a selective, accurate, precise, fast, and a simple method for determination of rifampicin in the plasma using HPLC system with UV detection.

2. MATERIAL AND METHOD

2.1 Reagents and chemicals

Rifampicin, acetanilide, and ammonium acetate were purchased from Sigma-Aldrich (Germany). The purity of all of these chemicals was above 99%. HPLC grade acetonitrile was purchased from J.T. Baker (China). Deionized water was obtained from Direct-Q UV3 (Millipore, France).

2.2 Chromatography

HPLC system consists of 1525 binary pumps (waters), 2489 UV/Visible detector (waters), 7125 Rhyenod manual injector, connected by 20 µL stainless steel tubes to Zorbax C18 column (150 × 4.6 mm., particle size 5 µm) equipped with zorbax C18 guard column. The column was held at room temperature. The mobile phase consists of HPLC grade acetonitrile as solvent A and 20mM ammonium acetate (pH 4.7, adjusted with acetic acid) as solvent B.

The mobile phase was delivered as 30% solvent A. and 70% solvent B. for the first 2 minutes, then percentage of acetonitrile was increased linearly to reach 50% by minute 6. This composition was maintained for a further 1 minute. At minute 7, the percentage of acetonitrile was decreased linearly to reach its original composition by minute 8. This later composition was maintained until the end of the run at minute 10. The flow rate was set at 1mL/min. The volume of injection was 20 µL, and the signals were monitored at 254 nm from the beginning of the run until 5 minutes, after which the wave length was changed to 334nm.

2.3 Preparation of calibration standards and quality control samples

Stock solution of rifampicin was prepared in methanol at concentration of 1g/mL. Stock solution of acetanilide was prepared in acetonitrile at concentration of 1 mg/mL. These stock solutions were kept in amber glass bottles and stored at -20 C°. Calibration standards (CS) of 0.75, 2, 5,10, 20, 30, and 40 mg/L and quality control samples (QCs) of 1.25, 25, and 38 mg/L of rifampicin in plasma were prepared in volumetric flasks by spiking the blank human plasma with an appropriate amount of rifampicin stock solution.

2.4 Sample preparation

To a 100 µL aliquot of the plasma, a100 µL of 4 mg/L of IS in acetonitrile was added. After mixing on a vortex shaker for 20 seconds, the mixture was centrifuged at 3000 rpm for 7 minutes. From the supernatant, a 20 µL was taken and injected directly into HPLC system.

2.5 Method Validation

- Selectivity: Selectivity of the method was assessed by analyzed six different batches of pooled human plasma.
- Calibration curve was constructed by plotting the peak height ratio of rifampicin to the IS (*y-axis*) *versus* nominal concentration of rifampicin (*x-axis*). Calibration equations and the correlation coefficients were generated by the least square linear regression.
- Limit of detection (LOD) and Lower limit of quantification (LLOQ): The limit of detection (LOD) was defined as the lowest concentration with a signal-to-noise (S/N) ratio of 3 or more. The LLOQ was defined as the lowest concentration on the calibration curve with a signal-to-noise ratio of 5 or more, and a precision with relative standard deviation (RSD, %) of less than 20% and accuracy with a relative error (RE, %) of less than 20%.
- Accuracy and precision: Three quality control samples at; low concentration (1.25 mg/L), midrange concentration (25 mg/L), and high concentration (38 mg/L) together with LLOQ (0.75 mg/L) were used to assess the accuracy and precision of the method. Each of these QC samples was injected at least 5 times daily to assess the intra-day accuracy and precision. This process was repeated for three days to assess the inter-day accuracy and precision. The accuracy was expressed as the relative error (RE, %), and the precision was expressed as the relative standard deviation (RSD, %).
- Recovery: Recovery was assessed by comparing the peak height of the 3 QCs, with their corresponding concentration of rifampicin in water. Five replicates of each concentration were carried out. Recovery of the internal standard was assessed by comparing its peak height at concentration of 4 mg/L in plasma samples with its peak height at the same concentration dissolved in pure water.

3. RESULTS

3.1 Chromatographic separation

Under programmatic gradient elution described above, acetanilide eluted first at 3.7 minute. Then by increasing the percentage of acetonitrile in the mobile phase, rifampicin eluted at 6.5 minute. Both peaks were with good shape and symmetry. There was no interfering from endogenous compounds in the plasma, or from other drugs, that were taking concurrently with rifampicin. Chromatograms of blank plasma, plasma spiked with known concentration of rifampicin and IS, and a plasma from tuberculosis patients are presented in figures1-3.

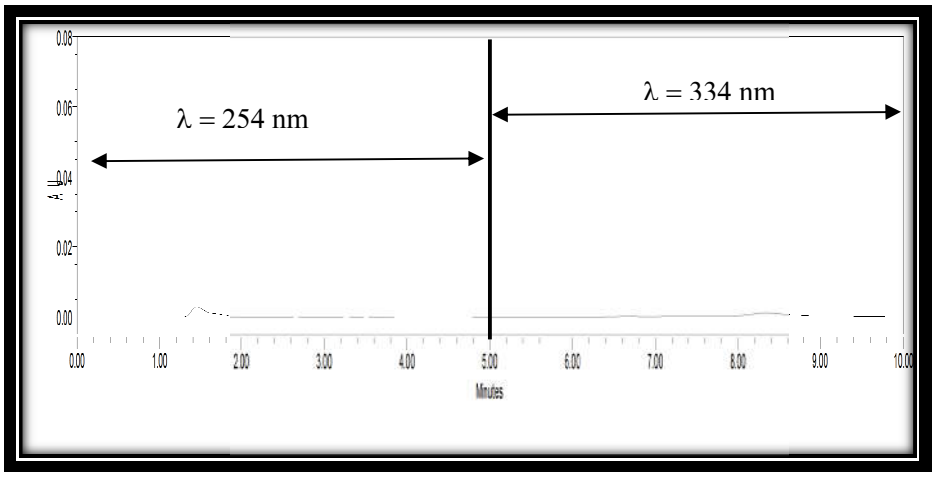

Fig.1 Typical chromatogram of blank human plasma processed without the internal standards
λ = wave length

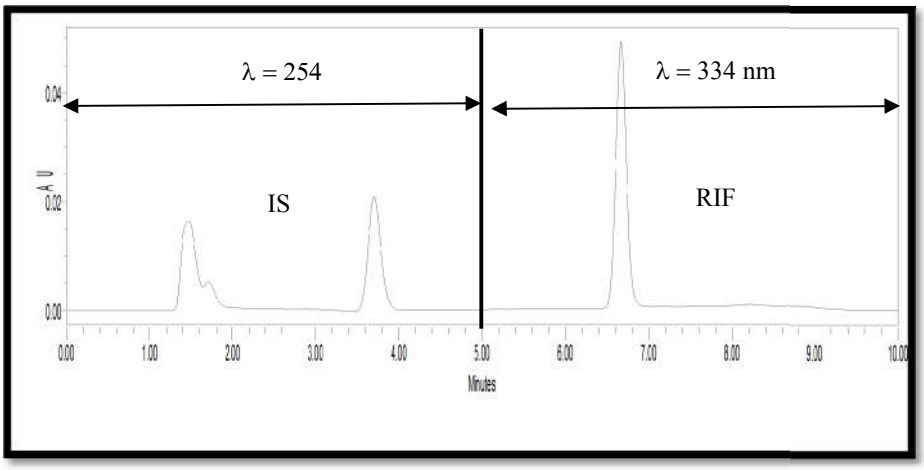

Fig. 2 Typical chromatogram of blank plasma processed with the IS and spiked with 4 mg/L IS, and 20 mg/L RIF

λ = wave length, IS = Internal standard, RIF = Rifampicin

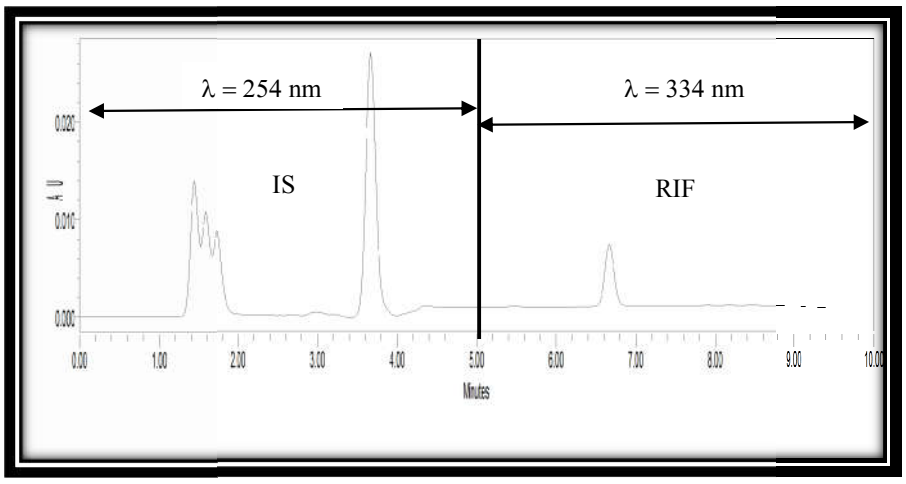

Fig. 3 Typical chromatogram of the plasma of tuberculosis patient processed with the IS

λ = wave length, IS = Internal standard, RIF = Rifampicin

3.2 Method validation

- Selectivity of the method: by analyzing six different batches of human plasma there was no interfering peaks at, or close to, the retention time of rifampicin or the IS. No interfering peaks were seen from the plasma of tuberculosis patients who were taking isoniazid, pyrazinamide, ethambutol, and other drugs in addition to rifampicin.
- The calibration equation of the calibration curves was $y = (0.126 \pm 0.0045)\ x + (0.0016 \pm 0.006)$, and the correlation coefficient was $r^2 = 0.997 \pm 0.002$ (n =3). The relationship between the concentration of rifampicin and its peak height to the IS peak height ratio was linear in the range of 0.75 to 40 mg/L. None of the calibration standards was deviated by 15% or more from their true concentration. Figure 4 shows the

calibration curves generated in three different days.

- Limit of detection (LOD) was 0.5 mg/L. Lower limit of quantification LLOQ was 0.75 mg/L.
- Accuracy and precision: Intra-day accuracy (RE %) was between - 6.54 and 2.87%, and inter-day accuracy (RE %) was between - 1.45 and 5.77%. Intra- and inter-day precision (RSD, %) was in the range of 2.95 to 8.06 % and 4.88 to 7.03% respectively. Details of intra- and inter-day accuracy and precision are shown in table 1.
- Recovery of the method: The mean recovery of rifampicin was 118.8%. The mean recovery of the IS was 99.6%. Table 2 shows the recovery of rifampicin and the internal standard

3.3 Application of the method to tuberculosis patients

The method described here, was successfully applied for determination of rifampicin in plasma samples, collected from 25 patients with pulmonary tuberculosis. Five blood samples were taken at; 0.5, 1, 2, 3, and 4 hours after witnessed ingestion of rifampicin along with other antitubercular drugs. The blood samples were collected in evacuated blood collection tubes contains K2 EDTA as the anticoagulant. The dose of rifampicin was based on patients' body weight, in the range of 7.4 - 11.8 mg/Kg/day (300 - 600 mg/day). Plasma was separated immediately and stored in freeze at -20 °C until the analysis which was undertaken within 2 months of blood collection. For every patient, the rifampicin C_{max} is the highest concentration of rifampicin in the five blood samples. The T_{max} of rifampicin is the time at which the rifampicin C_{max} was achieved. A wide variation of the rifampicin C_{max} was observed. Rifampicin C_{max} was ranging from 1.3 to 20.8 mg/L, (mean ±SD; 7.29 ± 4.31). The median Tmax was 2 hours and most frequent T_{max} was 3 hours. Comparing with a published reference range9 of (8 - 24 mg/L), 20 patients (80%) have a low C_{max} of rifampicin, 5 patients (20%) have normal Cmax, and no patient has high C_{max} of rifampicin.

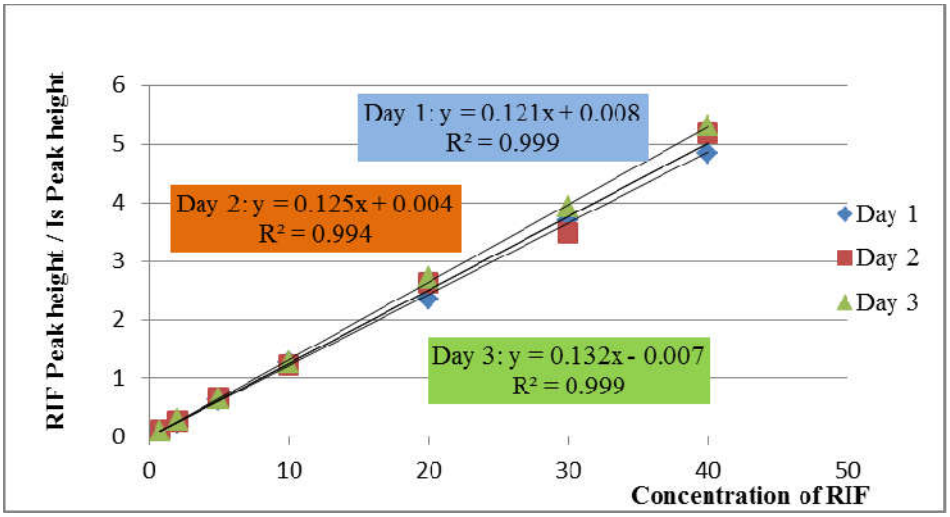

Fig. 4: The calibration curves of RIF in three different days

RIF = rifampicin, IS = internal standard

Table 1: Intra- and inter-day accuracy and precision of the method for rifampicin in the plasma

Concentration spiked mg/L	Intra- day (N=5)			Inter- day (N=15)		
	Concentration found (mean ± SD)	Accuracy (RE, %)	Precision (RSD, %)	Concentration found (mean ± SD)	Accuracy (RE, %)	Precision (RSD, %)
0.75	0.772 ± 0.041	2.87	3.72	0.793 ± 0.518	5.77	6.535
1.25	1.261 ± 0.102	0.85	8.06	1.231 ± 0.086	- 1.45	7.028
25	25.49 ± 0.969	1.96	3.8	24.816 ± 1.2	-0.733	4.838
38	35.516 ± 1.05	- 6.54	2.95	37.487 ± 2.485	-1.35	6.63

RSD, % = relative standard deviation, RE, % = relative error

Table 2: Recovery of rifampicin and the internal standard

	Concentration	Recovery
Rifampicin	1.25	118.9%
	25	114.7%
	38	123%
	Mean	118.8%; (RSD = 2.85%)
Internal Standard	4	99.6%; (RSD= 4.9%)

RSD, % = relative standard deviation, RE, % = relative error

4. DISCUSSION

During the development of this method, various composition and flow rates of the mobile phase have been tried. The best result in terms of peak symmetry, peak height, retention time, and S/N ratio was obtained by using acetonitrile with 20 mM ammonium acetate at pH 4.7 in gradient flow described above. Under this condition, acetanilide was eluted first at 3.7 minute, then by increasing the percentage of acetonitrile in the mobile phase, rifampicin eluted at 6.5 minute. Both peaks were with good shape and symmetry. Various wavelengths for both acetanilide and rifampicin have been tried during the development of this method; however, we found that 254 nm and 334 nm are the best wavelengths, in terms of peak height, and S/N ratio for acetanilide, and rifampicin respectively.

For protein precipitation, the ratio of acetonitrile to plasma was chosen to be 1:1. It has been found that ACN can precipitate as much as 93% of plasma proteins at this ratio[15]. Increasing the ACN to the plasma ratio, would increase the percentage of protein removed from the plasma to up to 98% at 4:1 ratio [15]. However, the use of such large volume of ACN will lead to dilution of rifampicin in the supernatant, and necessitate sample drying and reconstitution, which will make sample preparation, a time consuming, and expensive process. We found that using of acetonitrile at a ratio of 1:1 to the plasma is good enough to obtain a clear supernatant

suitable for HPLC injection and to avoid the evaporation and reconstitution steps.

The choice of calibration standards was made based on the range of rifampicin concentration, expected to be found in TB patients. The expectation of such range was based on previous studies in which rifampicin concentrations were measured in tuberculosis patients or healthy volunteers [5, 7, 16, 17]. The concentrations of the quality control samples was chosen based on the recommendation of FDA guideline for bioanalytical method validation[18], which recommend the use of three different concentrations as quality control samples. The low quality control sample (LQC) is to be within 3 times the LLOQ (1.25 mg/L), the middle quality control sample (MQC) is to be in midrange of the calibration curve (25 mg/L), and the high quality control sample (HQC) should approach the upper limit of the calibration curve (38mg/L). Acetanilide was prepared and used at concentration of 4 mg/L in acetonitrile, since we found that, the peak height of acetanilide at this concentration is close to the peak height of rifampicin in midrange concentration.

The C_{max} of rifampicin found in tuberculosis patients participated in this study is in line with previous studies. Kimerling et al.,[7], reported that, the rifampicin concentration was less than 8 mg/L in 64% of 22 patients.

Van Crevel et al.,[16], reported that, the plasma concentration of rifampicin in 2 hour post dose samples, was below 8 mg/ L in 70% of 62 tuberculosis patients.

Tappero et al.,[17], measured the plasma concentrations of rifampicin in 91 patients, the blood samples were taking at 1, 2, and 6 hours after the ingestion of the drug. Rifampicin concentration was less than 8 mg/L in 78% of the patients.

In conclusion**,** the method presented here is selective, accurate, precise, simple, fast and affordable in simple HPLC system. These features make this method suitable for analysis of a large number of samples. The C_{max} of rifampicin was below the reference range in most of the tuberculosis patients.

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AUTHORS' CONTRIBUTIONS

All the authors have made substantial contribution in development of the method and preparation of the manuscript

CONFLICT OF THE INTEREST

None of the authors have any conflict of interest

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