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Note

Identification and quantitation of rifamycins by reversed-phase high-performance liquid chromatography

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Tuberculosis and leprosy are among the major communicable diseases in the developing countries. It is estimated that every year about 10 million people develop tuberculosis and there are about 12 million people suffering from leprosy[1]. In the chemotherapy of both these diseases one very successful drug is rifampicin[2–5]. Rifampicin is a semisynthetic derivative of the microbial metabolites belonging to the ansamycin group of antibiotics, the rifamycins[6]. Among the new drugs being explored for a better treatment of mycobacterial infections, rifamycin derivatives have great potential[7].

Microbial production of these rifamycins yields a mixture of many closely related members, among which the rifamycins B, O, S and SV are of commercial importance. Although some methods of identification[6,8–10] and quantitation[11] exist, none really offers complete analysis. Using high-performance liquid chromatography (HPLC) a method which effectively separates and quantitates these rifamycins (Fig. 1) has been achieved.

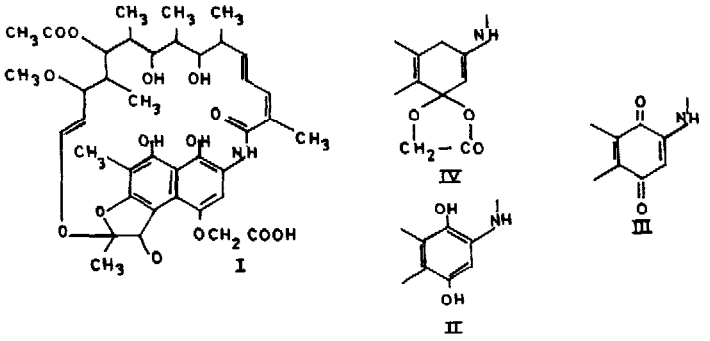

Fig. 1. Structures of rifamycin: I = B; II = SV; III = S and IV = O.

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EXPERIMENTAL

Reagents and chemicals

The rifamycin standards B, S and SV were obtained by courtesy of Professor J. Nuesch (Ciba Geigy, Basle, Switzerland) and rifamycin O from Dr. Egidio Marchi (Alfarecerche, Rome, Italy). *Nocardia mediterranei* (ATCC 21271) was obtained from the American Type Culture Collection, U.S.A. All reagents and chemicals used were of analytical grade.

Chromatographic conditions

The chromatographic apparatus consisted of a Beckman 342 dual-pump HPLC system equipped with a Model 165 detector set at 254 nm. The detector signals were recorded on a Shimadzu (Kyoto, Japan) CR3A integrator. The rifamycins were separated on a reversed-phase, Altex Ultrasphere ODS column (particle size 5 μm; 25 cm $\times$ 4.6 mm). A rotary injection valve with a 20-μl injection loop was used and the column temperature was ambient. Mobile phase: solvent A was 0.05 *M* ammonium formate in deionized water adjusted to pH 7.2 by addition of 0.1 *M* NaOH; solvent B was HPLC grade methanol (LKB Biochrom, Cambridge, U.K.). Both solvents were passed through a 0.2-μm Millipore filter and degassed before use. The solvent programme was a 10-min linear gradient from 50 to 75% solvent B in A, and then held at 75% B for 15 min. At the end of each experiment the initial conditions were reestablished and the column was equilibrated for 10 min. The flow-rate was 1.0 ml/min in all experiments.

Standards and sample preparation

Standard solutions of the rifamycins B, O, S and SV were prepared separately in ethyl acetate. Each rifamycin component was chromatographed individually in triplicate to determine the exact retention time. The components were later mixed in appropriate proportion and subsequently subjected to HPLC with varying parameters till all the four rifamycins were clearly resolved. For the identification of the rifamycins produced by *Nocardia mediterranei* ATCC 21271[12,13] a 5-ml aliquot of the beer was withdrawn from the fermenter and acidified to pH 4.5 with 5 *M* sulphuric acid. After acidification, 1 ml of ethyl acetate was added and then shaken vigorously on a vortex mixer for 1 min. It was then centrifuged at 1000 *g* for 5 min and an aliquot of the ethyl acetate extract analysed.

RESULTS AND DISCUSSION

To determine the optimum conditions for the separation of the rifamycins, an isocratic methanol–0.05 *M* ammonium formate (60:40, v/v) system was first tried. This system took about 50 min to give well separated rifamycin components, with the rifamycin O peak tending to tail. In order to shorten the elution time, a gradient system was devised (as described in Experimental). This gave a distinct separation in 20 min. Fig. 2a shows a typical chromatogram of a mixture (200 ng each) of the four different rifamycins B, SV, S and O clearly resolved on the Ultrasphere ODS column. Having established the separation method, the ethyl acetate extract from a fermentation broth of *Nocardia mediterranei* ATCC 21271, a known producer of rifamycin

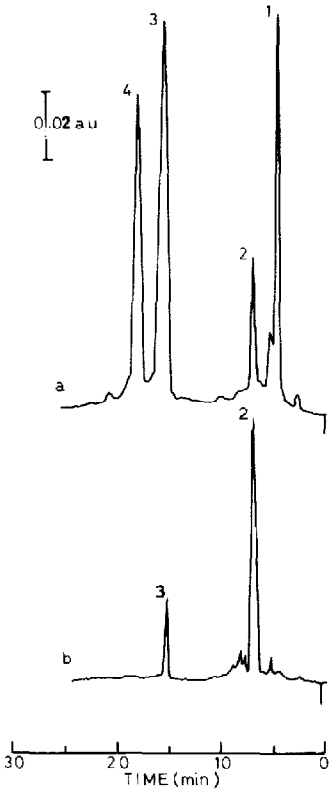

Fig. 2. HPLC of (a) 200 ng each of standard rifamycins (peaks: I = B; II = SV; III = S; IV = O) and (b) of an ethyl acetate extract from *N. mediterranei* fermentation broth (peaks: II = SV; III = S).

SV[12], was injected. The results presented in Fig. 2b clearly show that the broth which had a substantial amount of rifamycin SV (1.6 mg/ml) also had a significant amount (0.2 mg/ml) of rifamycin S. To estimate the recovery and precision of the method, five replicate injections of control broth spiked with 200 ng/ml of each of the rifamycins

TABLE I

ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF RIFAMYCINS FROM SPIKED CONTROL BROTH

Each value represents the mean ± C.V. (%) of five determinations per concentration.

Rifamycin	*Concentration spiked (ng/ml)*		
	200	*400*	*800*
B	198.2 ± 4.5	401.0 ± 0.8	800.6 ± 0.8
SV	196.7 ± 5.0	393 ± 1.1	792 ± 0.4
S	192.7 ± 0.7	391 ± 1.3	791.8 ± 0.3
O	200.3 ± 2.1	401.5 ± 1.8	804.2 ± 1.0

were made. Recovery values of 99, 98, 96 and 100% for rifamycin B, SV, S and O were obtained. These values were fairly constant over a wide range of concentration. The coefficient of variation (C.V.) was less than 5% for both inter and intra assay for all the rifamycins spiked at a concentration of 200 ng/ml (Table I).

Calibration graphs generated in the concentration range 50–2000 ng showed good linearity with a correlation coefficient of 0.993 for rifamycin B, 0.988 for rifamycin SV, 0.998 for rifamycin S and 0.999 for rifamycin O. Based on a signal-to-noise ratio of more than 3, the limit of detection is *ca.* 20 ng of each rifamycin in an injection volume of 20 μl.

The method presented here will be of use in the accurate analysis of fermentation broths and would not only reveal the quantity but also the presence of other rifamycins produced at a particular stage. It can also be adapted to study other intermediates of the biosynthetic pathway of the rifamycins.

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