ABSORPTION AND METABOLISM OF TRITIUM-LABELLED FACTOR M_1 OF VIRGINIAMYCIN BY ORAL AND PARENTERAL ADMINISTRATION

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After both intravenous injection and oral administration, the factor M_1 of virginiamycin was rapidly diluted or absorbed. It then disappeared from the plasma stream at a first order exponential rate with a half life of approximately 5 hours following a process of both tissue fixation and body excretion. The bile was the main route by which the antibiotic was excreted from the body. The ratio C_bM_1/C_pM_1 was nearly one, indicating that factor M_1 was not actively transported from the liver to the bile though it is extensively metabolized to large polar fragments. After oral ingestion 15~18 % of the administrated factor M_1 was absorbed through the gastro-intestinal tract. Neither bile nor the lymphatic system seemed to play a role in that absorption. The experimental data emphasized the importance of the plasmatic compartment as a reservoir for the antibiotic, the rapidity of tissular fixation and the special affinity of the factor M_1 for the skin. They correlated very well with the clinical trials which revealed the efficiency of the drug in the treatment of the gram-positive bacterial infections and the rapid onset of its action.

Virginiamycin is an antibiotic produced by a strain of *Streptomyces virginiae*, isolated in 1954 by DE SOMER and VAN DIJCK[9]. By its physicochemical properties and its antimicrobial spectrum it belongs to the family of streptogramin[19]. In the crude antibiotic, two groups of substances have been identified: the group of factors M or "micrococcus" factors and the one of factors S or "bacillus subtilis" factors[18]. They show a definite synergistic action. Factors of the group S significantly enhance the potency of factors M, *in vitro* as well as *in vivo*.

Most of the antimicrobial activity of virginiamycin is due to factor M_1 which is also quantitatively the main component. Its chemical structure appears very similar if not identical to ostreogrycin A[7,8], a macrocyclic unsaturated lactone containing an oxazole ring. Gram-positive bacteria, *Neisseria gonorrhoeae* and *Hemophilus pertussis* are sensitive to the bacteriostatic effect of factor M_1 with factor S. This antimicrobial action seems to be related to a blockade of the protein biosynthesis at the ribosomal site[11,12].

The present publication reports the pattern of absorption, tissue distribution and elimination of the antibiotic in the rat after oral and parenteral administration. Some correlations are made between the results obtained with the two modes of administration.

Material and Methods

Male Wistar rats weighing 200~350 g and fasted 24 hours before the experiments were used.

Blood samples were collected by puncturing the caudal vein.

Bile samples were obtained by inserting a cannula (polyethylene tubing P.E 50) into the biliary duct, under a slight anesthesia by ether, according to the method described by D'AMOUR and BLOOD (1963). In order to minimize the physiological disturbances resulting from the interruption of the enterohepatic cycle, the bile was never collected over more than 3 hours[4]. During this period of time the mean value of the measured biliary flow remained constant and reached 42 mg/min./kg. The lymph was obtained from the thoracic duct according to the technique reported by BOLIMAN *et al*[2].

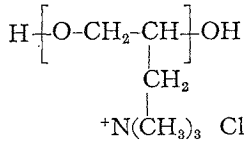
As previously described[15], factor M_1 was injected into the femoral vein at a dose of 1 mg/kg as a plasmatic solution or administrated *per os* as an aqueous suspension at a dose of 3 mg/kg by means of a gastric tubing without anesthesia.

The radioactivity of the samples was measured by liquid scintillation counting after combustion in a Parr bomb or a Schöniger flask, hyamine digestion or direct dissolution in the solvent-scintillators system. Counting efficiency was determined using *n*-hexadecane ($^3H_2$-1,2) or (^{14}C-1) as an internal standard.

Chromatographic analysis of factor M_1 and of the bile extracts were carried out by thin-layer chromatography. (Adsorbent: silical gel HR and HF_{254} "Merck"; solvent CH_2Cl_2-CH_3OH 9/1 V/V[13]).

The antibiotic was assayed spectrophotometrically after reaction at 70°C in 2.5 N HCl with ERHLICH reagent[18]).

A quaternary ammonium compound of general structure



was used at a dose of 2 mg/ml bile to destroy its micellar structure.

The reverse isotopic dilution method applied to the bile samples was essentially identical to that previously described for plasma[16].

The confidence limits of the curves presented in this paper were calculated according to the equation

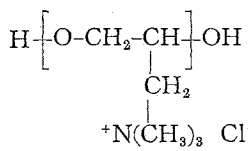
$$Y_x = Y_x \pm t_{1/2}\alpha \cdot s_{xy}\sqrt{1+\frac{1}{N}\cdot\frac{(x-\bar{x})^2}{(N-1)s_x}} \qquad (10)$$

Pure and crystallized factor M_1 was kindly supplied by RIT Laboratories, Belgium.

Radioactive compounds were prepared either by biological synthesis using ^{14}C labelled precursors: acetate ($-^{14}COO^-$) and L-methionine ($^{14}CH_3$)[15] or by tritiation[14] according to the WILZBACH method. The specific activities were respectively 5 μCi/mmole for the ^{14}C labelled factor M_1 and 60 to 80 μCi/mmole for the tritiated one.

The enzymes β-D-glucuronidase and aryl sulphatase (helix pommatia) were purchased from Koch-Light Lab.

The quaternary ammonium



was kindly supplied by Dr. M. CLAESEN, Rega Institute, Louvain, Belgium.

Results

A. Absorption and Distribution of Factor M_1

1. Time course of antibiotic concentration changes in plasma.

After oral administration or intravenous injection, the tritiated and/or ^{14}C labelled factor M_1 was rapidly absorbed or diluted. Radioactivity present in the blood after both ingestion and injection was located for its major part (respectively 80 % and 90 %) in the plasmatic compartment where it was distributed between a protein bound (75 %) and a free (25%) fraction. This latter part of the radioactivity was identified as unmetabolized factor M_1[16].

The absorbed or diluted radioactivity disappeared from the plasma stream at a first order exponential rate with a half life of approximately 5 hours (Fig. 1).

2. Excretion from the body

The bile was the main route by which the antibiotic was excreted from the body. During the first hours following the oral administration the radioactive bile concentration (C_b) was high and reached between 15 to 25 μg/ml and from the given 0.75 mg dose, 30 μg were excreted between 0 and 3 hours (Table 1).

Three hours after intravenous injection 20 % of the dose was already found in the intestine and/or the caecum[15]. The rate of decline of factor M_1 concentration in the bile has been studied over a 2-hour period. During this interval the bile outflow remained unchanged. The variance analysis of the logarithmically transformed data (5 experiments) showed two linear regressions (Fig. 2).

On the other hand, the elimination by the kidney was low, the radioactivity in the uring (C_u) corresponded to a concentration of

Fig. 1. Evolution in time (min.) of the plasma concentration (μg/ml) of radioactivity (C_p) of factor M_1 (^3H) after
a) intravenous injection
b) oral administration
• mean experimental values ± s.e. t 0.05

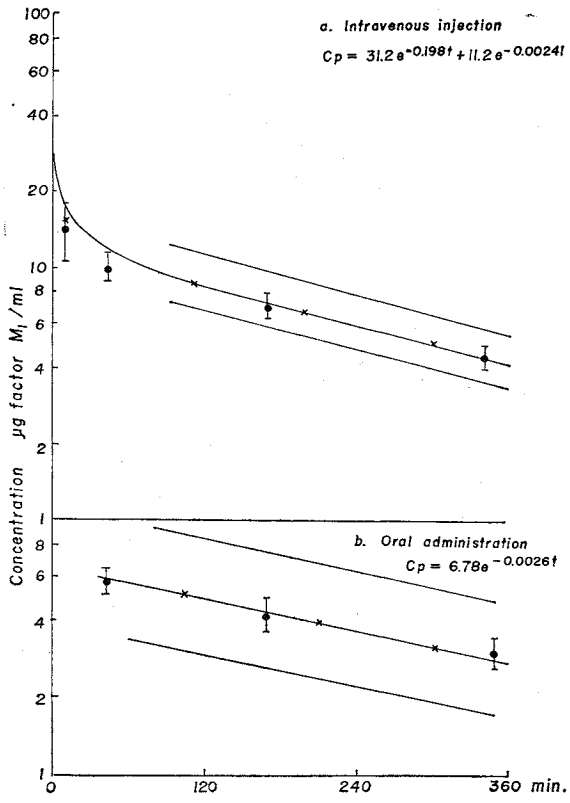


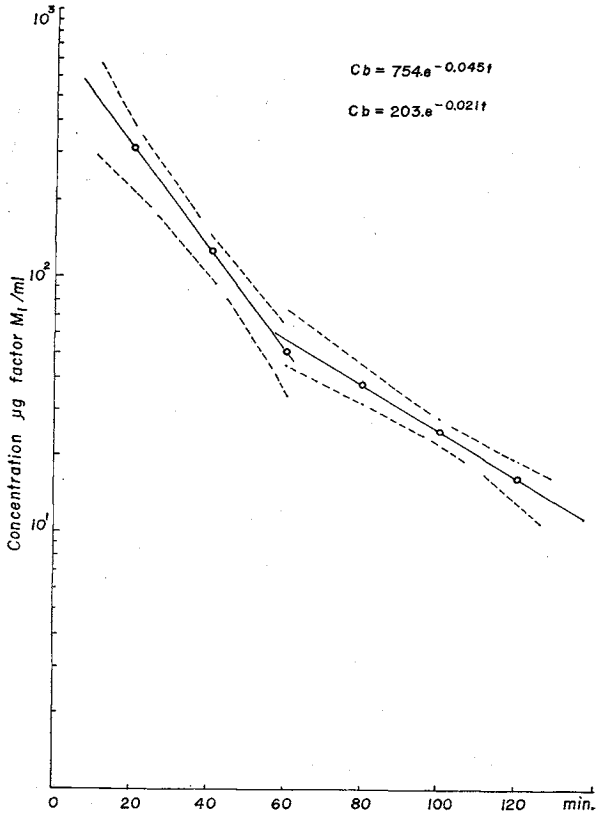
Table 1. Biliary and urinary excretion of radioactivity after oral administration of factor M_1 (^3H) at a dose of 3 mg/kg.
(in brackets : number of experiments)

	Period of time (min.)	total μg	μg/ml
			C_{bi}
Bile	0~40 (12)	4.5±1.1	14.7±3.2
	0~100 (12)	16.4±2.5	20.8±3.1
	0~170	29.7±4.1	24.2±4.0
			C_u
Urine	0~40 (4)		0.7*
	0~340 (3)		0.6*
	0~1,440 (14)		5.4±0.5

mean values ± s.e. $t_{0.10}$ * mean values

factor M_1 less than 1 μg/ml for the first 6 hours after oral administration. The total excreted amount in 24 hours did not exceed what was found in the bile 3 hours after the ingestion. Following the intravenous injection, the urinary excretion was less than 10 % in 24 hours[15].

Fig. 2. Kinetics of biliary excretion after intravenous injection of factor M_1(^{3}H).
C_b=bile concentration in μg/ml



3. Distribution in tissues

While being excreted by the bile and the urine, the factor M_1 was taken up by the rat's tissues (Fig. 3). All the tissues were involved. The liver (3.4 μg/g), the skin (1.4 μg/g), the small intestine (1.7 μg/g) and the kidneys (1.4 μg/g) showed however a real selective affinity by comparison with the other tissues whose values ranged from 0.2 to 0.3 μg/g.

The fixation was a rapid phenomenon which appeared to be reversible. After 40 minutes maximum concentration was reached most of the times. Muscle concentration (0.10 μg/g) was practically identical whatever the route of administration.

B. Route and Efficiency of the Oral Absorption

After oral administration, the highest radioactive blood concentration was so rapidly reached that it seemed important to search for the participation of the gastric mucosa in the absorption of the antibiotic. At the same time, taking into account the well-known factor M_1 chemical reactivity at acidic pH *in vitro* analysis of the extracts from the gastric juices was also carried out.

Finally, the lipid-soluble character of factor M_1 should suggest that its intestinal absorption, if any, might be influenced by the bile or might occur through the lymphatic system. Those two parameters were also investigated.

1. The stomachic absorption and the stability in the gastric juice.

The orally administered factor M_1 was artificially either maintained in the stomach up to 160 minutes by ligation of the duodenum or deviated from the intestine by insertion of a cannula into the stomachic cavity through the pylorus. The radioactive plasmatic (C_p) and biliary (C_b) concentrations were measured at various times after the ingestion. The results presented in Table 2 showed the presence or radioactive material in both plasma and bile samples. They clearly indicated a real but probably minor role, in more physiological conditions, of the stomachic mucosa in the absorption of the antibiotic. The radiochromatographic analysis as well as the chemical assay of

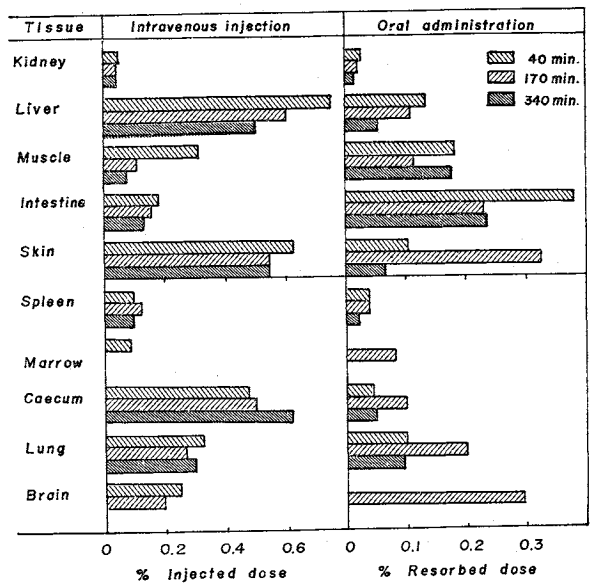
the dichloromethane extracts from the gastric juice revealed no important degradation. The procedure extracted 70~75 % of the total radioactivity from which 95 % were intact factor M_1.

2. Influence of bile and the lymphatic system on the intestinal absorption.

Coming out from the stomach, the factor M_1 reached the intestine where its absorption continued. When the bile was deviated out of the animal by means of a cannula directly inserted into the biliary duct, there was no significant decrease in the radioactive blood concentration measured at 40 or 170 minutes after the ingestion as compared to control animals (Table 3).

Fig. 3. Tissue distribution of radioactivity at various time after oral administration and intravenous injection of factor M_1 (^3H)

For oral administration the values were calculated on the bases of a 15 % absorbed dose (cf § Bc)



The radioactive lymph concentrations (Cl) were always much lower than the corresponding blood levels, which would indicate the secondary, if any, role of this system in the antibiotic absorption (Table 4).

Table 2. Stomachic absorption of factor M_1 (^3H)

	C_p (μg/ml)*				C_b (μg/ml)*		
	20 min.	40 min.	80 min.	160 min.	0~40 min.	40~100 min.	100~160 min.
Ligation of the duodenum	0.13	0.17	0.34	0.23	5.0	36.0	42.0
	0.12	0.16	0.13	0.16	5.7	10.0	12.0
	0.12	0.12	—	—	9.0	14.0	
Insertion of a cannula into the pylorus	0.14	0.16	0.17	0.21	11.0	22.0	25.0
	—	0.14	0.21	0.17	22.0	26.0	26.0
	0.15	0.20	0.23	0.29	12.5	35.0	52.0

* C_p and C_b (μg/ml) are measured at various times after the ingestion.

Table 3. Influence of bile on the intestinal absorption of the factor M_1 (^3H)

Time after ingestion (min.)	Radioactive plasma concentration (μg/ml)	
	Control rats	Rats from which the bile was deviated out
40	4.32±1.50 (6)	4.28±2.05 (4)
170	4.26±1.08 (6)	4.30±2.23 (4)

Mean values±s.d. (in brackets number of rats)

Table 4. Influence of the lymphatic system on the absorption of the factor M_1 (^3H)

Time after injection (min.)	C_l	C_{bl}
40	0.34	6.05
100	0.60	5.12
140	0.72	3.72
180	0.61	4.19
240	0.52	3.63

C_l : Radioactive lymph concentration as expressed in μg/ml of factor M_1

C_{bl} : Radioactive plasma concentration (μg/ml).

C. The Absorbed Dose

In order to estimate the total amount of factor M_1 (^3H) absorbed through the gastro-intestinal tract we used three different calculations: (1) the total amount of radioactivity present in all tissues, organs and body fluids accounted for 17.7±1.4 %* of the ingested dose. (2) after oral administration 29.7±4.1 μg were excreted by the bile between 0 and 170 minutes. For the same period the excretion rate after intravenous injection reaches 39.2±4.7 % of the dose. Assuming that the excretion process was identical in both cases it could be calculated that 29.7 μg would correspond to a total absorbed amount of 76±23 μg or 12.6 % of the ingested antibiotic to which approximately 6~7 μg excreted by urine were to be added. (3) Forty minutes after the administration, the difference between the ingested dose and the amount of radioactivity assayed in the gastro-intestinal tract and corrected for biliary excretion during this period represented 15~17 % of the dose.

It can therefore be concluded that some 15~18 % of the administered factor M_1 was absorbed through the gastro-intestinal tract, which, for 250 g rat, was equivalent to 110~135 μg of antibiotic.

D. Nature of the Compounds Excreted by the Bile

After intravenous injection of a mixture of factor M_1 (^3H) and M_1 (^{14}C), the biliary excretion of radioactivity was always equivalent for either tracer. The ratio dpm ^3H to dpm ^{14}C remained nearly constant for all the time (Table 5). Since the pattern of labelling of these two radiotracers was basically different (WILZBACH tritiation was largely a random precess, while ^{14}C biosynthesis was a specific one) the present observation might indicate that the antibiotic was not broken up before its passage through the liver and that the biliary excretion affects intact molecule or, at least, large fragments.

Thin-layer chromatographic analysis was carried out in order to identify the non-metabolized factor M_1 in the bile. Before and after enzymatic hydrolysis or physico-chemical destruction of the micellar structure, diluted samples of bile corresponding to the 0~40 minutes and to the 40~100 minutes period were extracted with dichloromethane and chromatographed. As presented in Table 6, 40~45 % of the total radioactivity passed into the dichloromethane extract but the radioscanning of their thin-layer chromatograms showed an overlapping of several peaks, with but a small proportion of factor M_1.

The previously described reverse isotopic dilution technic was then applied to the bile samples in order to quantitatively estimate the amount of the unchanged antibiotic.

Table 5. Biliary excretion of ^3H and ^{14}C radioactivity after intravenous injection of factors M_1 (^3H) (Wilzbach) + M_1 (^{14}C) (methionine)

	0 to 40 min.				0 to 100 min.			
	total μg	% dose	μg/g	$^3H/^{14}C$	total μg	% dose	μg/g	$^3H/^{14}C$
^3H	45.8±3.6	24.8±1	202±44	6.92±0.48	69.6±4.2	37.9±1	110± 8.5	5.53±0.11
^{14}C	58.1±2.6	23.9±1	257±57		96.5±3.9	39.7±1	155±13.5	

number of rats: 3. mean values±s.d.

* ±Mean value±s. $et_{0.10}$.

Table 6. Percentages of CH_2Cl_2 extracted radioactivity from bile samples after intravenous injection of factor M_1 (^3H)

Bile	First extraction	Second extraction after			Total extracted radioactivity
		Enzymatic hydrolysis		Physico-chemical destruction of the micelles	
		arylsulfatase	β glucuronidase		
0~40 min.	42.0±3.6 (8)	7.5 (2)	6.7 (2)	9.1 (4)	50.1±3.3 (8)
40~100 min.	40.1±3.6 (6)	6.7 (2)	6.5 (2)	14.3 (3)	50.1±7.4 (6)

mean values+s.e. number in brackets are the number of experiments

Table 7. Reverse isotopic dilution analysis of the biliary excreted radioactivity

Period	C_b		1 st extrration	2 nd extraction		
				β-D Glucuronidase	Arylsulfatase	Micelles destruction
0~40 min.	107.6±26.5	Extract.	42.0 %			
		Fact. M_1	8.6 %	0.4 %	0.4 %	0.4 %
			9.2±1.2 μg	0.45 μg	0.45 μg	0.45 μg
40~100 min.	44.7±12.2	Extract.	40.1 %			
		Fact. M_1	8.3 %	0.4 %	0.4 %	0.3 %
			3.7±1.3 μg	0.16 μg	0.18 μg	0.12 μg

Mean values from last 4 experiments±s.e.

Only 8~9 % of the total radioactivity corresponded to the factor M_1 which represented approximately 20 % of the total extracted labelleds compounds. None of the applied treatments enhanced this percentage (Table 7).

Table 8. Comparison between the values of angular coefficients and periods after intravenous and oral administration of factor M_1 (^3H).

Route of administration dose	Angular coefficient	T 1/2 min.
Intravenous 1 mg/kg	−1.050±0.095+	290±25
Per os 3 mg/kg	−1.114±0.145+	270±35

± mean values±s.e. $t_{0,10}$

Discussion

Whatever the route of administration of the labelled factor M_1, the angular coefficient and the period for the plasma elimination curves (cf. Fig. 1) were very similar if not identical (Table 8). In both cases the bile played the preponderant role in the elimination of drug and the antibiotic was simultaneously taken up by the tissues in a reversible step.

With the equations reported in Figs. 1 and 2 representing respectively the kinetics of factor M_1 plasmatic elimination and biliary excretion it was possible to calculate the theoretical plasmatic (C_p) and biliary (C_b) concentrations in terms of radioactivity at various times after the intravenous injection of factor M_1 at a dose of 1 mg/kg. Taking into account the fact that the plasmatic free fraction corresponded essentially to the unchanged drug[16] and that 8~9 % of the radioactivity excreted in the bile was due to non-metabolized molecules, it was also possible to calculate the (C_bM_1) and (C_pM_1) values for Factor M_1. The C_b/C_p and C_bM_1/C_pM_1 ratios times the value of biliary flow (mean measured value : 0.042 g/kg) gave an estimate of the excretion rate as a "biliary clearance" as defined by Cook *et al.*[5] *i.e.* "the plasma volume containing the amount of factor M_1 excreted per minute". In the present case, we observed indeed (cf. Fig. 1) that there was practically no latency between the time of injection and the first appearance in the

bile. Moreover, the biliary concentration (cf. Fig. 2) reached its maximum at the earliest collection period and fell logarithmically thereafter.

In terms of total radioactivity the ratio C_b/C_p was in favor of the bile for all the period of time explored. On the other hand the C_bM_1/C_pM_1 ratio, high at the beginning, decreased rapidly to a near unit value. In both cases the excretion rate slowed down with time. After oral administration the values of C_b/C_p and "clearance" were higher than after intravenous injection (Table 9).

Table 9. Values of C_b/C_p and C_bM_1/C_pM_1 and of "clearance" total$=C_b/C_p\times0.042$ and "clearance" factor M_1 $C_bM_1/C_pM_1\times0.042$ at various time after intravenous or *per os* administraction of factor M_1 (^3H).

Mode of administration	time in min.	Total radioactivity		Unchanged factor M_1	
		C_{bi}/C_p	Total "biliary clearance"	$\frac{C_b/C_p}{M_1\ M_1}$	Total "biliary clearance"
Intravenous injection	20	23.3	0.9	7.4	0.31
	40	11.8	0.50	3.8	0.18
	60	5.3	0.22	1.8	0.08
	80	4.2	0.18	1.2	0.05
	100	3.0	0.13	1.0	0.04
	120	2.1	0.09	0.5	0.02
per os ingestion	40	58	1.76	18.5	0.56

By reference to BRAUER (1959)[3] factor M_1 of virginiamycin should thus be classified in the category A grouping the compounds for which their bile to plasma concentration ratio is nearly one. The drug has little, if any, affinity for the hepatic transfer mechanisms. It penetrates into the bile as a result of a simple diffusion or osmotic filtration process. Among the antibiotics, streptomycin, neomycin and chloramphenicol behave similarly[18].

As reported by THOMSEN[17] a bacterial species is sensitive to virginiamycin if its growth is inhibited *in vitro* by a concentration of the antibiotic corresponding to 1/3 or 1/5 of the plasma level, a ratio which, according to our observations, corresponds to the value of the plasmatic free fraction. The resistance limit is fixed at $\pm5\,\mu g/ml$ and in general the staphylococci are sensitive to concentrations below or equal to $0.2\,\mu g/ml$. Forty minutes after oral administration the factor M_1 plasmatic free concentration reaches 0.6 to $0.7\,\mu g/ml$ and after intravenous injection around $2.0\,\mu g/ml$. At the same time the average tissue levels are respectively 0.6 and $1.2\,\mu g/g$.

In connection with our present results, the clinical trials reveal the efficiency of the drug in the treatment of the Gram-positive bacterial infections and the rapid onset of its action. Among all the reported cases it is interesting to emphasize in particular the great utility of the *per os* treatment against cutaneous infections[1,20] or septicemiae.

All those observations might be interpreted in terms of the importance of the plasmatic compartment as a reservoir for the antibiotic, the rapidity of tissue fixation and the special affinity of the factor M_1 for the skin.

After oral administration the blood and tissue levels are essentially lower than those obtained after intravenous injection but high enough. Indeed the extreme rapidity of the absorption and the tissular distribution throughout the plasma can account for the therapeutic efficiency of this antibiotic.

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