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Determination of rifampin in human plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple, specific and sensitive high-performance liquid chromatographic (HPLC) method was developed for the determination of rifampin in human plasma. Rifampin and sulindac (internal standard) are extracted from human plasma using a C_2 Bond Elut extraction column. A 100-μl volume of 0.1 *M* HCl is added to the plasma before extraction to increase the retention of the compounds on the extraction column. Methanol (1 ml) is used to elute the compounds and 0.5 ml of 3 mg/ml ascorbic acid in water is added to the final eluate to reduce the oxidation of rifampin. Separation is achieved by reversed-phase chromatography on a Zorbax Rx C_8 column with a mobile phase composed of 0.05 *M* potassium dihydrogen phosphate–acetonitrile (55:45, v/v). Detection is by ultraviolet absorbance at 340 nm. The retention times of rifampin and internal standard are approximately 4.4 and 7.8 min, respectively. The assay is linear in concentration ranges of 50 to 35 000 ng/ml. The quantitation limit is 50 ng/ml. Both intra-day and inter-day accuracy and precision data showed good reproducibility.

Keywords: Rifampin

1. Introduction

Rifampin 3-(4-methylpiperazin-1-ylimino-methyl)rifamycin SV, is a semisynthetic drug widely used in the treatment of tuberculosis and other infectious diseases. Rifampin metabolizes in the liver of man with principle metabolites of 25-desacetylrifampicin [1] and 3-formylrifampicin [2].

Rifampin is widely used in the treatment of tuberculosis and other infectious diseases in AIDS patients. It is important to study the interaction of rifampin with other potential drugs for treating the acquired immune deficiency syndrome. A number of high-performance liquid chromatographic (HPLC) [3–10] and thin-layer chromatographic (TLC) [11] methods have been developed for the determination of rifampin and its metabolites in biological fluids. These methods use solvent extraction [3,6], solid-phase extraction [5] and protein precipitation [4,8] for sample cleanup. However, these methods lacked sensitivity (100 ng/ml) and specificity. A simple, sensitive and specific method is described in this paper.

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2. Experimental

2.1. Materials

Rifampin and internal standard (sulindac) were obtained from Sigma (St. Louis, MO, USA) and USP (Rockville, MD, USA), respectively. Atevirdine (U-87,201) and its metabolite (U-89,255); and delavirdine (U-90,152) and its metabolite (U-96,183) were provided by The Upjohn Company. Heparinized human plasma was donated by Corning Hazleton Inc. (CHI) employees. Acetonitrile, HPLC grade, was obtained from Fisher (Fairlawn, NJ, USA). Hydrochloric acid (GR grade) and potassium dihydrogen phosphate anhydrous were obtained from EM Science (Gibbstown, NJ, USA). Methanol, HPLC grade, was obtained from Burdick and Jackson (Muskegon, MI, USA). Ascorbic acid, analytical reagent grade, was obtained from Mallinckrodt (Paris, KY, USA). Deionized water was processed through a Milli-Q water purification system, Millipore Corporation. The Bond Elut C_2 (1 ml) extraction was obtained from Varian (Harbor City, CA, USA).

2.2. Chromatographic systems

The HPLC system consisted of an SSI 222C micro pump (State College, PA, USA), a Perkin-Elmer ISS 100 autoinjector (Norwalk, CT, USA), and ABI/Kratos spectroflow 783 UV detector (San Jose, CA, USA) at 340 nm. The analytical column was a Zorbax RX C_8, 250×4.6 mm I.D., 5 μm particle size (Mac-Mod, Chadds Ford, PA, USA) protected by a Brownlee RP-8 pre-column (15×3.2 mm I.D., 7 μm particle size, ABI, San Jose, CA, USA). Data collection and calculations were conducted with an HP1000 Model A900 computer with a 3350A Laboratory Automation System (Hewlett-Packard, Palo Alto, PA, USA). The mobile phase was 0.05 *M* potassium dihydrogen phosphate–acetonitrile (55:45, v/v) with a flow-rate of 1 ml/min at ambient temperature.

2.3. Preparation of standard solutions

A stock standard solution of rifampin (2 mg/ml) was prepared by dissolving 50 mg of rifampin in 25 ml of methanol (containing 1 mg/ml ascorbic acid). A stock solution of internal standard (100 μg/ml) was prepared by dissolving 10 mg of sulindac in 100 ml of water. Working solutions of rifampin (0.5–350 μg/ml) were prepared by diluting the stock solution with methanol (containing 1 mg/ml ascorbic acid in water). The internal standard working solution (20 μg/ml) was prepared by diluting the stock solution with water. The rifampin standard solutions were stored at −20°C in glass vials protected from light, and the internal standard solutions were stored at 5°C. These solutions were stable for at least six months.

2.4. Quality control samples

Pooled quality control samples (controls) were prepared to determine the precision and accuracy of the method, and to evaluate the stability of samples. Over-curve control pools were also prepared to evaluate parallelism when specimens required analysis at partial volume.

Plasma control pools (150, 4000, and 20 000 ng/ml) were prepared by diluting 75 μl of 100 μg/ml, 100 μl of 2 mg/ml, and 500 μl of 2 mg/ml rifampin, respectively, to a 50-ml volume, using blank human plasma. An over-curve control (70 000 ng/ml) was prepared by diluting 350 μl of 2 mg/ml to a 10-ml volume with blank human plasma.

All control pools were aliquoted into 6-ml polypropylene vials and stored at approximately −70°C.

2.5. Sample preparation

Calibration standards were prepared by adding 50 μl of rifampin working solutions to 0.5 ml of blank human plasma. Clinical specimens and controls were prepared by aliquoting 0.5 ml of plasma into glass tubes. Calibration standards, clinical specimens and controls were processed by adding 25 μl of internal standard, and 100 μl of 0.1 *M* hydrochloric acid. The samples were mixed by vortexing briefly and centrifuged at approximately 630 *g* for 5 min before loading onto the Bond Elut columns.

Each C_2 Bond Elut extraction column was conditioned with 1 ml of methanol, followed by 1 ml of

water. The plasma mixture was loaded onto the column and the column was washed with 1 ml of water. The analytes were eluted with 1 ml of methanol into 10×75-mm tubes that contain 500 μl of 3 mg/ml ascorbic acid. Aliquots of 150 μl were injected onto the HPLC system.

2.6. Validation

Duplicate calibration curves were analyzed on each of three days. One reagent blank (water substituted for plasma), blank plasma, control zero (blank plasma spiked with internal standard) and triplicate controls at each concentration (0.15, 4, and 20 μg/ml of rifampin in plasma) were analyzed with each calibration curve. The calibration curves were obtained by weighted ($1/C^*C$) least-squares linear regression analysis of the peak height ratios of rifampin/internal standard versus the concentration of rifampin. The equations of the calibration curves were then used to calculate the concentration of rifampin in the samples and controls from their peak height ratios.

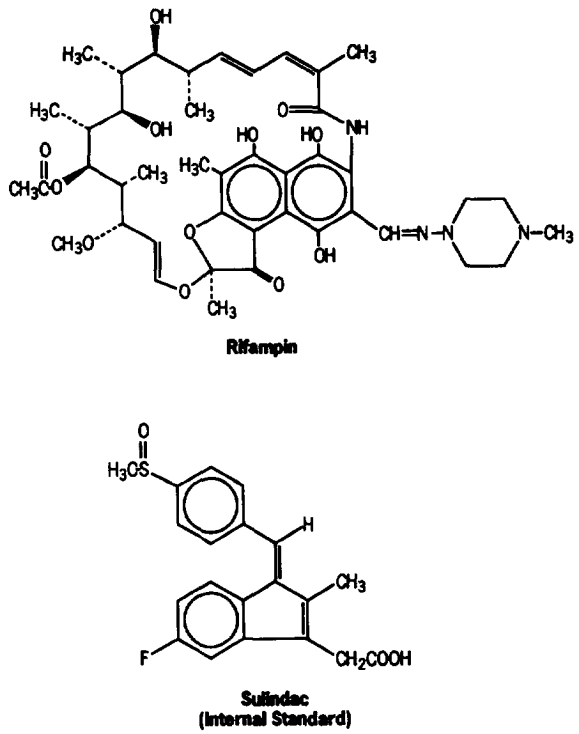

Fig. 1. Molecular structures of rifampin, sulindac (internal standard).

3. Results and discussion

3.1. Separation

The molecular structures of rifampin and sulindac (internal standard) are shown in Fig. 1. The two compounds were well separated from each other as shown in representative chromatograms (Fig. 2). The mean retention times of rifampin, and the internal standard were 4.4, and 7.8 min, respectively.

3.2. Specificity

Blank plasma from seven pools was tested for endogenous interferences. When commercial plasma was used, the internal standard region was clean for all of the lots tested; however, the rifampin region was not clean. The screening of the plasma donated by CHI employees was clean in both rifampin and internal standard regions.

An assay specificity check with AIDS drug candidates atevirdine and its metabolite (U-89,255), as well as of delavirdine and its metabolite (U-96,183) was also conducted. Controls at each of the three concentration were spiked to give 10 and 100 μM of these four compounds, and analyzed in duplicate. The retention times of atevirdine, U-89,255, delavirdine and U-96,183 are approximately 15.8, 6.2, 9.4 and 3.8 min, respectively. All four compounds were well separated from rifampin and the internal standard. The presence of these four compounds did not significantly affect the accuracy of the control results.

3.3. Linearity, precision, and accuracy

Calibration curve data and parameters for rifampin are in Table 1 and Table 2. Calibration curves for rifampin in plasma were linear over the concentration range of 50 to 35 000 ng/ml, with correlation coefficients (r) greater than 0.9979 for all curves.

Data from the spiked control samples are shown in

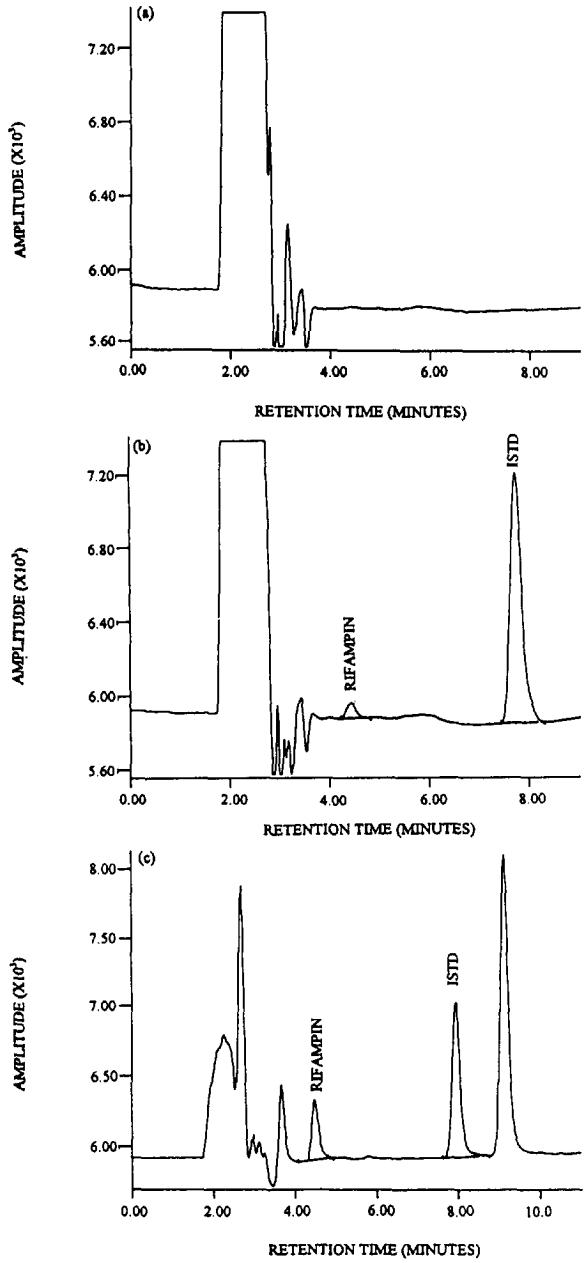

Fig. 2. Chromatograms of (a) blank human plasma, (b) 50 ng/ml calibration standard in human plasma, and (c) plasma from a patient taken 1 h after dosing with rifampin.

Table 3. The within-day precision of the method as measured by the relative standard deviation (R.S.D.) of the daily mean ($n=6$) was less than 6.1% at the three control concentrations in human plasma. The overall precision was 6.0%, 5.5%, and 3.3% R.S.D.

Table 1
Calibration curve parameters for rifampin in human plasma

Day	Curve	Slope	Intercept	r
1	1	$1.13\cdot10^{-3}$	$6.24\cdot10^{-3}$	0.9987
	2	$1.05\cdot10^{-3}$	$9.14\cdot10^{-3}$	0.9991
2	3	$1.10\cdot10^{-3}$	$1.13\cdot10^{-2}$	0.9985
	4	$1.06\cdot10^{-3}$	$5.16\cdot10^{-3}$	0.9979
3	5	$1.13\cdot10^{-3}$	$5.60\cdot10^{-4}$	0.9989
	6	$1.12\cdot10^{-3}$	$3.21\cdot10^{-3}$	0.9989

Results of validation study over three-day periods.

($n=18$) for the 150, 4000, and 20 000 ng/ml rifampin controls, respectively.

The accuracy of the method was determined by comparing the means of the measured concentrations with the nominal (theoretical) concentrations of rifampin in the plasma controls. All of the daily mean ($n=6$) and overall mean ($n=18$) values for the controls were within 7.5% of their expected values.

3.4. Limit of quantitation

The limit of quantitation (LOQ) was set at 50 ng/ml of rifampin in plasma. Six replicates of the lowest standard (50 ng/ml) were analyzed to evaluate the LOQ. At the LOQ, the R.S.D. ($n=6$) of the peak height ratios was 6.0%, the R.S.D. ($n=6$) of the measured concentrations was 7.5%, and the deviation of the mean ($n=6$) of the measured concentrations from their nominal value was 0.8%.

3.5. Absolute recoveries

Absolute recoveries were determined by comparing the peak heights of extracted calibration standards with the peak heights of recovery standards prepared by mixing 25 μl of internal standard working solution and 50 μl rifampin working solutions in 1 ml methanol and 0.5 ml of 3 mg/ml aqueous ascorbic acid mixture. The mean ($n=14$) recoveries for rifampin, and the internal standard were 97% and 93%, respectively.

3.6. Parallelism

An over-curve control pool containing 70 000 ng/ml rifampin was prepared and analyzed at the partial volumes of 100 and 200 μl. The mean ($n=6$) values

Table 2
Calibration curve data for rifampin in plasma

Calibration standard concentration (ng/ml)	Calculated concentration (mean±S.D., n=6) (ng/ml)	R.S.D. (%)	Deviation (%)
50	50.3±1.6	3.2	0.7
100	100±6.44	6.4	0.0
250	245±12.4	5.1	−2.2
1000	962±23.8	2.5	−3.8
2500	2420±41.8	1.7	−3.2
10000	10200±350	3.4	2.0
17500	18100±679	3.8	3.4
35000	36200±523	1.4	3.4

Results of validation study over a three-day period.

Table 3
Precision and accuracy of the assay for rifampin in plasma

Control concentration (ng/ml)	Calculated concentration (overall mean±S.D., n=18) (ng/ml)	R.S.D. (%)	Deviation (%)
150	156±9.31	6.0	3.7
4000	4070±223	5.5	1.8
20000	21200±690	3.3	6.0

Results of validation study over a three-day period with six determinations per day.

for both partial volumes were within 3.9% of their expected values. The precision was better than 5.8% R.S.D. (n=6) at both partial volumes.

In addition, the high control pool which contains 20 000 ng/ml of rifampin was prepared and analyzed at the partial volumes of 100 and 200 μl. The mean (n=6) values for both partial volumes were within 8% of their expected values. The precision was better than 3.7% R.S.D. (n=6) at both partial volumes.

3.7. Stability

Stock and working solutions of rifampin in methanol (containing 1 mg/ml ascorbic acid) were stable for at least six months when stored at −20°C and protected from light. Ascorbic acid was added to prevent air oxidation of rifampin. The stability of rifampin was determined by measuring the concentration changes in the control samples over time. The plasma controls stored in polypropylene at −70°C were stable for more than five months. Stability was tested by subjecting the plasma controls to two and three freeze/thaw cycles, and storage for 24 h at room temperature. The thawing and refreezing of controls, and the storage of controls at room temperature had little effect on the precision or accuracy of the results. The mean (n=3) value was within 11.5% of their expected values.

Process stability was tested by extracting two sets of calibration standards with triplicate controls. One

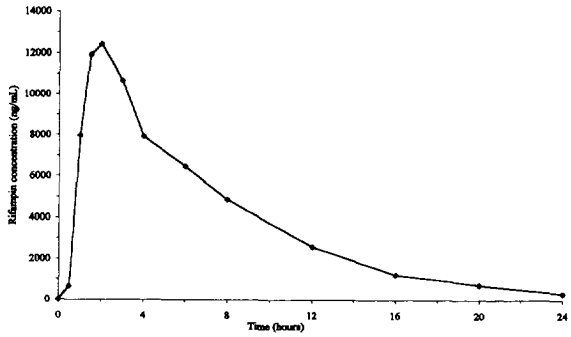

Fig. 3. Mean rifampin plasma pharmacokinetic profile from a clinical study.

set was stored overnight at room temperature, and the other at 5°C before analyzing. The storage of extracted samples at room temperature or 5°C had little effect on the accuracy of the control results. The mean value was within 3.1% of their expected values.

3.8. Application of the method

This new assay procedure is precise and accurate for the quantitation of rifampin in human plasma. The ruggedness of the assay has been demonstrated by applying the method to a clinical study in which subjects were dosed with rifampin. Fig. 3 presents the mean rifampin pharmacokinetic profit from the study.

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