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Development and validation of new RP-HPLC method with UV detection for the determination of rifampicin in plasma

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ABSTRACT

To standardize a high-performance liquid chromatography (HPLC) method for the determination of rifampicin in plasma by external standard method.**Material and Methods:** A simple, specific and sensitive HPLC method was developed for the determination of rifampicin in plasma. Separation was achieved by reverse phase chromatography on a C18 column with a mobile phase composition of phosphate buffer pH 7.4: methanol (75:25 v/v) at 475 nm.**Results**: The retention time of rifampicin was 2.54 minutes. The assay was linear from 0.05 to 20.0 µgm /mL for plasma. Both intra-day and inter-day accuracy and precision data showed good reproducibility. Recoveries (extraction efficiency) for drug were greater than 90% in plasma .The plasma method was precise (total coefficient of variation ranged from 1.46 to 6.71%) for the analytes.**Conclusion:** The HPLC method described is sensitive, selective and linear for the wide range of concentrations for rifampicin in plasma. Thus, the method developed is well suited for the pharmacokinetic studies.

Keywords: Rifampicin, HPLC, Reversed phase chromatography

INTRODUCTION

Tuberculosis is one of the major communicable diseases in the developing countries. The therapeutic potential of rifampicin (RIF) in tuberculosis is well recognized due to its unique ability to kill semi dormant tubercule bacilli (*Mycobacterium tuberculosis*), when they undergo sporadic bursts of metabolism and growth[1-3].

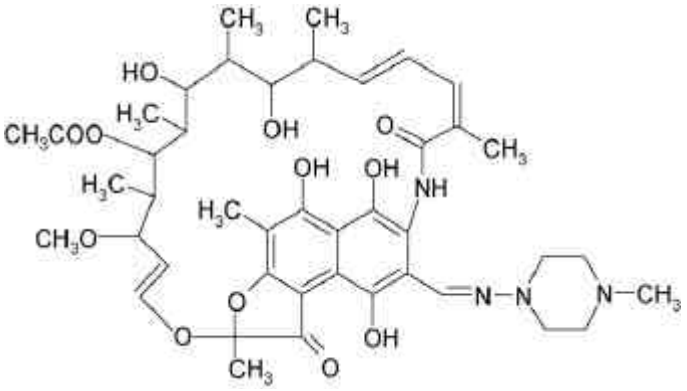

Figure 1: Structure of rifampicin

Rifampicin, 3-[[4-methyl-1-piperazinyl)-imino]-methyl]- rifamycin SV, (RIF) (Fig. 1) is a semi-synthetic derivative of Rifamycin SV.[4,5] It can be used alone or in combination with other drugs, such as isoniazid (INH) and pyrazinamide, in treatment of tuberculosis, leprosy and other infectious diseases specially those resulting from AIDS. Rifampicin is an important first line drug prescribed throughout TB therapy[6], often as part of fixed dose combination (FDC) tablets, which may also contain isoniazid and pyrazinamide. Although FDCs simplify the prescribing process and encourage compliance[7], the absorption of RIF from these formulations may vary, especially in tablets containing isoniazid[8], and contribute to treatment failure. Treatment failure and the development of drug resistance may be attributed to non-compliance with the treatment regime, poor bioavailability of RIF in some preparations, including some FDCs as described; counterfeit preparations, or malabsorption of RIF. While directly observed therapy (DOT) may address the first issue, it cannot address treatment failure due to the latter two causes. Currently, plasma levels of RIF are not monitored routinely in TB patients but it is clear that this would be advantageous if a simple and effective quantitative test were available. A number of methods for the determination of RIF in plasma have been reported[9-16]. These methods are characterized by lengthy sample preparation procedures[13,15], non-ideal chromatographic retention parameters (low analyte capacity factor)[9,10,15] and poor selectivity in the presence of the major metabolite (desacetlyrifampicin (DRIF))[10,11,13] and the degradant (RIF-quinone)[9-13,15,16].

The high occurrence of tuberculosis in HIV infected subjects makes the management of HIV treatment complex. RIF is a very active antituberculosis drug that accelerates the metabolism of protease inhibitors.[17,18] Due to the increasing necessity to monitor plasma concentrations in HIV patients with tuberculosis, different methods such as spectroscopy,[19-23] fluorometry,[24-26] gas chromatography,[27] polarography[28] amperometry[29] and high performance liquid chromatography[30-35] have been developed to measure RIF alone or in the presence of INH. These methods are expensive and need more expertise in experimentation.

Earlier, RIF in plasma/serum was quantified by microbiological methods [36-38] but these methods lacked precision and selectivity [39,40]and so does not permit separate determination of rifampicin in biological

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fluids[41] .In the recent past, several HPLC procedures have been reported in the literature for quantitative estimation of RIF and its metabolite, in serum/plasma or urine[42-49]. But many of these methods suffer from limitations such as lengthy and tedious procedures, high plasma/serum sample volumes required, large quantities of solvents involved etc. The present study was undertaken with the objective to develop and validate a simple, sensitive HPLC assay procedure for the determination of rifampicin in plasma by modifying certain experimental conditions of the existing methods to enable good resolution of rifampicin peak with shorter run time. After a number of trials using different combinations, we arrived at the present mobile phase and wavelength.

MATERIAL AND METHODS

Pure rifampicin standard was obtained from Lupin laboratories, India. Methanol used was of HPLC grade obtained from Merck (Mumbai, India). The chemicals, potassium dihydrogen orthophosphate, disodium hydrogen phosphate, and ascorbic acid were of analytical grade. Triple distilled water filtered through 0.45 mm membrane filter was used in all the experiments.

Instrumentation

A gradient HPLC (Waters HPLC system, Millipore, Billerica, USA) with two 515 and 717 pumps, a rheodyne manual injector (Rheodyne, Cotati, CA, USA) attached with a 20 µL sample loop was used for loading the sample. a variable wavelength programmable UV/VIS Detector (Waters, Milford Massachusetts, USA)., an SCL-10A VP system controller (waters) , and a reversed phase C -18 column (250m×4.0mm ID; particle size 4 µm)(waters associates) were used. The HPLC system was equipped with the MILLENNIUM32 software (version 3.05.01) was used for data acquisition and processing.

The mobile phase was prepared with methanol and phosphate buffer pH 7.4(with 18.7 ml of 0.02 M KH_2PO_4 and 80.3 ml of 0.02 M $Na_2HPO_4.2H_2O$.) in the ratio of 75:25 . The components of the mobile phase were filtered before use through 0.45 µm membrane filter and degassed for 15 minutes and the respective solvent reservoir were pumped to the column at the flow rate of 1.5mL/min . The column temperature was maintained at 30^0 C and the volume of the injection loop was 20µl. The column was equilibrated for atleast 30 minutes with the mobile phase. The eluent was detected by UV detector at 475nm for rifampicin[50] .The chromatogram was run for 5 min. Unknown concentrations were derived from the linear regression analysis of the peak height Vs. concentration curve. Linearity was verified using estimates of correlation coefficient (r).

Preparation of standard solution

Primary stock solution of 1mg/mL of rifampicin was prepared in methanol. Appropriate dilutions of rifampicin from stock solution were made in mobile phase to produce working stock solutions of 0.05, 0.1, 0.5, 1, 2 , 5, 10, 20 µgm/mL. These dilutions were used to spike plasma in the preparation of a calibration curve.

Rifampicin spiked plasma samples were prepared by mixing 1 mL blank plasma with appropriate volumes of the standard rifampicin solutions (100µL) on the day of analysis. A blank was also prepared containing 1 mL blank plasma. The samples were estimated for rifampicin by HPLC on the same day to avoid any degradation. Samples for the determination of recovery, precision and accuracy were prepared by spiking blank plasma with blanks of appropriate concentrations (2, 5 and 10 µgm/mL) of rifampicin. After preparation, the samples were stored at -20 ^oC till the time of analysis.

Extraction procedure

Plasma was spiked with varying quantities of rifampicin stock solution prepared as above, so as to give a series of drug concentrations ranging from 0.05-20 µg/ml.100µL of plasma was deproteinated by adding 100µl acetonitrile.[51,52]. The mixture was vortexed for 3 minutes and centrifuged at 3000*g* in a micro centrifuge (Spinwin, India) for 5 minutes. The supernatant was neutralized with 0.2ml of 1M NaoH and 20 µL directly injected into the HPLC.

Linearity, Limit of detection and Limit of quantification

The calibration samples were prepared by spiking 1 mL of blank plasma with appropriate amount of rifampicin on the day of analysis. The limit of detection (LOD) is the lowest level of the drug that can be detected in sample. The limit of quantification (LOQ) was defined as the lowest concentration at which the coefficient of variation (CV) and deviation from the nominal concentration were less than 20%.

Assay validation

The intra- and inter- run precision and accuracy of the assay (n= 6) were determined by percent coefficient of variation based on reported guidelines[53] like FDA guidelines. Samples containing 2.5, 5 and 10 µgm/mL concentration were prepared for the determination of precision and accuracy.

Extraction efficiency

The efficiency of the extraction method to recover rifampicin from plasma was tested using samples containing 5, 10 and 20 µgm/mL rifampicin. These samples were then subjected to the sample preparation procedure explained above. The recovery of rifampicin after sample preparation was measured by comparing the peak area found in plasma sample with the peak area obtained by direct injection of pure standard with equivalent amounts of rifampicin.

RESULTS AND DISCUSSION

Chromatography

The chromatograms of blank plasma and plasma samples containing rifampicin are shown in figures 2 and 3. in this analytical process, rifampicin were resolved with good symmetry and retention time of rifampicin was 2.54 mins(as shown in figure 3). No endogenenous interfering peaks were observed in the individual blank plasma at the retention time of rifampicin, thereby conforming the specificity of the analytical method.

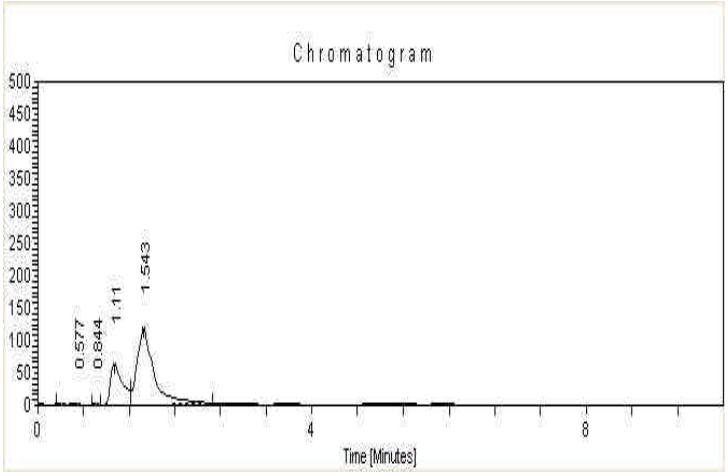

Figure 2: Typical HPLC chromatogram for analysis of blank pla

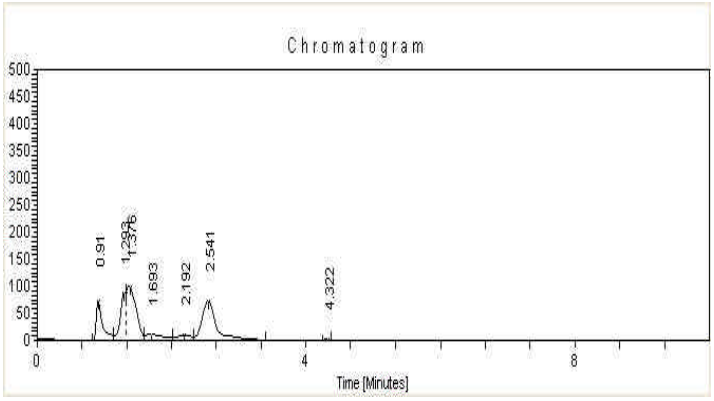

Figure 3: Typical HPLC chromatogram of rifampicin. Retention time of rifampicin is 2.541 min

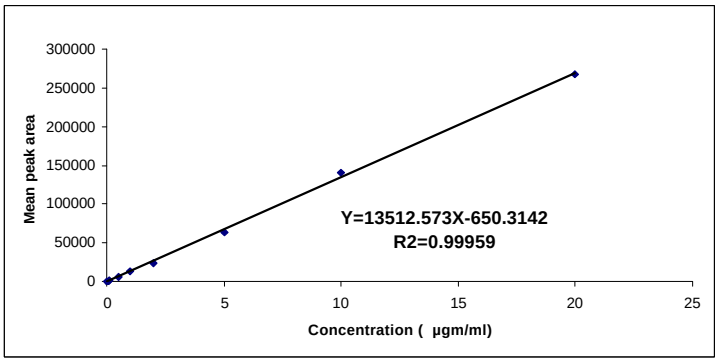

Figure 4: Calibration curve for rifampicin in plasma (n=6)

Table 1: Intraday and interday accuracy and precision of the assay.

Rifampicin concentration (µgm/mL)	Amount of rifampicin (µgm/mL) found on Intra-day (% CV) , n=6	Inter-day (%CV), n=6
2.5	2.39 (2.31)	2.46 (3.4)
5.0	4.22 (1.55)	4.93(6.71)
10.0	9.65 (1.71)	9.97 (1.46)

Table 2: Recovery and accuracy of the proposed method.

Amount of drug added (µgm/mL)	Mean (± S.D) amount (µgm/mL) recovered (n=6)	Mean (± S.D) % accuracy (n=6)
5	4. 14 ±0.06	82.80 ±1.98
10	9..64 ±0.29	96.40± 1.09
20	19.41 ±0.33	97.05 ± 0.52

Limit of quantification

The peak area of rifampicin was used for the quantification of rifampicin in plasma samples. The calibration curves were linear in the concentration range 0.05-20 µg/mL.The type of regression equation is y=mx+c, where y represents the peak area of rifampicin, x represents the concentration of rifampicin, m is the slope of the curve and c is the intercept. The equation of the calibration curve obtained from 8 points ranging from 0.05-20 µg/mL is Y=13512.573X-650.314. The correlation coefficient (R^2) between rifampicin concentration and peak area of rifampicin in plasma is 0.9995.

The LOQ, established by determining the concentrations of rifampicin in three spiked calibration standards having reproducibility with a coefficient of variation less than 20% (in case of LOD it should be <25%) and an accuracy of 80-120%, was found to be 0.05 µg/ml . The LOD was 0.025 µgm/mL with coefficient of variation (%CV) of 21% . The intra day precision of the assay was determined by analyzing plasma samples at each concentration on the same day. For the determination of inter day precision, the samples were analyzed on five different days. The intra day and inter day coefficient of variation (%CV) values are shown in Table 1. These values are within the limits specified for inter and intra day precision [54].The recovery of rifampicin from plasma was estimated at 5, 10and 20 µgm/mL concentrations. Plasma samples (in triplicate) containing rifampicin were extracted and were analyzed. Triplicate samples containing similar concentrations of rifampicin in mobile phase were directly injected, and peak areas were measured. Recovery was calculated by comparing the peak areas of pure samples spiked with the same amount of rifampicin and proceeded similarly. The recoveries ranged from 4.14-19.41 µgm/mL and are shown in Table 2. The accuracy of the method was verified by comparing the concentrations of rifampicin measured in extracted plasma with the actual concentration added. Accuracy ranged from 82.80-97.05 %

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CONCLUSION

The assay procedures described are suitable for the quantification of rifampicin in plasma and these experiments confirm that the present method for determination of rifampicin in plasma is simple, selective, sensitive, specific, accurate and precise. The calibration curve is linear in the concentration range of 0.05-20 µgm/mL. Hence such a method would be ideally suitable for the estimation of rifampicin in pharmacokinetic studies and these methods could be used for therapeutic drug monitoring of rifampicin in TB patients.

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