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### Note

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**Quantitative assay of rifampicin and three of its metabolites in human plasma, urine and saliva by high-performance liquid chromatography**

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Rifampicin (R) is partly metabolised in man, its principal metabolite being 25-desacetyl-rifampicin (DR) [1]. It was recently reported [2] that rifampicin could also give sizable amounts of 3-formylrifamycin SV (FR), while 3-formyl-25-desacetyl-rifamycin SV (FDR) and N-desmethylrifampicin could be expected in low concentrations. A quinone metabolite has also been suspected, but not detected because it would have been reduced in the presence of the ascorbic acid required to protect rifampicin from air oxidation.

The microbiological assay of rifampicin and most of the physicochemical procedures described [1, 3-5] do not permit the separate determination of R and its metabolites. A high-performance liquid chromatography (HPLC) technique for the quantitative analysis of R and DR down to 0.1 µg/ml of plasma has been reported [6], but its specificity as regards FR and FDR has not been demonstrated.

This paper describes a simple and sensitive HPLC assay procedure for the simultaneous and specific determination of R, FR, DR and FDR in biological fluids.

## EXPERIMENTAL

### *Materials*

R, FR, DR and FDR were supplied by Ciba-Geigy, (Basle, Switzerland). The solvents and reagents used were all of analytical grade: dichloromethane

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(E. Merck, Darmstadt, G.F.R., Cat. No. 6050), isooctane (Merck, Cat. No. 4727), ethanol (Prolabo, Paris, France, Cat. No. 20821).

The buffer solution used consisted of 2 g ascorbic acid (Merck, Cat. No. 127) and 10 g anhydrous sodium sulphate (Merck, Cat. No. 6649) made up to 50 ml with concentrated buffer solution pH 6 (Merck, Cat. No. 9886) and continuously mixed in a 50-ml volumetric flask. Fresh batches were prepared each day.

### *Instruments*

Chromatography was performed on a Model 1010 high-performance liquid chromatograph (Hewlett-Packard), equipped with a U6K valve injector (Waters Assoc.) and a Model 1036 A fixed-wavelength (254 nm) UV absorbance detector (Hewlett-Packard). The detector was connected to a CRS 204 electronic integrator (Infotronics) and to a 3012 potentiometric recorder (W+W, Kontron).

### *Column*

The chromatographic column was a stainless-steel tube (10 cm X 7.5 mm I.D.) filled with LiChrosorb Si 60 (5  $\mu$ m particles) (Merck) by the slurry technique [7, 8]: 3 g of LiChrosorb were suspended in 15 ml of the mobile phase and the slurry was forced into the column under pressure. The flow-rate must be high enough to give a pressure around 150 bars at the end of filling. The column is ready for use as soon as filled. Its efficiency is 2000–3000 theoretical plates for the DR peak.

### *Assay procedure*

To 1 ml of plasma, urine or saliva in a 5-ml glass centrifuge tube are added 50  $\mu$ l of methanol, 1 ml of buffer solution and 1 ml of isooctane–dichloromethane (3:2, v/v). The tube is stoppered and mechanically shaken for 10 min at 350 rpm with an Infors shaker, then centrifuged for 5 min at 2000 g. A 100- $\mu$ l portion (5  $\mu$ l when the concentration of R is higher than 20  $\mu$ g/ml and 200  $\mu$ l when it is lower than 0.1  $\mu$ g/ml) of the supernatant organic phase is injected into the chromatographic column.

The mobile phase, dichloromethane–isooctane–ethanol–water–acetic acid (36.6:45:16.8:1.65:0.002), is pumped at a constant flow-rate of about 3 ml/min under a pressure of about 40 bars at room temperature.

Every sample is analysed in duplicate, and one aliquot of each assay is chromatographed. If the peak areas of the compounds are not within  $\pm 5\%$ , a third analysis is performed.

### *Calibration*

Calibration samples are prepared by measuring 100  $\mu$ l of convenient R, FR, DR and FDR dichloromethane solutions into 10-ml tubes. Dichloromethane is evaporated under nitrogen, the compounds are redissolved in 50  $\mu$ l of methanol and 1 ml of plasma, urine or saliva is added. Three to four samples containing 0.2–20  $\mu$ g of the compounds are prepared. The calibration graphs (peak area against concentration on a log–log graph) are straight lines. The complete calibration is repeated every day.

## RESULTS AND DISCUSSION

### Specificity

Fig. 1A shows that R is clearly separated from its three metabolites. Under the prescribed conditions, some batches of LiChrosorb may result in a poor separation of either R and FR or FR and DR. In such cases, a good separation is achieved by increasing or decreasing the dichloromethane content of the mobile phase by 1–2%: increasing the dichloromethane content lengthens the retention times of FR and FDR. Plasma components do not interfere (Fig. 1B and C). The same is true of urine and saliva.

### Reproducibility and accuracy

Various spiked human plasma, urine and saliva solutions were analysed repeatedly. Table I shows that the proposed procedure permits the quantitative assay of R, FR, DR and FDR down to 0.1  $\mu\text{g/ml}$ ; if a higher sensitivity is needed, it is possible to inject a larger volume of extract (200–300  $\mu\text{l}$ ). The absolute detection limit is 1 ng R per injection when the peak height is about five times the background noise.

Table II demonstrates the good reproducibility of the chromatographic step. It is to be noted that the coefficient of variation calculated from the ratio (peak area of R/peak area of DR) is not better than when it is obtained from either the R or DR peak area. This shows that an internal standard would not improve accuracy. The yields ( $\pm$  S.D.) of extraction from plasma or urine are  $96 \pm 5\%$  for R,  $86 \pm 6\%$  for FR,  $72 \pm 6\%$  for DR and  $75 \pm 6\%$  for FDR.

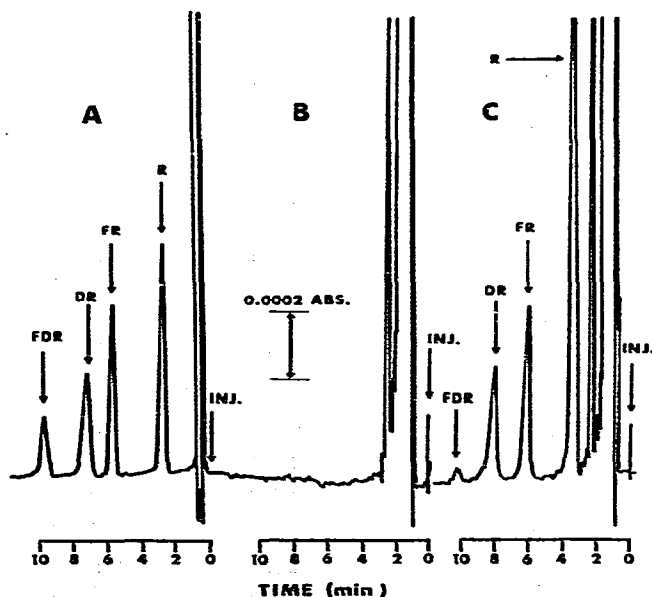


Fig. 1. (A). Chromatography of a synthetic mixture of rifampicin and three of its metabolites. R, Rifampicin; FR, 3-formylrifamycin SV; DR, 25-desacetylrifampicin; FDR, 3-formyl-25-desacetylrifamycin SV. (B). Chromatography of a blank plasma extract (C). Chromatography of a plasma extract of a subject given an oral dose of rifampicin. R, found: 1.45  $\mu\text{g/ml}$ ; FR, found 0.41  $\mu\text{g/ml}$ ; DR, found: 0.45  $\mu\text{g/ml}$ ; FDR, found: 0.05  $\mu\text{g/ml}$ .

### Stability of R, FR, DR and FDR solutions

The presence of ascorbic acid in the organic solution is necessary to prevent oxidation of R and its metabolites. Dichloromethane calibration solutions can be stored for one month at +5°. Aqueous solutions were found not to be stable enough to be used for calibration, even with ascorbic acid. No decrease in the R content was observed in plasma and urine samples when stored frozen for three months at -20°.

### Speed of analysis

The analytical technique is fast: one single extraction is needed before chromatography, which takes about 10 min.

### Application

The described procedure was used in the quantitative assay of R, FR, DR

TABLE I

### REPRODUCIBILITY AND ACCURACY OF THE ASSAY IN HUMAN PLASMA, URINE AND SALIVA

100 µl of extract injected.

Compound	Sample (1 ml)	Amount of compound added (µg)	Average of 6 assays (µg)	Coefficient of variation (%)
Rifampicin	Plasma	0.05	0.05	23.0
		0.10	0.10	7.6
		0.50	0.50	4.7
		1.00	0.96	4.9
		5.00	4.90	4.9
		20.00	19.90	5.9
	Urine	9.00	9.10	1.2
		18.00	18.50	2.3
	Saliva	2.00	2.00	8.6
25-Desacetylrifampicin	Plasma	0.10	0.11	9.2
		0.20	0.20	7.0
		0.50	0.50	6.5
		2.50	2.49	4.0
		5.00	4.84	4.0
		10.00	10.40	4.5
	Urine	4.50	4.60	1.8
		9.00	9.20	1.8
	Saliva	1.00	1.01	4.7
3-Formylrifamycin SV	Plasma	0.05	0.05	12.0
		0.10	0.10	5.4
		2.50	2.45	3.8
3-Formyl-25-desacetyl-rifamycin SV	Plasma	0.10	0.11	10.6
		0.20	0.21	7.2
		0.50	0.52	4.2

TABLE II

## INJECTION OF TEN SUCCESSIVE SAMPLES OF THE SAME R AND DR DICHLORO-METHANE SOLUTION

100  $\mu$ l solution injected.

Injection No.	Rifampicin peak area (arbitrary units)	25-Desacetylriofampicin peak area (arbitrary units)	Rifampicin peak area/ 25-Desacetylriofampicin peak area
1	184208	92369	1.99426
2	183845	91599	2.00706
3	185639	92321	2.01079
4	185746	91451	2.03109
5	184873	92167	2.00584
6	183810	92240	1.98876
7	184299	91891	1.99803
8	185390	94983	1.95182
9	184765	91161	1.98635
10	181082	92424	2.01069
Average	184366	92261	1.99847
Coefficient of variation	0.7	1.1	1.0

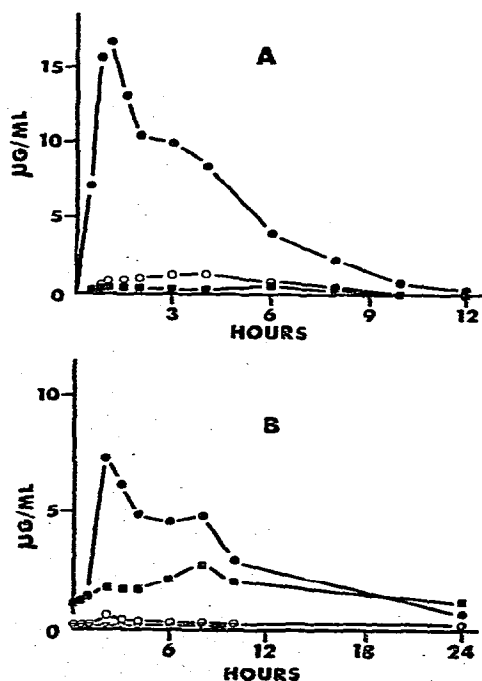


Fig. 2. Serum concentrations obtained: (A) in one healthy subject on the 1st day of treatment; (B) in one cirrhotic patient on the 7th day of treatment after daily administration of 600 mg of rifampicin. •, Rifampicin; ■, 3-formylrifampicin SV; ○, 25-desacetylriofampicin; △, 3-formyl-25-desacetylriofampicin SV.

and FDR in the serum of normal subjects and cirrhotic patients given 600 mg of rifampicin daily for seven days. Fig. 2 shows the serum concentrations obtained in one healthy subject on the first day and in one of the patients on the seventh day of treatment.

#### CONCLUSION

The proposed technique permits the separate determination of rifampicin and three of its metabolites in human plasma, urine and saliva. It is fast and more specific than the existing assay procedures. It can be used to monitor rifampicin concentrations in patients, and to ascertain whether any accumulation of metabolites takes place.

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