PHYSIOLOGICAL DISPOSITION OF A SERIES OF RIFAMYCINS IN RAT: A COMPARATIVE STUDY

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(Received for publication March 14, 1978)

The disposition of four C_3-substituted piperazinyl rifamycins was studied in the rat following the intravenous administration of 5 mg/kg of the ^{14}C-labelled antibiotics. Considerable quantitative differences in the pharmacokinetics of these antibiotics were shown in blood levels, tissue distributions and body clearances. Feces were largely the major route of elimination for the parent drug and metabolites. The results suggest that the liver compartimentalization, regulating the biliary excretion, is to be the kinetic parameter affecting the pharmacokinetic behaviour of this class of antibiotics.

C_3-Substituted rifamycins, a class of semi-synthetic antibiotics derived from a fermentation of *Nocardia mediterranei*[1], have been shown to be clinically useful in the treatment of tuberculosis and leprosy.[2,3] Since pharmacokinetics are known to be important in determining both the antibacterial activity *in vivo* and the toxicology of the antibiotics, a comparative kinetic study was carried out with one series of C_3-substituted piperazinyl rifamycins in order to elucidate what factors determine their physiological disposition. Previous pharmacokinetic studies with other rifamycins, including rifampicin, demonstrated that in animals[4,5] and in humans[6,7] as well as in the isolated perfused rat liver[8], these compounds are characterized by distinctive properties directly correlated with their liver uptake and their biliary excretion. In the present paper, these characteristics were investigated and more precisely defined with this new series of rifamycin derivatives.

Materials and Methods

Chemicals

38-^{14}C-AF/AMP (3-[(4-Methyl-1-piperazinyl)imino]methyl rifamycin SV) specific activity 10.77 mCi/mmole; 38-^{14}C-AF/AETP (3-[(4-ethyl-1-piperazinyl)imino]methyl rifamycin SV) specific activity 10.29 mCi/mmole; 38-^{14}C-AF/ACPP (3-[(4-cyclopentyl-1-piperazinyl)imino]methyl rifamycin SV) specific activity 10.21 mCi/mmole and 38-^{14}C-AF/ABDP (3-[(4-*cis*-aminobenzyl-2,6-dimethyl-piperazinyl)imino]methyl rifamycin SV) specific activity 12.15 mCi/mmole, were kindly supplied by G. SARTORI from our research laboratories.[9]

Animal treatment and sample collection

Male Wistar rats weighing 200~260 g were fasted for 18 hours before they were used. Rifamycins, dissolved in 0.3 ml of 0.1 M $NaHCO_3$, containing 10^{-3} M ascorbic acid and 2.5% N,N'-dimethylformamide, were administered intravenously *via* the femoral vein at a dosage of 5 mg/kg. Animals were kept in appropriate glass metabolic cages for collection of urines and feces. Bile was collected from urethane-anesthetized animals cannulated in the bile duct. Blood was sampled by puncture of the orbital sinus. Tissues were taken after exsanguination from the abdominal aorta; livers were excised, washed once with ice-cold 0.05 M Tris-HCl buffer, pH 7.4, weighed and homogenized with 4 volumes of buffer, containing 0.25 M sucrose, using a glass Teflon homogenizer. The homogenate was centrifuged

twice at 800 $g \times 15$ minutes for nuclei and cellular debris; at 18,000 $g \times 20$ minutes for mitochondria; and at 105,000 $g \times 60$ minutes for microsomes and the cytosol was decanted from the microsomal pellet. Protein concentrations were estimated by the method of LOWRY *et al.*[10]

For the *in situ* absorption studies, jejunal loops (7 cm) were tied in anesthesized and heparinized rats, so to interrupt the vascular connections to the neighbouring loops, while the vein which drained the loop was punctured and the blood collected in vials.

To maintain a constant intestinal blood flow the loss of blood was compensated for by an infusion of heparinized homologous blood.

Either rifamycin solutions (1 ml of KREBS-HENSELEIT medium, pH 7.4, containing 1 mmole of the drugs, 10^{-3} M of ascorbic acid and 2.5% N,N'-dimethylformamide), or the radioactive bile samples collected from treated and cannulated animals (1 ml of bile containing 0.08 mmole equivalents of labelled compound) were directly introduced into the loops with a syringe, and the venous blood sampled every 10 minutes.

The appearance rate in the intestinal venous blood was derived from the blood flow and the concentration in the blood collected. For details of the method see WINNE and REMISHOVSKY.[11]

Radioactivity determinations

Blood samples (200 μl) were digested by the addition of 0.5 ml of H_2O_2 (30%) and 1.5 ml of the mixture Soluene 350® (Packard Instrument) - isopropanol (1 : 1). After 1 hour, 15 ml of the scintillation cocktail Instagel® (Packard Instrument)-0.5 N HCl (9 : 1) were added for counting.

Lyophilized feces samples (20 mg) were rehydrated with 0.1 ml of water, and solubilized by 1 ml of Soluene 350® for 2 hours at 50°C. After cooling 0.5 ml of isopropanol and 0.2 ml of H_2O_2 (30%) were added and the samples were incubated again at 50°C for 2 hours. As scintillation cocktail, 16 ml of the mixture Dimilume T30® (Packard Instrument) - H_2O (5 : 3) were used.

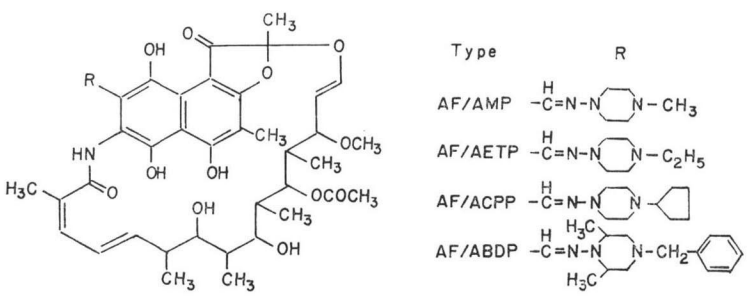
Lyophilized tissue samples (50 mg) were rehydrated with 0.3 ml of water, and digested for 4 hours at 50°C by adding 1.3 ml of the mixture Soluene 350® - isopropanol (5 : 1), and 0.2 ml of H_2O_2 (30%). As scintillation cocktail, 15 ml of Dimilume T30® was used. All other materials, either aqueous (*e.g.* plasma, urine, and bile) or organic solutions (*e.g.* eluates from TLC), were counted in Instagel®. Counts were obtained with an Intertechnique SL 30/300 liquid scintillation spectrometer.

Thin-layer chromatography (TLC)

Precoated silica gel plates F_{254}, 0.25-mm thick from Merck buffered at pH 6.0 with a solution of 0.4 M Na_2HPO_4, 0.2 M citric acid, 50% of methanol and 1% of ascorbic acid were used with a solvent system benzene - acetone - ethanol (10 : 8 : 4). Authentic AF/AMP, AF/AETP, AF/ACPP and AF/ABDP were run together with the assayed samples as reference compounds. TLC-autoradiographs were obtained by exposing Kodirex-ray-films to the plates from Eastman Kodak Co. for the necessary time periods. For the quantitative analysis of the plates, individual zones of radioactive products were scraped off and measured by liquid scintillation counting.

The calculation of the areas under the concentration time curves of the blood kinetics, were estimated by a graphical method, cutting out the areas under the curves and weighing them.

Fig. 1. C_3-Piperazinyl rifamycins, basic structure and kinds of substitution.



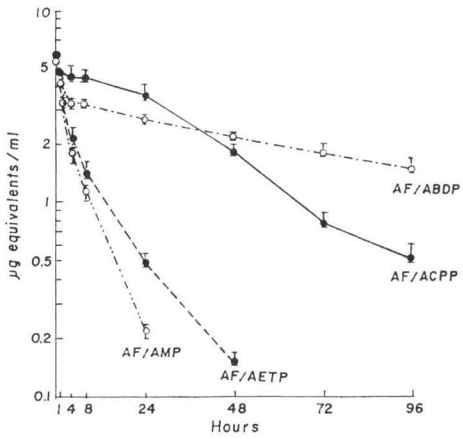
Results

Blood Levels

The blood levels after the intravenous administration of 5 mg/kg of ^{14}C-rifamycins AF/AMP, AF/AETP, AF/ACPP, and AF/ABDP were measured as total radioactivity. The results, expressed according to time as μg equivalents of the administered drug per ml of blood are presented in Fig. 2. Striking kinetic differences immediately arise, even when one makes a rough comparison of the ^{14}C-disappearance rates of the different rifamycins from the blood. At 24 or 96 hours after dosing, the comparison of the areas under the ^{14}C-blood concentration-time curves, the following results were obtained: AF/AMP=29.76 (24 hours) and 36.48 (96 hours); AF/AETP=35.52 (24 hours) and 46.08 (96 hours); AF/ACPP=94.08 (24 hours) and 196.8 (96 hours); AF/ABDP=75.36 (24 hours) and 215.04 (96 hours), μg equivalents hour/ml.

Fig. 2. Blood levels of radioactivity in the rat following a single intravenous dose (5 mg/kg) of ^{14}C-rifamycins AF/AMP, AF/AETP, AF/ACPP and AF/ABDP.

The average values ± standard error are reported (groups of 4 animals).



Tissue Distribution

Tissue distributions of total radio activity in brain, heart, kidneys, liver, lungs, muscle, pancreas, spleen, testes and thymus were measured at 0.25 and 24 hours after treatment. The earlier time was selected to compare the rates of distribution, of the 4 rifamycins from the blood compartment into the tissues and especially the liver. The 24-hour time was chosen both to evaluate the rates of disappearance of total ^{14}C, and to show, by the ratios of concentration between tissues and blood, any possible

Table 1. Tissue distribution of total at 15 minutes after dosing

Tissue	μg equivalents per g of dry tissue				μg equivalents per g of dry tissue/ μg equivalents per g of dry blood			
	AF/AMP	AF/AETP	AF/ACPP	AF/ABDP	AF/AMP	AF/AETP	AF/ACPP	AF/ABDP
Blood	20.3	25.4	20.0	28.1	1	1	1	1
Plasma	59.5	93.1	85.9	99.4	2.93	3.67	4.30	3.54
Brain	1.0	1.4	1.6	2.7	0.05	0.06	0.08	0.10
Heart	29.8	40.9	46.7	62.7	1.47	1.61	2.34	2.23
Kidneys	42.5	55.8	58.5	71.1	2.09	2.20	2.93	2.53
Liver	112.5	149.2	149.7	159.4	5.54	5.87	7.49	5.67
Lungs	29.9	39.3	40.3	49.7	1.47	1.55	2.02	1.77
Muscle	14.6	22.9	22.3	13.0	0.72	0.90	1.12	0.46
Pancreas	21.7	29.9	32.9	26.3	1.07	1.18	1.65	0.94
Spleen	22.7	30.7	36.3	53.3	1.12	1.21	1.82	1.90
Testes	3.2	2.9	3.0	3.0	0.16	0.11	0.15	0.11
Thymus	16.2	16.3	12.0	12.5	0.80	0.64	0.60	0.44

The values are the average of data obtained with two animals.

Table 2. Tissue distribution of total ^{14}C at 24 hours after dosing.

Tissue	μg equivalents per g of dry tissue				μg equivalents per g of dry tissue/ μg equivalents per g of dry blood			
	AF/AMP	AF/AETP	AF/ACPP	AF/ABDP	AF/AMP	AF/AETP	AF/ACPP	AF/ABDP
Blood	0.49	2.35	12.8	17.5	1	1	1	1
Plasma	1.09	7.38	59.5	64.2	2.22	3.14	4.65	3.67
Brain	0.17	0.18	2.06	2.67	0.35	0.08	0.16	0.15
Heart	0.37	2.64	23.0	34.1	0.76	1.12	1.80	1.95
Kidneys	0.75	6.25	29.8	44.8	1.53	2.66	2.33	2.56
Liver	6.55	35.5	71.5	82.2	13.37	15.11	5.59	4.70
Lungs	0.43	2.89	19.3	29.5	0.88	1.23	1.51	1.69
Muscle	0.26	2.64	11.3	14.6	0.53	1.12	0.88	0.83
Pancreas	0.83	2.36	20.2	36.6	1.69	1.00	1.58	2.09
Spleen	0.44	2.24	17.2	31.0	0.90	0.95	1.34	1.77
Testes	0.40	2.11	16.1	14.6	0.82	0.90	1.26	0.83
Thymus	0.42	1.84	17.1	27.9	0.86	0.78	1.34	1.59

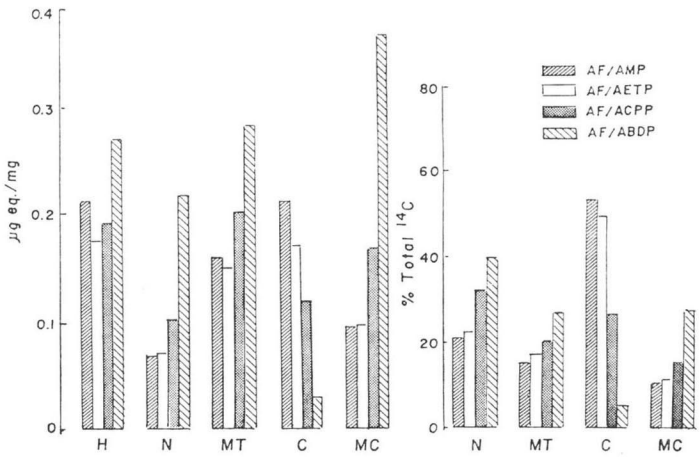
The values are the average of data obtained with two animals.

accumulation phenomena. Tables 1 and 2 summarize the results, expressed as μg equivalents of ^{14}C-rifamycins per g of dry tissue, and as the ratio between tissue and blood concentration. At 15 minutes after the administration, blood, plasma and tissue levels of ^{14}C for the different rifamycins were similar even though they increased in the order AF/AMP, AF/AETP, AF/ACPP, AF/ABDP. Highest radioactivity concentrations were found in liver. Lower but still greater than the blood concentrations were found in heart, lungs, pancreas, and spleen. At 24 hours after the administration the tissue levels of radioactivity differ largely within the series of rifamycins, while the main target tissues remain, liver, kidneys, pancreas and to a minor degree, the heart, lungs and spleen.

Fig. 3. Subcellular distribution of radioactivity in livers of rats 15 minutes after the intravenous administration of ^{14}C-rifamycins AF/AMP, AF/AETP, AF/ACPP, and AF/ABDP when the blood levels of total ^{14}C were respectively of 2.52, 3.72, 4.59 and 5.43 μg equivalents/ml.

The data averages of two animals are expressed as μg equivalents per mg of proteins, and as percentage of the total liver radioactivity.

H=homogenate, N=nuclei+cellular debris, MT=mitochondria, MC=microsomes, C=cytosol.



Liver Subcellular Distribution

Fifteen minutes after the intravenous administration of ^{14}C-AF/AMP, AF/AETP, AF/ACPP, AF/ABDP, the subcellular distributions of the total radioactivity, in the nuclei+cellular debris, mito-

Table 3. Urinary and fecal excretion of total radioactivity.

Time after administration in days	Cumulative excretion values, % of the administered dose ± S.E.							
	Urine				Feces			
	AF/AMP	AF/AETP	AF/ACPP	AF/ABDP	AF/AMP	AF/AETP	AF/ACPP	AF/ABDP
1	8.16±0.71	12.41±0.94	3.21±0.51	0.74±0.10	30.92±3.52	19.96±1.99	18.54±1.11	13.84±1.10
2	8.50±0.74	14.31±1.36	4.13±0.44	0.86±0.11	53.81±2.65	49.86±1.53	35.03±1.40	27.71±1.14
3	8.71±0.77	15.38±1.09	4.74±0.40	0.92±0.13	72.32±1.85	66.55±2.05	49.27±1.70	40.03±1.13
4	8.84±0.80	16.15±1.61	4.99±0.41	1.01±0.10	79.39±1.42	75.85±2.63	69.53±2.00	50.02±1.12
5	8.91±0.81	16.37±1.65	5.26±0.50	1.07±0.09	82.85±1.28	79.94±2.27	76.34±2.10	61.13±1.20
6	8.97±0.80	16.45±1.66	5.39±0.48	1.10±0.08	83.86±1.36	81.43±2.23	79.30±2.12	70.07±1.24
7	9.00±0.81	16.51±1.67	5.48±0.44	1.13±0.08	83.97±1.38	82.15±2.38	80.05±2.20	76.80±1.30
8			5.57±0.44	1.18±0.09			81.18±2.13	81.59±1.27
9			5.59±0.43	1.20±0.10			81.60±2.07	86.08±1.31
10			5.61±0.42	1.22±0.10			81.96±2.01	88.05±1.30
11			5.62±0.43	1.24±0.09			82.14±2.00	88.85±1.30

The values are the average of data obtained with 3 animals.

chondrial, microsomal and soluble fractions from rat livers were investigated. The results reported in Fig. 3 are expressed as μg equivalents of ^{14}C-rifamycins per mg of protein and as percentages of the total liver radioactivity.

The concentrations of rifamycins and their metabolites in the particulate fraction occur in the following increasing order, AF/AMP, AF/AETP, AF/ACPP and AF/ABDP. The opposite occurs in the soluble fraction with AF/ABDP being more than 90% localized in the particulate fraction.

Urinary and Fecal Excretions

The cumulative values of the urinary and fecal excretions of radioactivity for the four rifamycins under investigation are presented in Table 3.

Recovery of 50% of the administered radioactivity through these two elimination routes requires almost 35 hours for AF/AMP, 37 hours for AF/AETP, 64 hours for AF/ACPP, and 94 hours for AF/ABDP. Moreover at the 96th hour no residual radioactivity could be detected in the carcasses of the rats treated with AF/AMP, AF/AETP, while in the rats given AF/ACPP and AF/ABDP 1.28±0.2% and 7.66±0.34% of the dosages were found in the carcasses.

Biliary Excretion

In Table 4 the biliary excretion values relating to AF/AMP, AF/AETP, AF/ACPP and AF/ABDP for a time period of 4 hours after the intravenous administrations, and the plasma and liver concentrations of radioactivity measured at the 4th hour are summarized.

The results show within this series of rifamycins that rates of excretion were very high for AF/AMP and AF/AETP, and much lower for AF/ACPP and AF/ABDP. These excretions are in agreement with the above kinetic results.

Table 4. Biliary excretion.

Type	Biliary excretion (0~4 hours) % of the dose (mean±S.E.)	μg eq/ml or g of dry tissue* (mean±S.E.)	
		Plasma	Liver
AF/AMP	38.00±2.70	1.66±0.25	79.30±2.30
AF/AETP	27.65±1.40	2.39±0.14	89.60±1.31
AF/ACPP	8.4 ±0.60	4.74±0.96	93.34±1.64
AF/ABDP	2.4 ±0.10	4.88±0.26	114.51±2.63

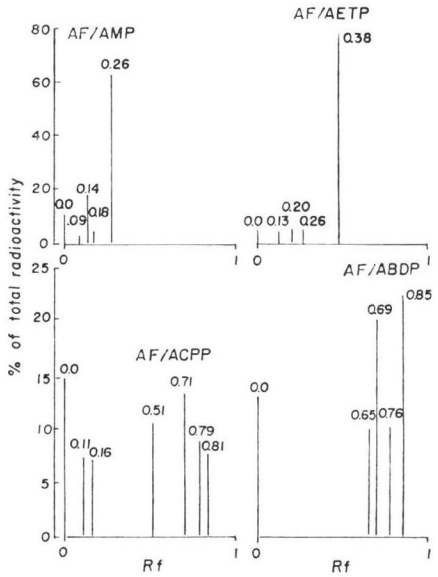
* Concentration at the 4th hour after treatment.

Bile Metabolic Pattern

An investigation aiming at analysis and quantification of total radioactivity in the bile from treated animals (0~4 hours fractions) was performed by TLC separation, as illustrated in Fig. 4. For all the rifamycins studied the percentage of the total radioactivity was small for AF/AMP, and AF/AETP, large for AF/ACPP and AF/ABDP. This is attributed to their metabolites. Data for AF/AMP and AF/ABDP are in agreement with previous observations.[12,13]

Fig. 4. Metabolic pattern of the radioactivity, after TLC of bile samples collected from cannulated rats given 5 mg/kg of ^{14}C-rifamycins AF/AMP, AF/AETP, AF/ACPP, and AF/ABDP.

On the abscissa, the chromatographic partition coefficients, on the ordinate, the percentage of total radioactivity.



Intestinal Absorption

The data concerning the intestinal absorption of rifamycins AF/AMP, AF/AETP, AF/ACPP and AF/ABDP and of the radioactivity in the administered bile are summarized respectively in Tables 5 and 6. Rifamycins AF/AMP, AF/AETP and AF/ACPP are absorbed and appear in the intestinal blood at practically the same rates which, at the steady state, range between 17 and 21 μg equivalents per g blood per minute. Rifamycin AF/ABDP seems to be less rapidly absorbed with an appearance rate of 5~6 μg equivalents per g blood per minute*.

The radioactivity absorptions from the bile samples were compared at molar concentrations twelve times lower than that chosen for the original rifamycin solutions, since this was the highest biliary concentration that could be obtained when AF/ABDP was administered to a rat at a dose of 15 mg/kg. The results reported in Table 6 for the time intervals of 10~40 minutes after the intestinal injections, demonstrate that for the 4 antibiotics there is a reabsorption of the biliary total radioactivity or at least of some components of it. When these data are compared with those obtained for the original solutions at the higher concentrations, and taking into account the diverse ratios between the

Table 5. Intestinal absorption of rifamycins.

Time interval after injection (minutes)	Appearance rate* μg eq/min/g**			
	AF/AMP	AF/AETP	AF/ACPP	AF/ABDP
10~20	18.8±3.8	17.1±0.9	21.2±1.7	5.9±0.8
20~30	17.9±2.2	17.4±0.7	19.5±1.9	5.5±0.6
30~40	17.7±1.7	15.1±1.1	17.0±1.3	5.5±0.3

* Values represent mean (±S.E.) of at least three experiments.
** Weight of wet intestinal tissue.

* The very low water-solubility of AF/ABDP caused the compound to precipitate on the intestinal wall after introduction into the intestinal loop, thus decreasing its molar concentration in the lumen.

Table 6. Intestinal absorption of the biliary radioactivity.

Time interval after injection (minutes)	Appearance rate* μg eq/min/g**			
	AF/AMP	AF/AETP	AF/ACPP	AF/ABDP
10~20	0.16	0.28	0.19	0.17
20~30	0.22	0.43	0.17	0.26
30~40	0.21	0.39	0.14	0.39

* Mean values of at least 2 or 3 experiments.
** Weight of wet intestinal tissue.

4 drugs and their metabolites, it is apparent there is non-linearity between the appearance rates and the luminal concentration values of these compounds.

Discussion

In agreement with the previously obtained pharmacological and toxicological data*, the results of the current study of a series of C_3-substitute piperazinyl rifamycins show considerable differences in the pharmacokinetics of these antibiotics. Above all there are differences in the rates of disappearance of the radioactivity from the blood within the series, with relative decreasing orders being AF/AMP, AF/AETP, AF/ACPP, and AF/ABDP.

The rates of disappearance from the various tissues and from the whole body reflect the blood kinetics. The major route of excretion is the biliary one.

The study of the biliary excretion for these rifamycins, as for others studied previously,[4,5,8] completely reflects the pharmacokinetics of these antibiotics, paralleling the rate of disappearance of the radioactivity from the blood and tissue compartments. The possibility of the existence of an enterohepatic cycle was shown by the studies of absorption *in situ* of the original rifamycins and of the radioactive compounds (or some of them) in the bile. Since, the data obtained suggest that the intestinal absorption is not linear with the luminal molarity over the range of concentrations found in the bile of treated rats, the reabsorption of the excreted radioactivity should practically affect the fecal eliminations of those rifamycins characterized by high biliary excretion rates (*i.e.* AF/AMP and AF/AETP).

Of course, the reabsorption from the bile of the total radioactivity depends also on the nature of the metabolites.

It does not appear that biliary excretions are limited by liver uptake of the compounds from the blood compartment since the liver concentrations of radioactivity at 15 minutes after intravenous administration were essentially the same for all 4 rifamycins. On the other hand the distribution of the radioactivity in the subcellular compartments of the liver differed considerably, and the data seem to suggest the existence of a dependence of the biliary excretion rates to the liver subcellular distribution of total ^{14}C, *i.e.*, the rates of excretion appear to be directly proportional to the rifamycins concentrations in the cytosol, and reversely to those in the nuclear, mitochondrial and microsomal fractions.

In previous studies[15] we showed that these antibiotics bind to bovine and human serum proteins in increasing order from AF/AMP, AF/AETP, AF/ACPP and AF/ABDP. This is the reverse order of that for the biliary excretions, but since the hepatic uptake was the same for all the rifamycins, these differences in binding should not influence the excretion rates. However, it may be that differences in the binding of the rifamycins or their metabolites to the different subcellular fractions of the liver play an important role in determining biliary excretion.

A further hypothesis which arises from the biliary metabolic patterns, suggests that a relationship might exist between the extent of metabolism and the biliary excretions. However, this possibility does not exclude or contrast with the above mentioned interpretation.

* Unpublished data.

In conclusion, these data provide a clear insight into the pharmacokinetic behaviour of this class of antibiotics, showing that the striking quantitative differences both in blood levels and tissue distributions are probably due to the liver compartimentalization and/or to a degree metabolism, both affecting the liver drainage through the biliary excretion.

Acknowledgements

The authors are indebted to Prof. ssa L. T. TENCONI for helpful suggestion and criticism.

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