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Note

Quantitative assay of rifampicin and its main metabolite 25-desacetylrifampicin in human plasma by reversed-phase high-performance liquid chromatography

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The microbiological assay of rifampicin does not allow separate determination of the drug and its main metabolite, 25-desacetylrifampicin [1] in biological fluids. Methods able to measure both substances are essential when information about the metabolism of the antibiotic is needed. A thin-layer chromatographic method for the simultaneous assay of rifampicin and some of its transformation products has been developed, but it was applied to urine samples only, in which the amounts to be measured are relatively large [2]. A more sensitive method, which uses high-performance liquid chromatography (HPLC) on silica columns, was recently set up for the analysis of rifampicin and 25-desacetylrifampicin in plasma, down to 0.1 μg/ml [3].

Another recent method based on the same technique for the quantitative assay of rifampicin, 25-desacetylrifampicin, 3-formylrifamycin SV, and 3-formyl-25-desacetylrifamycin SV in human plasma, urine and saliva, down to 0.1 μg/ml, has been reported [4].

Reversed-phase HPLC was recently used for the separation of rifampicin from its degradation products (among them, 25-desacetylrifampicin and 3-formylrifamycin SV) in pharmaceutical formulations [5]. The present method uses the same technique, because reversed-phase columns are less affected by the humidity of the solvents than silica columns, can be more easily washed free of pollutant substances present in the biological fluid extracts and possess a longer life-span. This method allows the separation of rifampicin, 25-desacetylrifampicin, 3-formylrifamycin SV, 3-formyl-25-desacetylrifamycin SV, and N-desmethylrifampicin (see Figs. 1 and 2), but quantitative recovery studies were carried out only for the first two compounds, as they are present in the largest amounts in the plasma of patients treated with the drug. In fact there are negligible quantities in plasma extracts of 3-formylrifamycin SV and 3-formyl-25-desacetylrifamycin SV [6], minute

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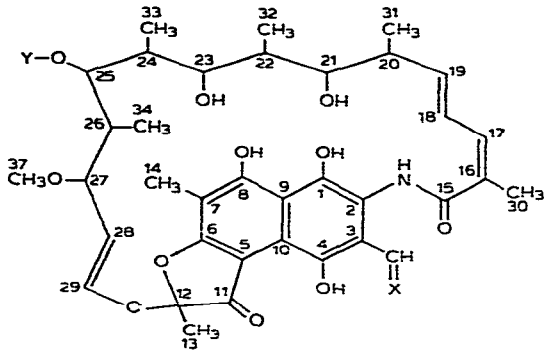


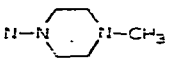
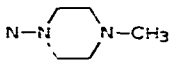
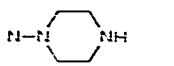
	X	Y
Rifampicin		CH_3CO
25-Desacetylrifampicin		H
3-Formylrifamycin SV	O	CH_3CO
25-Desacetyl-3-Formylrifamycin SV	O	H
N-Desmethylrifampicin		CH_3CO

Fig. 1. Structures of the compounds separated as shown in the chromatogram of Fig. 2. The carbon atom numbering system used here is conventional and does not follow the IUPAC recommendations.

amounts in bile and urine extracts [7], and N-desmethylrifampicin is found only in the urine [2].

EXPERIMENTAL

Materials

Rifampicin, 25-desacetylrifampicin, 3-formylrifamycin SV, 3-formyl-25-desacetylrifamycin SV, and N-desmethylrifampicin were Lepetit working standards of appropriate high purity. Butyl-*p*-hydroxybenzoate from Eastman-Kodak (Rochester, NY, U.S.A.) was used as internal standard. All the solvents and reagents were from Merck (Darmstadt, G.F.R.) or Carlo Erba (Milan, Italy), high-purity grade. The distilled water was filtered through the Millipore Mille-Q system. All glassware, previously washed, was cleaned by heating at 500°C for 6 h. The plasma blanks were obtained from healthy volunteers.

Standard solutions

Rifampicin, 25-desacetylrifampicin and the internal standard, butyl-*p*-hydroxybenzoate, were dissolved in acetonitrile—2-propanol (1:1, v/v) containing 0.5 mg/ml ascorbic acid to prevent oxidation of the compounds to be measured.

Extraction procedure

A 0.5-ml aliquot of heparinized plasma was pipetted into a screw-cap tube in which 10 μl of a solution of the internal standard in acetonitrile—2-propanol (1:1, v/v) had previously been placed. The amounts of the internal standard varied from 0.5 to 5 μg, depending on the expected amounts of the compounds to be determined. The sample was then diluted with 4.5 ml of 1 *M* KH_2PO_4, containing 1 mg/kg sodium ascorbate, and the pH adjusted to 4 with 1 *N* HCl. The diluted sample was extracted with 15 ml of ethyl acetate for 10 min at 300 inversions per minute on a Continental Alter 2864 shaker. After centrifugation at 2500 *g* for 10 min, 14 ml of the organic phase were transferred to a screw-cap tube. The organic phase was then taken to dryness under a stream of nitrogen at 37°C and the residue dissolved in 3.5 ml of 90% aqueous acetonitrile. After extraction with 3 ml of *n*-heptane, the sample was centrifuged and the *n*-heptane phase discarded. Then 3 ml of the acetonitrile phase were transferred to a conical tube and taken to dryness at 37°C under nitrogen. The residue was redissolved in 25—100 μl of acetonitrile—2-propanol (1:1, v/v) according to the expected content of the compounds, and introduced into the microvials of the HPLC automatic sampler.

Instrumentation

Two liquid chromatographs, Hewlett-Packard and Waters, were used during the standardization and application of the analytical method.

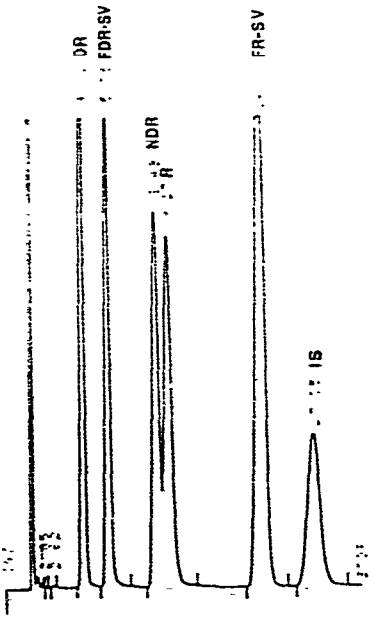


Fig. 2. Chromatogram of a mixture of 1 μg each of 25-desacetylrifampicin (DR), 3-formyl-25-desacetylrifamycin SV (FDR-SV), N-desmethylrifampicin (NDR), rifampicin (R), 3-formylrifamycin SV (FR-SV), and butyl-*p*-hydroxybenzoate (as internal standard, IS) dissolved in acetonitrile—2-propanol (1:1, v/v). The conditions are those described in the section Waters operating conditions.

Hewlett-Packard operating conditions. Instrument: H.P. Model 1084B equipped with a Model 79870A fixed-wavelength (254 nm) UV absorbance detector and a Model 79841A automatic sampler. Injection: 10 µl, containing about 50—250 ng of the compounds. Column: RP-8, 10 µm, 25 cm × 4.6 mm, Brownlee Labs. (Santa Clara, CA, U.S.A.). Elution: isocratic, 38% of B in A, where A = 0.1 *M* KH_2PO_4, pH 3.5 with 0.2 *M* H_3PO_4; B = acetonitrile. Flow-rate: 2 ml/min. Temperature: 30°C.

Waters operating conditions. Instrument: Waters Associates equipped with Model 660 solvent programmer, a Model 440 fixed-wavelength (254 nm) UV absorbance detector and a Model 710A sample programmer W.I.S.P. Injection, column, and flow-rate: see previous section. Elution: isocratic, 40% of B in A, where A = 0.1 *M* KH_2PO_4, pH 3.5 with 0.2 *M* H_3PO_4; B = acetonitrile containing 10% water. Temperature: ambient. Quantification: Hewlett-Packard Model 3380A computing reporting integrator.

RESULTS AND DISCUSSION

Known amounts of rifamycin and 25-desacetylrifamycin using standard solutions prepared as described in Experimental, were added to human plasma samples in order to have the same concentrations of each compound. Three concentration levels were established, based on the amounts of rifamycin and 25-desacetylrifamycin expected to be in the plasma: 0.5, 2, and

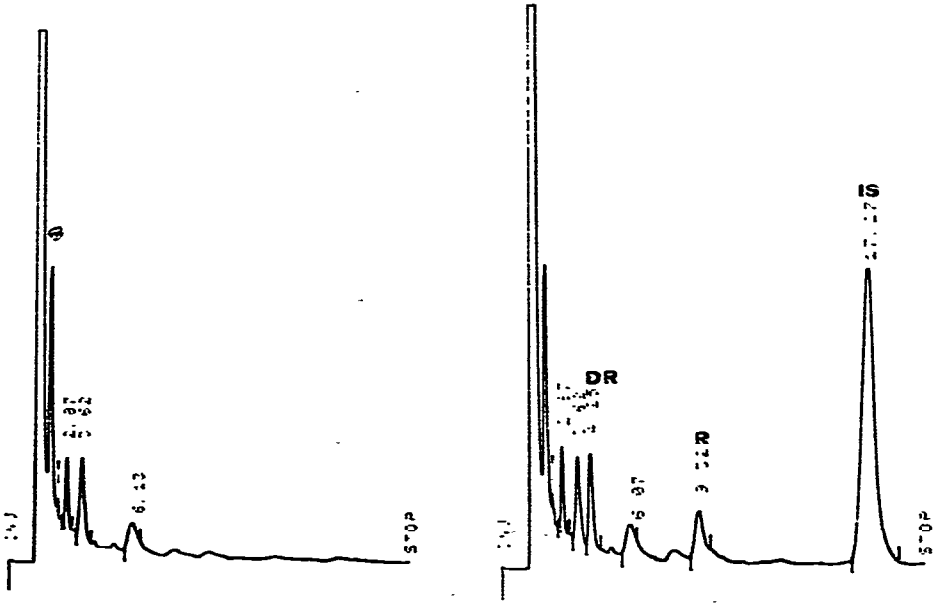


Fig. 3. Chromatogram of an extract of blank human plasma. The amount of extract injected is equivalent to 0.2 ml of plasma. (See Hewlett-Packard operating conditions and Results section.)

Fig. 4. Chromatogram of a sample prepared as described under Experimental. Amount of extract injected is equivalent to 0.2 ml of plasma with 25-desacetylrifampicin (DR, 0.5 µg/ml), rifampicin (R, 0.5 µg/ml), and internal standard (IS, 2 µg/ml) added. The conditions are described in the section Hewlett-Packard operating conditions.

TABLE I

RESULTS OF RECOVERY TRIALS

Different amounts of rifampicin (R) and 25-desacetylrifampicin (DR) were added to 0.5 ml of human plasma.

Added (μg)	Found (μg)				Average found (μg)	Standard deviation (S.D.)	Relative S.D.	Variance	Standard error	Average recovery (%)
R 0.236	0.191	0.186	0.212	0.192	0.195	0.011	5.875	$1.31 \cdot 10^{-4}$	0.006	82.6
DR 0.229	0.202	0.198	0.195	0.202	0.199	0.003	1.708	$1.15 \cdot 10^{-5}$	0.002	87.3
R 0.976	0.957	1.016	1.030	1.038	1.010	0.037	3.627	0.001	0.018	103.5
DR 0.910	0.851	0.894	0.905	0.895	0.886	0.024	2.710	0.001	0.012	100.0
R 4.95	5.00	4.84	4.87	4.90	4.903	0.069	1.417	0.005	0.035	99.0
DR 4.84	4.38	4.15	4.32	4.32	4.293	0.099	2.309	0.010	0.050	88.6

10 μg/ml. Four samples were prepared for each concentration level, extracted and analyzed as previously indicated under Experimental.

The chromatograms for two human plasmas are shown in Figs. 3 and 4.

The results of the recovery trials are listed in Table I. The precision of the measurements is good, even at the lowest concentrations, for both the compounds. In fact, the relative standard deviation for 25-desacetylrifamycin was always less than 3, while that of rifamycin was 5.87 at 0.47 μg/ml, and was less than 4 at the higher concentrations. The average recovery values were in the range of 80—90% for samples of about 0.5 μg/ml and in the range of 88—103% for the other samples. The linearity of the recovery was very good over the range of concentration 0.5—10 μg/ml, as demonstrated by the equations $y = -0.00196 + 0.99219x$ ($r^2 = 0.99936$) for rifamycin and $y = 0.03782 + 0.88051x$ ($r^2 = 0.99878$) for 25-desacetylrifamycin. The limit of detection was 0.2 μg/ml.

The method was used in the assay of rifamycin and 25-desacetylrifamycin in the plasma of healthy volunteers orally given a single dose of 600 mg per day. Fig. 5 shows the plasma concentrations of rifamycin and 25-desacetylrifamycin found in one subject during the first 12 h of treatment.

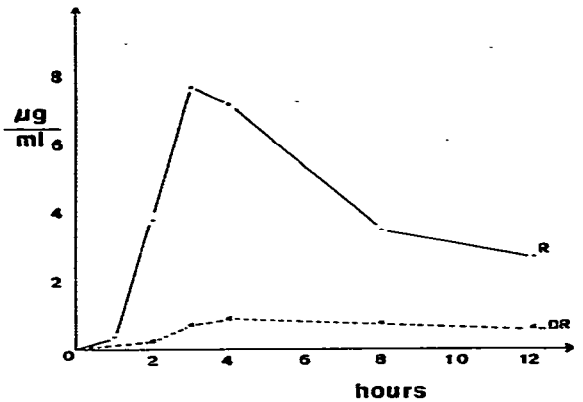


Fig. 5. Concentrations (μg/ml) of rifampicin (R) and 25-desacetylrifampicin (DR) in the plasma of one healthy volunteer after oral administration of 600 mg of rifampicin, during the first 12 h of treatment.

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