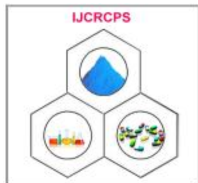

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RESEARCH ARTICLE

A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF RIFAMPIN IN PLASMA OF NON-HIV-INFECTEDTUBERCULOSIS PATIENTS

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Abstract

Rifampin (rifampicin) is a bactericidal for *Mycobacterium tuberculosis*. It inhibits mycobacterial DNA-dependent RNA polymerase synthesis by blocking RNA transcription (Ref). Most strains of *Mycobacterium tuberculosis* are inhibited in vitro by rifampin concentrations of 0.5 µg/ml (Ref). Rifampin is quickly absorbed from the gastrointestinal tract with peak serum concentrations of 6-8 µg/ml occurring 1.5-2.0 hours after administration. It is 75% bound to plasma proteins and it is metabolized in the liver to an active metabolite, deacetylrifampin and undergoes enterohepatic recycling. Preliminary data suggest that patients infected with mycobacteria have altered pharmacokinetic profiles for rifampin due to malabsorption of the drug, which contributes to the observed low rifampin levels in the serum of these patients . The resulting subtherapeutic levels may seriously affect the therapy outcome. For these reasons, therapeutic drug monitoring (TDM) of rifampin in tuberculosis patients may play a strategic role in improving the therapeutic outcome in selected patients . The other important indications for TDM of rifampin are the management of interaction between rifampin and other antituberculosis drugs and the evaluation of antituberculosis drug compliance in these patients . The reported methods for the assay of rifampin in the plasma of patients with active tuberculosis are lacking sensitivity and specificity. Many of these methods involve a time-consuming sample preparation and extraction and a very lengthy elution time.

Keywords: Rifampin, *Mycobacterium tuberculosis,* plasma proteins, enterohepatic recycling.

Introduction

Mycobacterium tuberculosis

Acid-fast, obligate aerobic, non-motile, rod-shaped bacterium, this is the causative agent of tuberculosis. Tuberculosis is, to this day, according to the WHO, the leading killer of adults, with approximately 2 million deaths annually worldwide. It is estimated that 8 million people are infected each year. A large part of its success as a pathogen is due to its ability to persist in a dormant or latent form for years or even decades, with a concomitant absence of clinical symptoms. This non-replicating persistent form is refractory to most drugs, it may be induced by hypoxia (oxygen depletion) and/or nitric oxide exposure. Up to one-third of the world's population is thought to be latently infected. An additional problem is the emergence of drug resistant strains, mainly because people do not complete their treatment plans or have been incorrectly treated and so have remained infectious. Mycobacteria have an unusual outer membrane approximately 8nm thick, despite being considered Gram-positive. The outer membrane and the mycolic acid-arabinoglactan-peptidoglycan polymer form the cell wall, which constitutes an efficient permeability barrier in conjunction with the cell inner membrane.

Chemotherapy today

Following streptomycin, p-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954),

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cycloserine (1955), ethambutol (1962), and rifampin (rifampicin; 1963) were introduced as anti-TB agents. Aminoglycosides such as capreomycin, viomycin, kanamycin, and amikacin, and the newer quinolones (e.g. moxifloxacin, levofloxin, ofloxacin, and ciprofloxacin) are effective but usually are used in drug resistance situations. Newer drugs and combination therapies are being studied by the Global Alliance for TB Drug Development in an effort to shorten treatment.

Three properties of anti-TB drugs are important: antibacterial activity capacity to inhibit the development of resistance, and capacity to kill intracellular, persisting organisms. The initial combination of isoniazid, rifampin, pyrazinamide, and ethambutal provide effective TB treatment.
With the proper four-drug regimen, there should be a rapid clinical improvement and a significant decrease in infectiousness. After a month, the patient should be without a fever, feel well, and have regained weight. Coughing and sputum should have decreased and improvements will be visible on the X-rays. Although bacteria may still be present in the smears, they will become more and more difficult to culture.

The absence of radiological improvement in the first three months should be grounds for concern and indicate that a change in therapy may be needed. Patient adherence and the bacteria's drug sensitivity should be reevaluated. Relapses usually occur within six months of the end of treatment, and in most cases are due to poor patient adherence or a clinician's inadequate treatment. Patient adherence must be monitored throughout treatment; through directly observed therapy, which should be the standard of care in every setting for every patient.

If TB becomes active again in a previously treated patient, there is a high chance that the bacteria will now be drug resistant. Any current therapy must be suspended until multiple drugs are found to which the pathogen is fully sensitive, and treatment can be resumed with the addition of these drugs to the original regimen. Never add a single drug to a failing regimen. If the microorganism is resistant to the standard drugs, then it will be necessary to administer more toxic medications such as Ethionamide, Cycloser

The chemotherapy of infectious diseases, using sulfonamide and penicillin's, had been underway for several years, but these molecules were ineffective against Mycobacterium tuberculosis. Since 1914, Selman A. Waksman had been systematically screening soil bacteria and fungi. In 1939 had discovered the marked inhibitory effect of certain fungi, especially actinomycetes, on bacterial growth. In 1940, he and his team at Rutgers University in New Jersey were able to isolate an effective anti-TB antibiotic, actinomycin, however, this proved to be too toxic for use in humans or animals.

A rapid succession of anti-TB drugs appeared in the following years. These were important because with streptomycin mono therapy (one drug treatment), resistant mutants began to appear with a few months, endangering the success of antibiotic therapy. However, it was soon demonstrated that this problem could be overcome with the combination of two or three drugs.

Rifampin (rifampicin) is a bactericidal for *Mycobacterium tuberculosis*. It inhibits mycobacterial DNA-dependent RNA polymerase synthesis by blocking RNA transcription (Ref). Most strains of *Mycobacterium tuberculosis* are inhibited in vitro by rifampin concentrations of 0.5 µg/ml (Ref). Rifampin is quickly absorbed from the gastrointestinal tract with peak serum concentrations of 6-8 µg/ml occurring 1.5-2.0 hours after administration. It is 75% bound to plasma proteins and it is metabolized in the liver to an active metabolite, deacetylrifampin and undergoes enterohepatic recycling.

Preliminary data suggest that patients infected with mycobacteria have altered pharmacokinetic profiles for rifampin due to malabsorption of the drug, which contributes to the observed low rifampin levels in the serum of these patients . The resulting subtherapeutic levels may seriously affect the therapy outcome. For these reasons, therapeutic drug monitoring (TDM) of rifampin in tuberculosis patients may play a strategic role in improving the therapeutic outcome in selected patients . The other important indications for TDM of rifampin are the management of interaction between rifampin and other antituberculosis drugs and the evaluation of antituberculosis drug compliance in these patients .
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Materials and Methods

Apparatus:

A HPLC system was used consisting of model LC-10AT liquid chromatograph , an autosampler model WISP-712 , model 480 UV/VIS variable wavelength detector set at 337 nm (Waters, Milford, MA, USA), in combination with a data module integrator model 746 . Chromatographic separations were accomplished using a µBondapak C_{18}, 10 µm, 150 mm×3.9 mm stainless steel column (Waters, Milford, MA, USA) with a guard precolumn of the same packing material.

Reagents

Rifampin was used as received from Ascorbic acid (as stabilizing agent) from Sigma chemicals , tetracycline hydrochloride (as internal standard) was obtained from Boehringer Mannheim GmbH. Methanol and phosphoric acid were HPLC grade . All other chemicals and reagents were of analytical grade.

Standard Solutions

Rifampin (50 mg) was dissolved in distilled water in a 50-ml volumetric flask. Ascorbic acid (25 mg) was added and volume was completed to 50 ml with water to produce a stock solution of 1 mg/ml. The internal standard, tetracycline HCL (10 mg), was dissolved in 10 ml of distilled water to give a 1-mg/ml stock solution. The solutions were stored at 4°C and they were stable for, at least, two weeks.

Chromatographic Conditions

The mobile phase consisted of methanol and 0.01 M phosphate buffer of pH 5.2, adjusted with phosphoric acid (55:45 v/v). The mixture was filtered through 0.22 µm membrane under vacuum, then degassed by flushing with nitrogen for 5 min. The mobile phase was pumped isocratically at a flow rate of 1.2 ml/min during analysis, at ambient temperature. The chromatograms were recorded and integrated at a chart speed of 0.25 cm/min. The effluent was monitored at 337 nm at sensitivity of 0.02 AUFS.

Analytical Procedure

Sample preparation and analysis were conducted at room temperature. Rifampin stock solution (1 mg/ml) was added to 15 ml graduated centrifuge tubes in volumes of 0.5, 10, 25, 50, 100 and 150 µL (5 mg of ascorbic acid was added to each tube). Drug-free human plasma was added to complete volume to 10 ml, and vortex-mixed for 30 sec to yield final calibration standard concentrations of 0 (no rifampin added), 0.5, 1.0, 2.5, 5.0, 10 and 15 µg/ml. Each of these standard solutions was distributed in disposable polypropylene microcentrifuge tubes (1.5 ml, eppendorf) in volume of 0.5 ml and stored at -70°C pending analysis.

For preparation of sample for injection onto HPLC system, a 10-ml aliquot of the internal standard (tetracycline HCL, 1 mg/ml) was added to 0.5 ml of plasma sample, and then 0.5 ml of methanol was added to the tube. The tube was shaken for 1 min on a vortex-mixer and then centrifuged at 13000 rpm for 20 min. Aliquots were loaded in the autosampler tray and volumes of 50 µL were injected into the chromatograph.

Clinical Study

The study was conducted on Saudi adult patients admitted to King Khalid University Hospital (KKUH) with active pulmonary tuberculosis. Treatment with rifampin was initiated and relevant clinical and laboratory information for each patient, including all concurrent medications, were recorded. Eight patients received standard therapy for tuberculosis for one week and plasma samples were drawn after 2 hours of the last dose of rifampin.

Simple & sensitive method of determining lipoic acid in human plasma using HPLC

Lipoic acid is a metabolic antioxidant synthesized in human and animal cells.

Crucial cofactor in the pyruvate dehydrogenase {PDH} & ketoglutamate dehydrogenase {KGDH} multienzyme complex responsible for the production of acetyl coA in metabolic pathway.

ROS reactive 0_2 species) also plays a role in protecting membrane by interacting with vitamin C & glutathione, which in turn recycles other antioxidant such as vitamin E.

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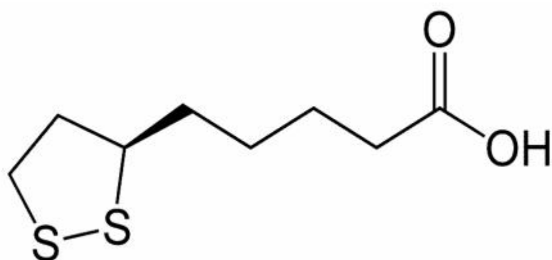
Chemical structure of lipoic acid



It is used as an effective therapeutic agent in various disease including diabetes, mitochondrial cytopathies, cardio vascular disease,hepatitis,cataract ,radiation damage, HIV infection ,neurodegenerative disorder & neurovascular abnormalities.

Although Liquid chromatography-Mass spectroscopy, Gas chromatography-Mass spectroscopy quantification of lipoic acid in biological fluid has been attempted.

Samples

Blood samples were collected in EDTA tubes .All the samples were centrifuged immediately. Plasma was separated and stored at -20°c until assay.

Estimation of plasma lipoic acid in all samples were undertaken with 1 to 2 days of blood collection.

Materials

chemical & reagents

-LIPOIC ACID powder
Methanol
Acetonitrile
Chloroform
Ethanol
Sodium phosphate
Deionized water
Pooled human plasma

Preparation of standard solution:

Stock standard (10mg/ml) of lipoic acid in 10 ml of methanol.
Working standard of lipoic acid (0.78 to 50μg/ml) prepared in human pooled plasma. Conc of standard (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 μg/ml).

Sample preparation

300 µl of sample (blank/std/test) + 300 µl of ethanol.

↓

1.8 ml of chloroform (vortex for 2 min).

↓

Centrifuge for 5 min at 2500 RPM.

↓

600 µl of supernatant was separated.

↓

Evaporated in nitrogen evaporater for 10 min.

↓

The residue was reconstituted with mobile phase + 50 µl of samples was directly injected to HPLC column.

Chromatographic condition optimization

The following chromatographic condition were optimized for the quantification of lipoic acid using HPLC-UV.

- Photodiode UV detector optimization.
- Mobile phase optimization.
- Flow rate optimization.
- Temperature optimization.
- Extraction solvent optimization.

Method ralidation

Proposed various parameter such as accuracy, linearity, precision, recovery, limit of detection (LOD),limit of quantification(LOQ), specificity and stability of sample was evaluated.

Accuracy & linearity:

Analyzing a set of standard (0.78 to 50µg/ml).

Unknown conc. Were derived from linear regression analysis of peak height of analyte vs concentration curve.

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Figure.1 Analysis of compounds by HPLC Instrument

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Linearity was verified using estimates of correlation coefficient.

Precision

Were carried out on the basis of injection repeatability and analysis

Our different concentration of lipoic acid were prepared in pooled plasma and analyzed in duplicate on three consecutive days.

Precision for plasma lipoic acid assay

Actual concentration. (µg/ml)	Found concentration µg/ml. Ratio ±SD (RSD %)
50	51.9 ± 0.55 (3.8)
6.25	5.87 ± 0.12(6.08)
0.78	0.64 ± 0.02(17.9)

Recovery :

The same-pooled human plasma samples containing previously determining concentration of lipoic acid were spiked with (0.39, 0.78, 1.56, 3.125, 6.25 µg/ml) of lipoic acid and assayed.

Recovery experiment were carried out on 3 different occasions.

Table: 3 Recovery of lipoic acid in plasma

Added (µg/ml)	Estimated (µg/ml) Mean ±SD	Recovery (%)
Baseline	1.39±0.18	
0.78	1.82±0.26	84%
Baseline	5.86±0.38	
3.125	10.12±1.06	113%

LOQ and LOD:

The limit of quantification (LOQ) was 0.78µg/ml and the limit of detection (LOD) was 0.84µg/ml for lipoic acid.

Stability

The stability of lipoic acid in human plasma when stored at -20°c was elevated by assaying plasma containing lipoic acid on days 1 to 30.

The stability studies shows that the standard solution of lipoic acid were stable for at least one month when stored at -20°c .However ,spiked plasma samples were stable for only one week when stored at -20°c .

Application of the method

Our developed and validated HPLC-UV method was applied for the measurement of lipoic acid levels in healthy volunteers and diabetic patients .This method is a part of biochemical analysis of blood samples collected from healthy volunteers and patients with diabetes .This method will be used for the determination of lipoic acid in clinical practice.

Results and Discussion

Mobile phase 50Mm Na_2HPO_4, adjusted to 2.4 :acetonitrile :methanol in the ratio of 50:30:20 offered a good resolution peak and better rotation time of analytes.

Liquid-liquid extraction with various organic solvent was applied for the extraction of lipoic acid from plasma.

Under this chromatographic condition, sufficient resolution was obtained with shorter chromatographic run time (5 min).

Similarly mobile phase flow rate was 1ml/min with better peak rate and separation.

Conclusion

In conclusion, the reported HPLC-UV method described here for the determination of lipoic acid in plasma is simple, sensitive, reproducible and precise with an accuracy of greater than 113 %. Through a spectrometric method has been

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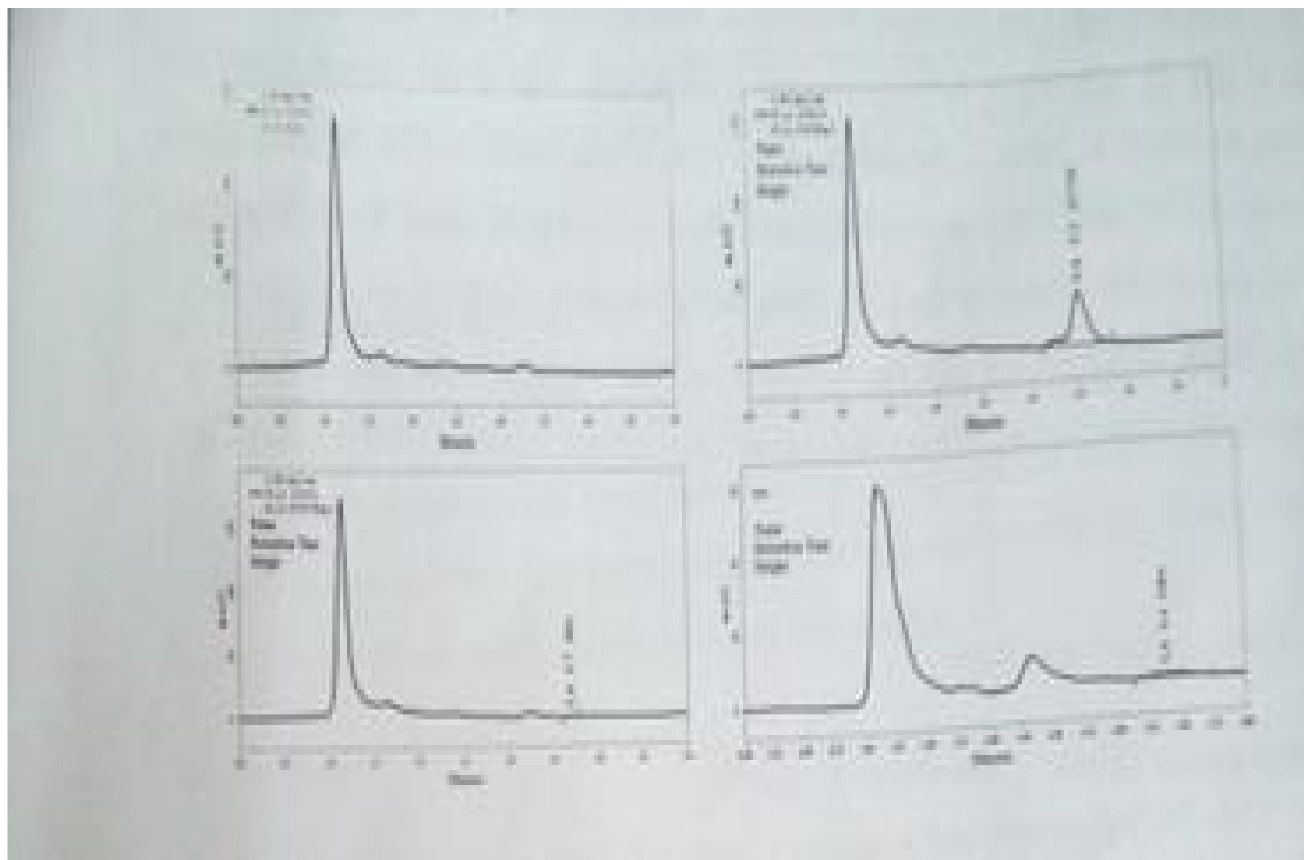
Figure 2. determination of lipoic acid in plasma by HPLC



developed very recently , the sensitivity was very low and range limit was a high concentration range .The assay is less laborious , economical and time consuming than other reporter methods .plasma concentration of lipoic acid was determined in human healthy volunteers.

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