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Original Research Article

Development and Validation of HPLC Method for the Determination of Rifampicin in Human Plasma

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Abstract

A simple and precise reversed-phase high performance liquid chromatography (HPLC) assay for the determination of rifampicin (RIF) in human plasma was developed and validated. Using hydrochlorothiazide (HCT) as an internal standard, separation was achieved on Atlantis dC18 column with a mobile phase composed of 0.01 M monobasic sodium phosphate and acetonitrile (60:40, v:v) and delivered at a flow rate of 1 ml/minute. RIF and HCT were extracted from plasma using methyl tert-butyl ether and dichloromethane (70:30%, v:v) and monitored using photodiode array detector set at 337 nm. Relationship between RIF concentration and peak height ratio of RIF to HCT was linear over the range of 0.3-25 µg/ml. Coefficient of variation and bias were ≤9.7% and ≤6.0%, respectively. Mean extraction recovery of RIF and HCT was 95% and 90%, respectively. Stability of RIF in extracted (24 hours at room temperature or 48 hours at -20 °C) and unprocessed (24 hours at room temperature, 8 weeks at -20 °C, or 3 freeze-thaw cycles) samples was ≥91%.

Keywords: Rifampicin, Hydrochlorothiazide, Human plasma, HPLC.

INTRODUCTION

Rifampicin (RIF) is an antibiotic used in treating bacterial infections, including those caused by Mycobacterium tuberculosis [1]. It is absorbed rapidly with a peak plasma concentration of 8-12 µg/ml within 1-2 hours after ingestion of a 600 mg dose [2]. Figure-1 depicts the chemical structures of rifampicin and hydrochlorothiazide (HCT), the internal standard (IS) used in the study.

Several analytical methods have been reported for the determination of RIF in biological fluids, alone [3-13], along with its metabolite, desacetyl rifampicin [14, 15], or in combination with others antibiotics, such as daptomycin [16], pyrazinamide and isoniazid [17, 18], daptomycin, amikacin and gentamicin [19], and ceftaroline, daptomycin and linezolid [20]. Most of the reported methods were based on liquid chromatography followed by ultra-violet (UV) detection [4-9, 13-18] and few on liquid chromatography with tandem-mass spectrometry [10-12, 19, 20]. For sample preparation, protein precipitation [7, 8, 11, 16], liquid-liquid extraction [3, 5, 9, 10, 14, 18], solid-phase (micro) extraction [6, 12-15, 19, 20] procedures were used. The reported methods variably had limitations that included relatively complex sample preparation [6], long run time [7], and high limit of quantification [8].

This paper describes a reversed-phase HPLC assay to determine RIF level in human plasma that has relatively simple sample preparation, short run time, and high sensitivity. The method was fully validated and successfully applied to assess RIF stability under various clinical laboratory conditions.

MATERIAL AND METHODS

Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 system (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. We used a reversed-phase Atlantis dC-18 (4.6 x 150 mm, 5 µm) column protected by guard column Symmetry C18 (3.9 x 20 mm, 5 µm). Data were collected with a Pentium IV computer, using Empower Chromatography Software.

Chemical and reagents

All reagents were of analytical-reagent grade unless stated otherwise. Rifampicin, hydrochlorothiazide, and monobasic sodium phosphate

were purchased from Sigma-Aldrich Co, Steinheim, Germany. Acetonitrile, methanol (both HPLC grade), methyl tert-butyl ether, dichloromethane, and phosphoric acid were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and further purified by passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of the King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia.

Chromatographic Conditions

The mobile phase was composed of 0.01 M monobasic sodium phosphate (pH= 4.5±0.1, adjusted with phosphoric acid) and acetonitrile (60:40, v:v) and delivered at a flow rate of 1.0 ml/minute at ambient temperature. The run time was 8 minutes. A photodiode array detector set at 337 nm was used.

Preparation of Standard and Quality Control Samples

Stock solution of RIF and IS (1.0 mg/ml) were prepared in methanol. They were further diluted with plasma or methanol to produce working solutions of 100 µg/mL for RIF and 500 µg/mL for IS. Nine calibration standards in the range of 0.3-25 µg/ml (in addition to blank and zero) and four quality control (QC) samples (0.3, 0.9, 12.5, and 22.5 µg/ml) were prepared in human plasma. Calibration standards and QC samples were vortexed for one minute and 0.5 ml aliquots were transferred into teflon-lined, screw-capped, borosilicate glass, 13 x 100 mm culture tubes and stored at –20°C until used.

Sample Preparation

Aliquots of 0.5 ml of calibration curve or QC samples were allowed to equilibrate to room temperature. To each tube, 50 µl of the IS working solution was added and the mixture was vortexed for 10 seconds. After the addition of 3 ml of methyl tert-butyl ether and dichloromethane, (70:30, v:v), the mixture was vortexed again for 3 minutes and then centrifuged for 15 minutes at 4200 rpm at room temperature. The organic layer was transferred to a clean tube, dried under a gentle stream of nitrogen at room temperature, and reconstituted in 200 µl mobile phase. The clean supernatant was transferred into an auto-sampler vial and 100 µl was injected into the HPLC system.

Stability Studies

Three QC samples (0.3, 0.9, and 22.5 µg/ml) were used for stability studies. Five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed (counter stability, 24 hours at room temperature), five aliquots were stored at –20°C for eight weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed and stored at room temperature for 24 hours or 48 hours at –20°C before analysis (autosampler stability). Finally, fifteen aliquots of each QC sample were stored at –20°C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to –20°C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

Method Validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [21].

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

After a number of trial runs using different combinations, we found a mobile phase composed of 0.01 M monobasic sodium phosphate (pH= 4.5±0.1, adjusted with phosphoric acid) and acetonitrile (60:40, v:v) and delivered at a flow rate of 1.0 ml/minute to be the best. A well-defined separation was achieved within 8 minutes of run time. The retention time of IS and RIF were around 3.0 and 5.0 minutes, respectively.

Specificity

In order to confirm method specificity, we screened six batches of blank plasma and eight frequently used medications (acetaminophen, omeprazole, ranitidine, nicotinic acid, ascorbic acid, caffeine, ibuprofen, and diclofenac) for potential interference. No endogenous component or potential medication co-eluted with RIF or the IS. Figure-2 depicts a representative chromatogram of drug free human plasma used in preparation of standards and QC samples.

Linearity, Accuracy and Precision

Linearity of the assay was evaluated by analyzing ten standard curves each containing blank and nine RIF concentrations over the range 0.3-25 µg/ml. Peak height ratios (RIF/IS) were subjected to regression analysis (regression equation, Y = 0.0981 X - 0.0070). The suitability of the calibration curves was confirmed by back-calculating RIF concentrations from the calibration curves (Table-1). All calculated concentrations were well within the acceptable limits (≤15%, except LLOQ ≤20%). Precision and bias were also determined for four QC concentrations (0.3, 0.9, 12.5, and 22.5 µg/ml). Intra-day (n=10) and inter-day (n=20, over three consecutive days) precision were ≤9.0% and ≤9.7%, respectively. Intra-day and inter-day bias were in the range of -6.9 to 1.1% and -6.0 to 5.2%, respectively. The results are summarized in Table-2. Figure-3 represents an overlay of chromatograms of extracts of RIF and HCT spiked human plasma.

Table-1: Mean back-calculated rifampicin levels from ten calibration curves

Nominal level (µg/ml)	Measured level (µg/ml)		CV (%)	Accuracy (%)
	Mean	SD		
0.3	0.2696	0.0222	8.2	90
0.6	0.5368	0.0164	3.0	89
1.2	1.1566	0.0918	7.9	96
1.8	1.8460	0.1237	6.7	103
3.6	3.4932	0.3039	8.7	97
7.5	6.9464	0.2691	3.9	93
10	10.2557	0.4726	4.6	103
20	20.5905	1.5196	7.4	103
25	26.3130	1.6906	6.4	105

Coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration x 100. Accuracy = measured level divided by nominal level x 100.

Table-2: Intra- and inter-day precision and bias of rifampicin assay

Nominal level (µg/ml)	Measured level (µg/ml)		CV (%)	Bias (%)
	Mean	SD		
Intra-day (n=10)				
0.3	0.2817	0.0252	9.0	-6.1
0.9	0.8796	0.0610	6.9	-2.3
12.5	11.6353	0.5900	5.1	-6.9
22.5	22.7553	0.7393	3.2	1.1
Inter-day (n=20)				
0.3	0.2806	0.0273	9.7	-6.0
0.9	0.8659	0.0688	7.9	-3.8
12.5	12.1963	0.8506	7.0	-2.4
22.5	23.6589	1.2574	5.3	5.2

Coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration x 100.
Bias = measured level - nominal level divided by nominal level x 100.

Table-3: Recovery of rifampicin and internal standard (IS) from human plasma

Concentration (µg/ml)	Human plasma*	Aqueous medium*	Recovery† (%)
Rifampicin 0.3	3300 (209)	3492 (232)	95
0.9	10961 (409)	11377 (750)	96
12.5	158646 (8549)	165049 (2188)	96
22.5	250093 (2538)	270591 (4472)	92
Internal standard 25	136312 (5450)	150805 (3115)	90

*Mean peak height (SD), n = 5. †Recovery = Mean peak height of rifampicin in human plasma divided by mean peak height in aqueous medium x 100. (Aqueous medium, methanol and water, 1:1, v:v)

Table-4: Stability of rifampicin under various clinical laboratory conditions

Nominal level (µg/ml)	Unprocessed		Processed		Freeze-thaw cycle		
	24 hrs RT	8 wks -20°C	24 hrs RT	48 hrs -20°C	1	2	3
0.3	91	104	103	96	89	85	111
0.9	98	93	98	89	110	99	115
22.5	101	100	83	90	103	104	102

Data represent stability (%) calculated as mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 24 hours at room temperature (24 hrs RT), after 8 weeks at –20ºC (8 wks, –20ºC), or after 1-3 cycles of freeze at –20ºC and thaw at room temperature; or processed and then analyzed after storing for 24 hours at room temperature (24 hrs, RT) or 48 hours at –20ºC (48 hrs, -20ºC).

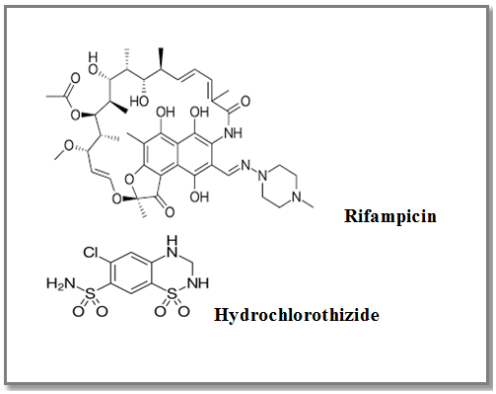

Fig-1: Chemical structure of rifampicin and hydrochlorothiazide (internal standard)

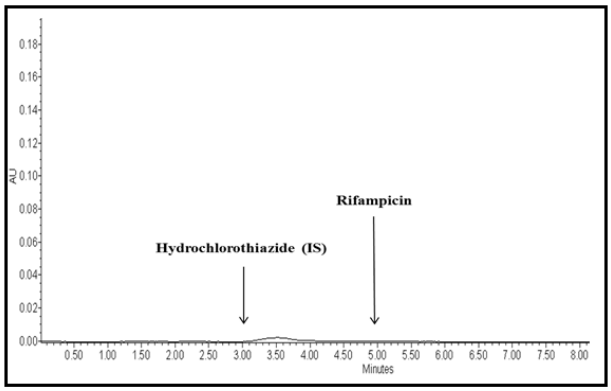

Fig-2: Representative chromatogram of drug-free human plasma sample

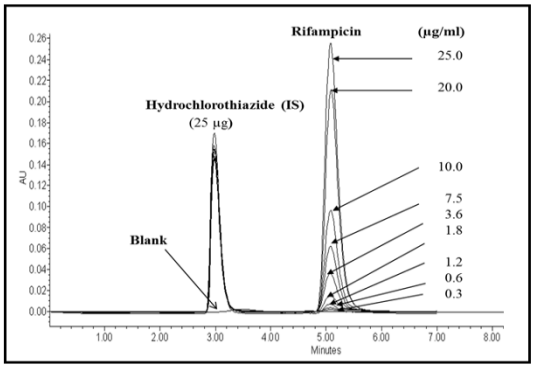

Fig-3: Overlay of chromatograms of extracts of human plasma spiked with the internal standard and rifampicin at various concentrations

Recovery

The absolute recovery of RIF was assessed by direct comparison of its peak height obtained from samples prepared in plasma and in aqueous medium, using five replicates for each of four QC samples (0.3, 0.9, 12.5, and 22.5 µg/ml). Similarly, the recovery of the IS was determined by comparing the peak height of the IS in 5 aliquots of 0.5 ml human plasma spiked with 50 µl of the IS working solution (500 µg/ml) with the peak height of equivalent samples prepared in aqueous medium. The results are presented in Table-3. Mean recovery of RIF and the IS were 95% and 90%, respectively.

Stability

RIF stability in processed and unprocessed QC samples (0.3, 0.9 and 22.5 µg/ml) was investigated. RIF was stable in processed samples for at least 24 hours at room temperature (≥83%) or 48 hours at -20°C (≥89%). RIF in unprocessed plasma samples was stable for at least 24 hours at room temperature (≥91%), eight weeks at -20°C (≥93%), and after three freeze-and thaw cycles (≥85%).

CONCLUSION

The described reversed-phase HPLC assay is rapid, precise, and accurate. It requires small volume of plasma and utilizes a simple and convenient method for sample preparation. The assay was fully validated and applied to monitor stability of RIF under various conditions generally encountered in the clinical laboratories.

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