Journal of Chromatography, 151 (1978) 199–205

CHROM. 10,545

ANALYSIS OF RIFAMPICIN AND OF ITS HYDROGENATED DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received February 28th, 1977; revised manuscript received August 1st, 1977)

SUMMARY

A rapid and complete separation of rifampicin from its quinone was accomplished in 7 min using a filled column of the MicroPak NH_2 type and a chemically bonded phase on silica gel (10 μm). The eluent was chloroform–methanol (97:3) under isocratic conditions. The same columns and conditions were used for a complete separation of all of the reaction products of the hydrogenation of rifampicin, including the dihydro and tetrahydro derivatives. The plate heights were optimally 0.5–1.9 mm. The method is particularly useful for the rapid control of the reaction products of hydrogenation.

INTRODUCTION

Rifamycins were isolated from cultures of *Streptomyces mediterranei* n.sp. as metabolic products and many of the derivatives were shown to possess important therapeutical activities, such as the commonly used rifamycin SV. Similarly, rifampicin or 3-(4-methylpiperazinyl)iminomethylrifamycin SV has been applied successfully in the treatment of various diseases. Some of its physico-chemical properties were described in 1966 by Maggi *et al.*[1], who compared some of the analytical methods available and examined the stability of rifampicin in different media. However, the analysis of rifampicin by liquid chromatography has not been described so far, although Schmit *et al.*[2] analyzed rifamycin SV by high-performance liquid chromatography on a column with ODS Permaphase, employing gradient elution. The mobile phase used was water–methanol. Schmit *et al.*[3] also mentioned the separation of 3-formylrifampicin SV from some contaminants on a Zipax–polyamide column with *n*-hexane–ethanol as eluent.

When following the course of hydrogenation of rifampicin[4], we used high-performance liquid chromatography and found that the product is not a unique dihydro derivative, as one might have expected, but that the reaction course is much more complex.

EXPERIMENTAL

Apparatus

A Varian Model 4100 high-pressure liquid chromatograph (Varian Aerograph, Palo Alto, Calif., U.S.A.) was used. The detector was a Variscan UV spectrophotometer (Varian) with a variable wavelength (190–900 nm). The 10×1 mm cell had a volume of 8 μl. Samples were injected with a Hamilton Type 701 SN (10 μl) micro-syringe using the stop-flow technique. UV spectra were recorded with the Variscan spectrophotometer and with a Specord UV–Vis spectrophotometer (Zeiss, Jena, G.D.R.).

Columns

Stainless-steel columns (25 cm $\times$ 2 mm I.D.) were filled with MicroPak Si-10 and Micro-Pak CN with chemically bonded alkylnitrile groups on silica gel, and with MicroPak NH_2 with chemically bonded alkylamine groups on silica gel. The silica gel was always 10-μm LiChrosorb.

Chemicals

The samples of rifampicin were obtained by purification of its drug-form Rifadin, produced by UMB Drugs (Bucharest, Rumania) under licence from Lepetit (Milan, Italy). The purification was described in detail by Hanuš *et al.*[5]. The hydrogenated derivatives were prepared in this laboratory[5]. The quinone form of rifampicin was obtained by preparative isolation on the silica gel column and its identity was checked by NMR spectroscopy.

The solvents used were methanol for UV spectroscopy (Lachema, Brno, Czechoslovakia) and analytically pure chloroform (Lachema), which were further purified on an activated silica gel column in the usual way.

Chromatographic analysis

Samples of rifampicin or its derivatives were dissolved in chloroform to a concentration of 200–400 μg/ml. The column was injected with 2–10-μl samples.

The mobile phase used was chloroform–methanol in various proportions and it was de-gassed before use. The elution flow-rates were 0.2–0.7 ml/min at pressures of 1.36–3.40 MPa.

Rifampicin and its derivatives were detected in the Variscan spectrophotometer at 334 nm.

Capacity factors (k') were calculated from $k' = (t_R - t_0)t_0$ where t_R is the retention time of the sample and t_0 the retention time of a non-retained compound (*n*-hexane in this work). The theoretical plate height (H) was calculated from the elution chromatogram according to $H = (L/16)\,(w_t/t_R)^2$ where L is the column length and w_t the peak width at the baseline.

RESULTS AND DISCUSSION

Analysis of rifampicin

Before analysis of the samples we removed the various additives, such as starch and magnesium stearate, which adversely affect the analytical results, by

extraction with diethyl ether and passage through a preparative column containing activated silica gel.

An adsorption column containing MicroPak Si-10 and columns filled with silica gel with a chemically bonded phase of the type MicroPak NH_2 and MicroPak CN were used for the analysis, MicroPak NH_2 being the most suitable. When using chloroform–methanol (97:3) as the mobile phase under isocratic conditions, the substance proper was preceded by a contaminant which was identified as a quinone form of rifampicin. The separation of rifampicin from its quinone is illustrated in Fig. 1.

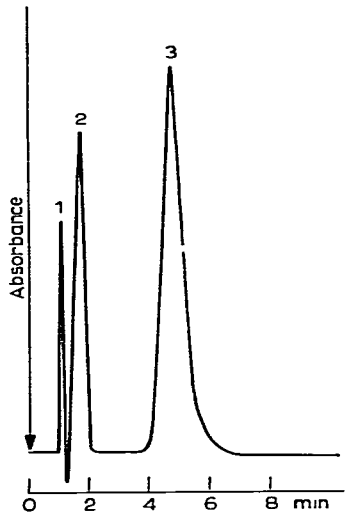

Fig. 1. Chromatogram of a mixture of rifampicin and its quinone form. MicroPak NH_2 (10 μm) 25 cm × 0.2 cm column, elution with chloroform–methanol (97:3), flow-rate 0.7 ml/min. Peaks: 1, *n*-hexane; 2, rifampicin quinone; 3, rifampicin.

In a silica gel adsorption column, rifampicin was separated from the quinone only on using a strongly polar eluent with a high methanol content. The chromatograms obtained were poorly reproducible and the efficiency of the column decreased rapidly. The MicroPak CN column possessed more suitable properties but the separation of the two compounds was not complete.

The contaminant was determined using a standard of rifampicin quinone and UV spectra. These were recorded in the Variscan spectrophotometer in an aqueous medium (pH 7.38) and the results were compared with published values. The spectra of the two substances are shown in Fig. 2.

Table I shows the retention times, capacity factors and the values of measured and reported absorption peaks.

The quinone form of rifampicin found as an impurity accompanying the starting preparation is formed readily by oxidation with atmospheric oxygen and is detected by a purple colour of the solution. The amount of quinone in rifampicin is hence dependent on the conditions and length of storage. In addition to these factors,

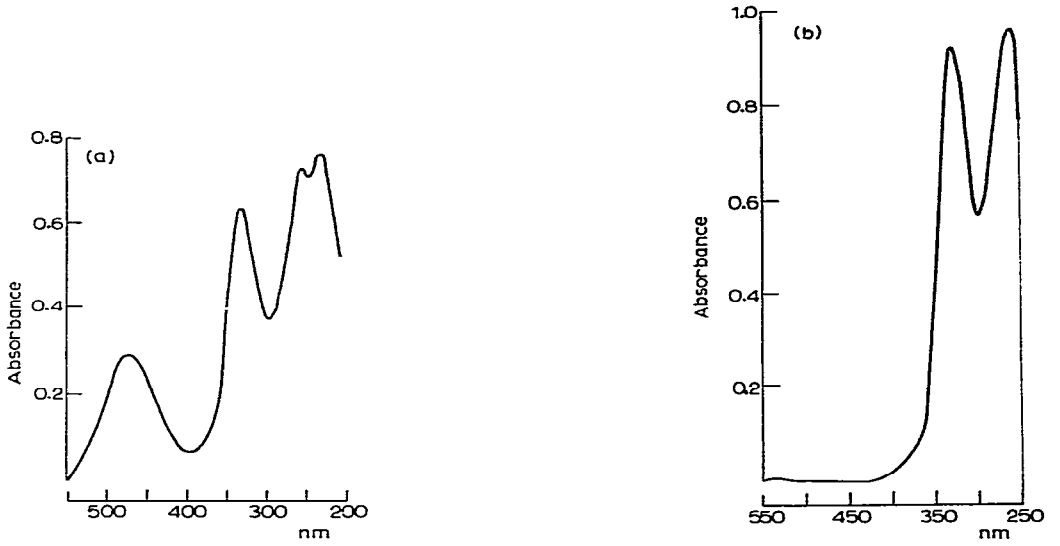

Fig. 2. UV spectrum of rifampicin (a) and its quinone (b) measured in a phosphate buffer of pH 7.38 in a Variscan spectrophotometer.

TABLE I

RETENTION TIMES (t_R), CAPACITY FACTORS (k') AND WAVELENGTHS OF MAXIMUM ABSORPTION ($\lambda_{max.}$) OF RIFAMPICIN AND ITS QUINONE

Compound	t_R *(min)* *	k' **	*Wavelength of maximum absorption (nm)* **	
			Measured values	*Published values*[1]
Rifampicin	4.7	3.7	235, 255, 333, 474	237, 255, 334, 475
Quinone	1.6	0.6	259, 332, 539	260, 332, 540

* Analyzed in a 25 × 0.2 cm column, MicroPak NH_2; elution with chloroform–methanol (97:3) at a flow-rate of 0.7 ml/min.

** Measured in a medium of pH 7.38.

the presence of the quinone form is affected by the preliminary purification procedures, whereupon its content decreases. The formulae of rifampicin and its quinone are shown in Fig. 3.

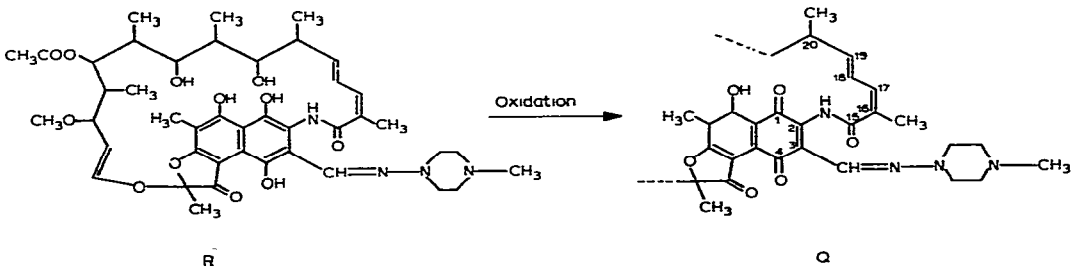

Fig. 3. Structural formulae of rifampicin (R) and its quinone (Q).

Analysis of hydrogenated derivatives of rifampicin

Rifampicin was hydrogenated at low and medium pressure on palladium(II) oxide. In following the course of hydrogenation and checking the purity of the reaction products, the best results were obtained with the MicroPak NH_2 column and chloroform–methanol (97:3) as the mobile phase. Depending on the reaction conditions, the products were hydrogenated at one or two double bonds.

Liquid chromatography showed that in all instances a mixture of four hydrogenated derivatives was formed, eluted from the column as peaks C, D, E en F. The proportions of these compounds differed, depending on the conditions and period of hydrogenation. The overall course of hydrogenation is illustrated in Fig. 4.

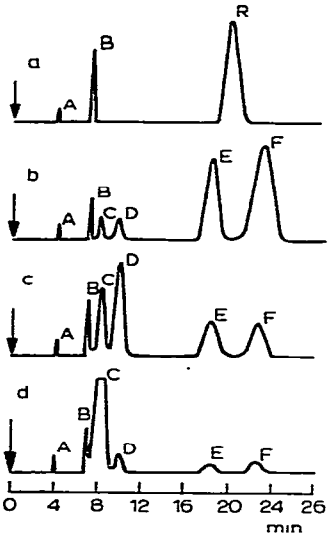

Fig. 4. Course of hydrogenation of rifampicin shown by liquid chromatography. (a) Rifampicin (R) and its quinone form (B). (b) Reaction products after first-degree hydrogenation. A, Solvent; B, quinone form; C, D, E and F, hydrogenated derivatives. (c) Reaction products after second-degree hydrogenation. Peaks as in (b). (d) Reaction products after total hydrogenation. Peaks as in (b).

After hydrogenation to the first degree the reaction products were mainly peaks E and F, fully separated, plus the minor peaks C and D and peaks of solvent A and the quinone form B (Fig. 4b). After hydrogenation to the second degree a product was obtained with major peaks C and D, minor peaks E and F and again peaks of the solvent A and the quinone B (Fig. 4c). After a prolonged total hydrogenation the final product was still a mixture of the substances with predominating peak C (Fig. 4d).

The individual eluates C, D, E and F were isolated and collected by the stop-flow technique and their individual UV spectra were recorded. Comparison of the spectra showed full agreement in the positions of all absorption peaks with the four derivatives. The spectra coincided with the spectrum of pure rifampicin (Fig. 5).

The analogies in the spectral analysis indicate that we are dealing with derivatives in which none of the principal conjugations were disturbed, *i.e.*, the naphthalene

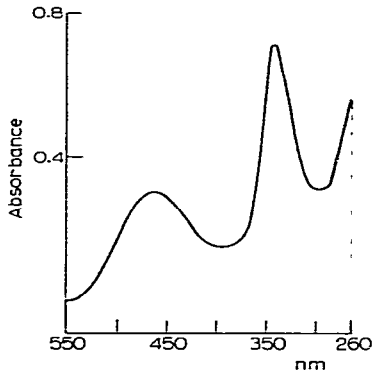

Fig. 5. UV spectrum of the four elution peaks C, D, E and F formed on hydrogenation of rifampicin, measured in ethanol in a Specord UV–VIS spectrophotometer. a, Starting rifampicin; b, hydrogenated derivatives C, D, E and F; c, quinone form of the hydrogenated derivatives.

skeleton was preserved, together with the conjugated C=N bond (see structural formulae in Fig. 3).

The degree of separation of the hydrogenated derivatives of rifampicin was found to depend on the proportion of methanol in the chloroform–methanol mobile phase. With less than 2% (v/v) of methanol, the compounds were not completely eluted from the column and the peaks were broad and tailed. At concentrations higher than 4.5% (v/v), peaks E and F were not separated, while at concentrations above 6% (v/v), even peaks C and D could not be resolved. The optimum concentration of methanol was 2–3% (v/v). Fig. 6 shows that the separation of eluates E and F is much more affected by the methanol concentration than is the separation of derivatives C and D.

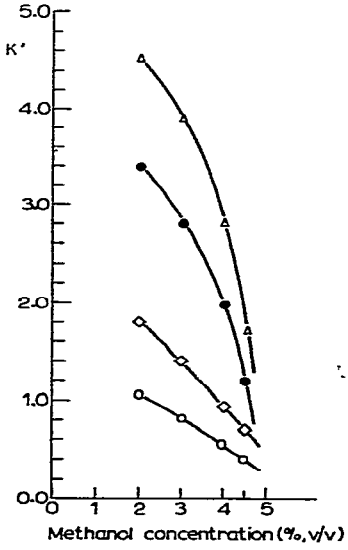

Fig. 6. Effect of methanol concentration in the mobile phase on the separation of hydrogenated derivatives of rifampicin.

TABLE II

DEPENDENCE OF RETENTION TIMES (t_R) AND CAPACITY FACTORS (k') ON CONCENTRATION OF METHANOL IN MOBILE PHASE

Analysis in a 25 × 0.2 cm column; MicroPak NH_2 (10 μm); elution with chloroform–methanol, flow-rate of 0.7 ml/min.

Compound	*Methanol concentration (%, v/v)*							
	2		*3*		*4*		*4.5*	
	t_R *(min)*	k'	t_R *(min)*	k'	t_R *(min)*	k'	t_R *(min)*	k'
B	—	—	1.4	0.4	—	—	1.1	0.1
C	2.05	1.05	1.8	0.8	1.55	0.55	1.4	0.4
D	2.8	1.8	2.4	1.4	1.9	0.9	1.7	0.7
E	4.4	3.4	3.8	2.8	3.0	2.0	2.3	1.2
F	5.5	4.5	4.9	3.9	3.8	2.8	2.8	1.7

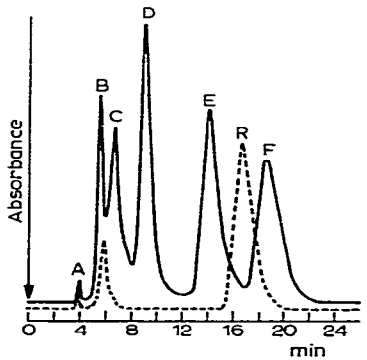

Fig. 7. Chromatograms of the hydrogenation products of rifampicin (solid line) and of rifampicin and its quinone (broken line). Peaks as in Fig. 4(b).

The retention times and the capacity factors of all of the hydrogenated products are summarized in Table II.

The starting preparation of rifampicin could not be separated in any of the systems tested from the hydrogenated forms E and F. The chromatogram in Fig. 7 shows the position of the two peaks E and F and that of rifampicin, lying between them.

The optimal efficiency of the MicroPak NH_2 column expressed as the theoretical plate height was 0.5–1.9 mm. After about 20 days, the efficiency of the column decreased.

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