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# Automated high-performance liquid chromatographic method for the determination of rifampicin in plasma

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ABSTRACT

Due to the unstable nature of rifampicin, a rapid automated high-performance liquid chromatographic method had to be developed for the analysis of a large number of plasma samples generated during a bioavailability trial. Extraction and injection of the samples were automatically done by a sample preparation system using $C_2$, 100 mg Bond Elut extraction columns. The extracts were chromatographed on a 4-$\mu$m reversed-phase $C_{18}$ column with a citrate buffer and acetonitrile as mobile phase. The analytes were detected at 342 nm. Calibration curves were linear to at least 20 $\mu$g/ml and the limit of quantification was 0.16 $\mu$g/ml.

## INTRODUCTION

Rifampicin,3-(4-methylpiperazin-1-yliminomethyl) rifamycin SV, a semisynthetic antibiotic drug, is widely used alone or in combination with other drugs such as isoniazid and pyrazinamide in the treatment of tuberculosis.

Rifampicin spontaneously oxidizes to a quinone derivative in atmospheric oxygen above pH 8. In aqueous solutions with lower pH values, rifampicin hydrolyses to 3-formylrifamycin SV and amino 4-methylpiperazine [1,2]. Rifampicin is extensively metabolized in the liver, especially during its first passage through the hepatoportal system, mainly to its active metabolite 25-desacetylrifampicin [3] and 3-formyl-25-desacetylrifampicin [4].

A number of microbiological [5,6], thin-layer chromatographic (TLC) [7] and high-performance liquid chromatographic (HPLC) [1,8–12] methods have been published for the determination of rifampicin and its metabolites in plasma and urine. These methods did not meet our requirements, since we needed a fully automated method to determine large numbers of samples generated during a bioavailability trial. Owing to the unstable nature of rifampicin we wanted to minimize the time between sample generation, extraction and chromatography of the samples.

This article describes a fully automated procedure for the quantitation of rifampicin in plasma using sulindac as internal standard.

## EXPERIMENTAL

### *Materials*

Rifampicin was obtained from Lennon (Port Elizabeth, South Africa) and sulindac from Adcock-Ingram (Johannesburg, South Africa). Ascorbic acid (BDH, Poole, UK) was used to protect rifampicin from oxidative degradation. All the other reagents were of guaranteed analytical grade, and were used as received. Water was purified by passing through a Millipore Milli-Q filtration system (18 m$\Omega$ cm resistivity) (Waters Assoc., Milford, MA, USA).

### *Analytical systems*

A modular HPLC system was used which consisted of a pump (Shimadzu LC-6A, Shimadzu, Kyoto, Japan), a Waters Radial pak, Nova Pak $C_{18}$, 4 $\mu$m particle size, 100 × 8 mm cartridge, held in an RCM 8 × 10 compression unit (Waters) protected by a Waters Guard Pak with a $\mu$Bondapak $C_{18}$ RCSS precolumn insert. A Shimadzu SPD-6A UV detector was used to measure the absorbance of the eluate. The chromatograms were recorded on a

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Spectra-Physics SP4290 integrator (Spectra-Physics, San Jose, CA, USA) and the data sent via a LABNET network to a Spectra-Physics ChromStation for automated data manipulation.

Sample processing and injections were done by an ASPEC system (automated sample preparation with extraction columns) from Gilson (Villiers Le Bel, France) utilizing Bond Elut (100 mg, $C_2$) extraction columns (Analytichem International).

*Chromatography*

The mobile phase consisted of acetonitrile–0.05 *M* sodium citrate buffer adjusted to pH 4.3 with 0.05 *M* hydrochloric acid (42:58) and pumped at a flow-rate of 2.3 ml/min at ambient temperature. The analytes were detected by a UV detector at 342 nm. Retention times for rifampicin and the internal standard sulindac were 4.65 and 3.07 min, respectively. A peak was found at 2.52 min, which was probably the 25-desacetylrifampicin metabolite.

*Sample preparation*

*Plasma standards.* An accurately weighed amount of rifampicin was dissolved in methanol and an aliquot immediately spiked into drug-free plasma to obtain a stock solution containing approximately 22 μg of rifampicin per millilitre of plasma. Subsequent dilutions with plasma were made to obtain adequate standards covering the expected range. All plasma samples contained 200 μg of ascorbic acid per millilitre as an antioxidant. Plasma standards were prepared freshly each week, divided into aliquots and stored in polypropylene tubes at −80°C.

The internal standard sulindac was prepared freshly each week in methanol to obtain an approximate concentration of 19 μg/ml.

*Samples from trial subjects.* Blood (10 ml) was sampled at predetermined intervals in heparinized Venoject tubes from twenty healthy male volunteers after receiving 450 mg of rifampicin in a double-blind, randomized, crossover design in a comparative bioavailability study. The samples were immediately centrifuged for 5 min at 4°C, and 2-ml plasma aliquots, to which 400 μg of ascorbic acid were added, were immediately frozen in solid carbon dioxide. The samples were stored at −80°C until analysed. All the samples were analysed within 4 days after sampling.

*Procedure.* Plasma samples (0.5 ml) were transferred to 5-ml glass tubes and mixed with 0.5 ml of 0.1 *M* hydrochloric acid to which 50 μl of the internal standard solution were added. The samples were briefly vortexed to obtain thorough mixing and placed in the ASPEC system.

The apparatus was programmed to condition each Bond Elut extraction column with 1 ml of methanol, followed by 1 ml of 0.1 *M* hydrochloric acid just before use. The plasma mixture was loaded onto the column and the column washed afterwards with 1 ml of 0.1 *M* hydrochloric acid to get rid of excess protein and plasma components. The analytes were eluted with 400 μl of methanol-acetonitrile (3:2) into a clean tube, mixed by repeatedly aspirating/dispensing the fraction, and a 20-μl aliquot automatically injected onto the HPLC column.

## RESULTS AND DISCUSSION

Fig. 1 shows representative chromatograms obtained from plasma determinations and demonstrates the lack of interfering endogenous compounds in the blank plasmas.

Quantifications was achieved using the peak-height ratio of rifampicin to the internal standard. All calibration curves were shown to be linear over a wide concentration range with the curves almost passing through the origin. Calibration curves were linear to at least 20 μg/ml and had good correlation coefficients with eight different standard concentrations. The mean recovery for rifampicin was 98% and for sulindac 73%.

The precision and accuracy of the method are indicated in Table I and are expressed as the relative standard deviation (%) and bias (%) for replicates of eight different concentrations covering the expected range. These results were obtained during the analysis of the trial samples. Although good results were obtained at 0.08 μg/ml with a signal-to-noise ratio of 9 during the validation of the method, the limit of quantification was set after the trial to 0.16 μg/ml (fifteen times base value) owing to the larger than expected coefficient of variation experienced in the day-to-day analysis of the low concentrations.

The extraction and chromatography procedures are fully automated and take only 9.5 min from in-

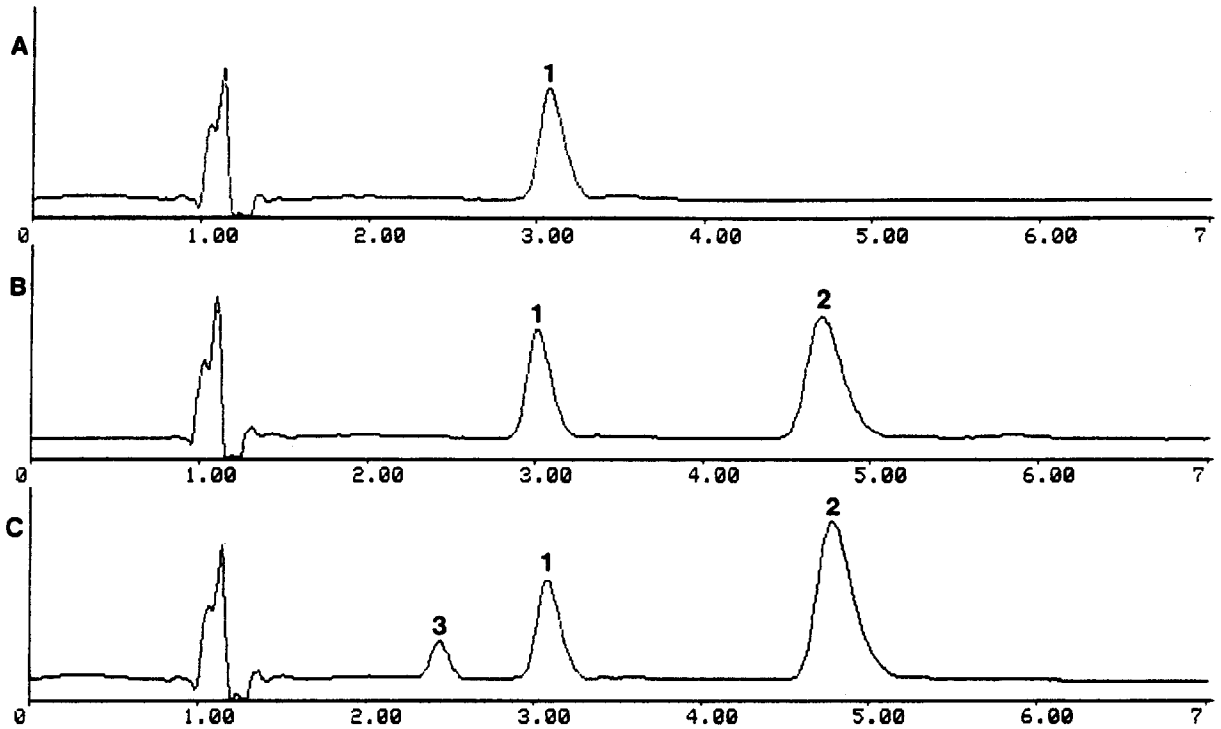


Fig. 1. Chromatograms: (A) blank plasma containing internal standard; (B) plasma standard of 5.2 μg/ml; (C) trial plasma sample equal to 7.2 μg/ml. Peaks: 1 = sulindac (internal standard); 2 = rifampicin; 3 = rifampicin metabolite. For HPLC conditions, see text.

jection of a sample to extraction and injection of a second sample. Minimum sample handling is required, and determinations can be done 24 h a day, as was done during the determination of the plasma samples during the trial. Fig. 2 represents a concentration–time profile of the mean plasma rifampicin values of twenty healthy volunteers after receiving an oral dose of 450 mg of rifampicin.

TABLE I

RELATIVE STANDARD DEVIATIONS (R.S.D.) AND BIAS OF QUALITY CONTROLS OBTAINED DURING THE TRIAL TO INDICATE THE PRECISION AND ACCURACY OF THE METHOD

| *n* | Rifampicin concentration (μg/ml) | Accuracy (bias, %) | Precision (R.S.D., %) |
|---|---|---|---|
| 6 | 22.19 | 3.1 | 3.6 |
| 7 | 12.06 | 3.6 | 4.0 |
| 6 | 5.80 | 2.4 | 2.5 |
| 7 | 2.93 | −3.2 | 4.7 |
| 5 | 1.40 | −5.4 | 3.3 |
| 7 | 0.69 | −5.0 | 3.8 |
| 6 | 0.32 | −8.9 | 5.9 |
| 6 | 0.17 | −2.5 | 5.0 |

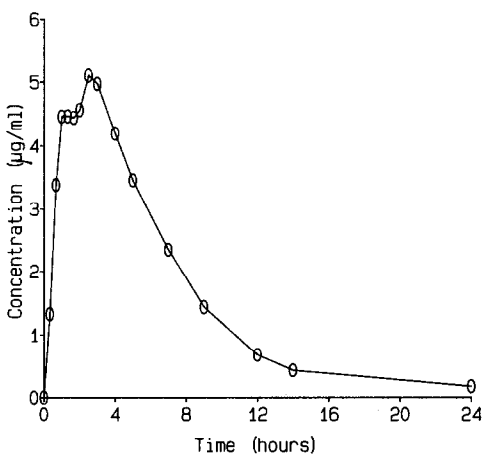


Fig. 2. Concentration–time profile of the mean rifampicin plasma values of twenty volunteers after each receiving a 450-mg rifampicin dose.

Since this method was used only during a study of comparative bioavailability, other substances were not tested for interference with rifampicin. However, no interference from plasma substances was found during chromatography.

Since we were perturbed about the stability of rifampicin when stored in plasma for some time, we checked the stability of rifampicin when plasma samples were stored at −80°C for 1 month. To these samples were added 200 μg of ascorbic acid per millilitre of plasma, and weekly determinations were carried out. No decrease in the rifampicin content was observed over this time. Degradation of rifampicin in organic solvents such as methanol occurs quite rapidly, so that these solutions should be kept for the shortest time possible.

The data from the integrators were sent to a ChromStation via a LABNET communication system. Calibration curves were automatically constructed from the eight standards processed with each batch using software developed in our laboratory. These standards and seven quality controls were scattered between the 64 samples of each batch to exclude possible variations due to degradation of rifampicin. No degradation peaks could be found in the standards or quality controls over this period. Calculations of the quality controls and trial samples were done from a linear calibration curve. Wherever deviations from linearity occurred, which is not unusual at low concentrations, a calibration curve including only the four lowest standards was constructed using the appropriate regression equation (usually a second order or power curve).

## CONCLUSION

The procedure provided us with a method that was sensitive enough to determine concentrations of rifampicin in low plasma volumes for 24 h after a single 450-mg oral dose. Determinations could be done 24 h a day since the extraction, injection, chromatography and data manipulation steps were completely automated.

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