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METABOLIC STUDIES OF PACTAMYCIN*

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Abstract—Pactamycin levels in serum or urine could be determined either by a tissue culture assay or the disc-plate assay using *Bacillus subtilis*. After intravenous administration, the antibiotic level in the sera of various animal species decreased rapidly, with complete disappearance of the activity within 30 min. This decrease was not attributable to inactivation by blood or irreversible binding by tissues. Excretion in urine accounted for less than 1 per cent of the total antibiotic injected. *In vitro*, pactamycin was degraded by liver slices of dog at the rate of 487 μg/hr per g liver slice. If the counterpart of the *in vitro*-system operates *in vivo*, then the rapid inactivation of pactamycin and low recoveries *in vivo* can be explained. Cancer patients treated with the drug also showed low blood levels and poor recovery from urine; however, patients suffering from extensive liver metastasis had higher blood levels and excreted more pactamycin than those with no liver involvement. This could be attributed to the inability of the metastatically involved liver to degrade the antibiotic and could account for the somewhat more violent reactions of these patients to the drug.

PACTAMYCIN (NSC 52947) is a new antibiotic active *in vitro* against KB human epidermoid carcinoma cells and a variety of gram-positive and gram-negative microorganisms. *In vivo*, it shows a broad spectrum of antitumor activity in mice and hamsters. The biological and chemical properties of the antibiotic have already been reported.[1, 2] Pactamycin is being tested clinically in cancer patients under the aegis of the Cancer Chemotherapy National Service Center. The work reported here describes the rapid disappearance of this antibiotic from the blood of various animals and its inactivation by liver and kidney tissues *in vitro*. Preliminary results of the determination of pactamycin levels in the blood and urine of cancer patients being treated with this antibiotic are also reported.

METHODS AND MATERIALS

Bioassay methods

Since pactamycin was active *in vitro* against KB cells and *Bacillus subtilis*, both systems could be used to assay antibiotic levels in biological fluids. It is possible that pactamycin might be converted in the body to a *B. subtilis*-active but KB-inactive compound, so that erroneous results might be obtained if only *B. subtilis* assay were used; accordingly, both assays were employed for selected samples.

The method of Smith *et al.*[3] was used to determine pactamycin activity against KB cells growing in tissue culture. The dose-response curves obtained when the anti-

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biotic was dissolved in water or in dog serum are shown in Fig. 1. The ID_{50} of pactamycin was 0·021 μg/ml in the presence of dog serum, plus calf serum, and 0·021 μg/ml in calf serum alone.

Since the *B. subtilis* disc-plate assay was simpler, more rapid, and correlated well with the tissue culture assay, it was routinely used in these experiments. Streptomycin assay agar medium inoculated with a suspension of *B. subtilis* spores was used to

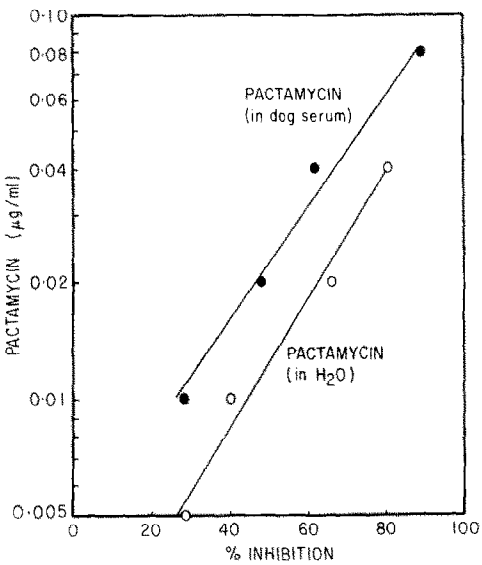

FIG. 1. Dose-response curves for pactomycin, diluted in either dog serum or water, in the tissue culture assay.

prepare the agar plates. Appropriate solutions of pactamycin were prepared with phosphate buffer (pH 6·5; 0·1 M) prior to use. Paper discs (13 mm diameter) containing 0·08 ml of a pactamycin solution (0·1 to 2 μg/ml) were applied to the agar plates; the zones of inhibition were measured after 18-hr incubation at 30°. Each determination was done in duplicate at three different dilutions. The zones produced by pactamycin added to biological fluids were not identical with those in the phosphate buffer, as shown in Fig. 2; therefore, standard curves for each series of determinations were obtained with appropriate dilutions of pactamycin in the biological fluid being assayed. The appropriate biological fluid was obtained prior to injection of the drug.

Animal studies

Dogs, monkeys, rats, rabbits, and hamsters were used in these experiments. All animals were injected with 0·5 mg pactamycin/kg body weight, unless otherwise specified. This dose level was lower than the LD_{50}.

Adult beagle mongrel dogs (8 to 10 kg in weight) were injected in the cephalic vein; blood was withdrawn from the jugular vein. New Zealand white rabbits (4 to 5 kg) were injected in the marginal ear vein and blood samples taken from the same vein. Upjohn Sprague–Dawley rats (0·2 to 0·3 kg) were injected in the tail vein and blood samples obtained by heart puncture. Rhesus monkeys (4 to 5 kg) were injected

in the saphenous vein and blood samples taken from the anterior vena cava. Syrian golden hamsters (male, 80 to 120 g) were anesthetized with CO_2, and a small incision was made to expose the external jugular vein near the pectoralis muscle. The hamsters were injected in the exposed vein, and blood samples were taken by heart puncture. Different hamsters were used for every blood sample taken, and the results obtained with two animals for each sampling time were averaged.

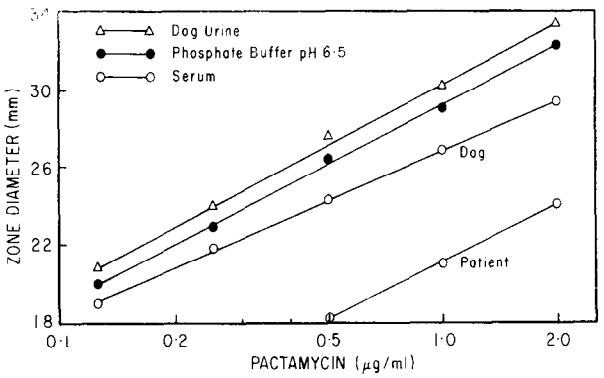

FIG. 2. Dose-response curves for pactamycin, diluted in either dog serum, dog urine, human serum, or phosphate buffer (pH 6·5; 0·1 M), in the *B. subtilis* disc-plate assay.

Dogs were used to determine the excretion pattern of pactamycin; they were housed in metabolism cages and given free access to water but no food. The urine was collected by indwelling catheterization, in bottles packed in ice, for 24 hr. Both dogs vomited several times after the injection; the vomit was assayed. The feces were assayed after homogenization in phosphate buffer (pH 6·5; 0·1 M).

Tissue metabolism of pactamycin

A Potter–Elvehjem homogenizer was used to prepare 10 per cent (wet weight per volume) tissue homogenates. Tissue slices of 0·3 to 0·5 mm thickness were prepared with a freezing microtome. One gram of tissue (either as homogenate or as slices) in 10 ml of various suspending media, was mixed with pactamycin and incubated on a reciprocating shaker at 37°. Samples were taken at intervals, and degradation of pactamycin by the tissue was stopped by pipetting the sample into 50% ethanol.

The following suspending media were used in these experiments: isotonic saline (0·9%), isotonic sucrose (0·25 M; 8·55%), Krebs–Ringer phosphate[4] supplemented with either glucose (0·27%), L-glutamic acid (0·078%), fumaric acid (0·036%), pyruvic acid (0·042%), ATP (0·001%), NAD (0·001%), or NADP (0·001%). L-Glutamic, pyruvic, and fumaric acids were neutralized with $NaHCO_3$ and included in the medium as sodium salts.

Chromatography

Qualitative identification of pactamycin was made by the method of Brodasky and Lummis.[5] When pactamycin in serum was chromatographed, it was necessary to precipitate the protein with 3 vol. ethanol, remove the alcohol under vacuum, and dilute the sample to its original volume; the results obtained with and without this

treatment are shown in Fig. 3. Such treatment of the urine samples was not necessary. Each sample was spotted alone, and mixed with a pactamycin standard; thus any variation in the Rf of pactamycin attributable to constituents of serum and urine was obviated.

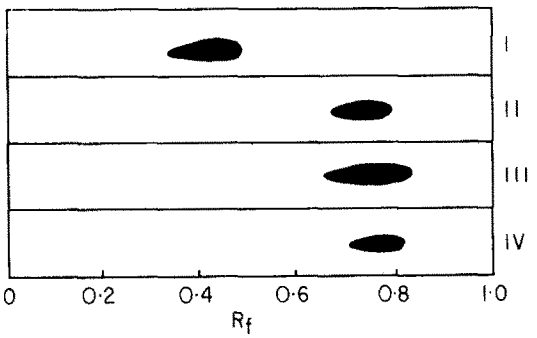

FIG. 3. Bioautography of pactamycin in untreated serum and serum treated with alcohol. The paper was developed in a solvent system consisting of n-butyl acetate:1-butanol-10% aqueous formic acid (4:1:5). I, Untreated serum; II, alcohol-treated serum; III, alcohol-treated serum plus pactamycin standard; IV, pactamycin standard.

Clinical dosage form of pactamycin

The clinical dosage form of pactamycin used in these experiments contained pactamycin, 5 mg; anhydrous citric acid, 1·72 mg; sodium chloride, 80 mg; and lactose, 120 mg. Prior to use, the preparation was dissolved in 10 ml of sterile water containing 0·9 w/v benzyl alcohol, to give a solution of pH 4·2 containing 500 μg pactamycin/ml.

RESULTS

Pactamycin stability

In view of the relative instability of pactamycin in aqueous solution, its recovery after storage at different pH values, temperatures, and in different suspending media was studied. Pactamycin, in human serum, urine, or phosphate buffer (pH 5 to 7), was stable for at least 7 days when stored frozen, for 1 day when stored at 4°, or for 2 hr at 37°. When pactamycin was stored at 37° for 24 hr in phosphate buffer of pH 5, 6, or 7 the percentage of activity recovered was 61, 54, and 6·5 respectively. Under the same conditions 36 per cent of the activity was recovered in human serum and 68 per cent in the clinical dosage form (in solution). The stability of the clinical dosage form might be accounted for by the weak acidity (pH 4·2) of the solution. Pactamycin (solid) stored for three months in the clinical dosage form at 4°, 25°, and 40° retained 90, 89, and 52 per cent of its activity respectively.

Blood level in different animals

Pactamycin was injected intravenously into different species of animals, and blood samples were taken at short intervals. The blood was allowed to clot and the serum assayed against the proper controls. The rapid disappearance of pactamycin from blood in all species studied is shown in Table 1. By 30 min the pactamycin level was below the limits of sensitivity of the bioassay ($< 0{\cdot}1$ μg/ml).

Since blood accounts for approximately 6 to 8 per cent of the body weight, if even distribution of pactamycin throughout the vascular system is assumed, at least 5 μg pactamycin/ml blood would be expected immediately after an intravenous injection of 0·5 mg/kg. The data show, however, that less than 10 per cent of the injected pactamycin was recovered in the serum of all species (except the monkey, in which 16 per cent recovery was obtained) 1 min after injection.

TABLE 1. LEVEL OF PACTAMYCIN IN SERUM OF DIFFERENT ANIMALS SPECIES*

Animal	Pactamycin (μg/ml) 1 min	5 min	10 min	30 min
Dog	0·3	0·2	0·15	<0·1
Monkey	0·8	0·46	0·2	<0·1
Rabbit	0·9	0·5	0·3	<0·1
Rat	0·34	0·14		<0·1
Hamster	0·49	0·12†	<0·1	

* All animals were injected intravenously with 0·5 mg pactamycin/kg body weight except the rabbit, which was given 1 mg/kg.
† Three-minute sample.

TABLE 2. RECOVERY OF ADDED PACTAMYCIN FROM BLOOD

Blood fraction	Added pactamycin (μg/ml)	Recovered pactamycin (μg/ml)	Recovery (%)
Serum	11·6	13·1	113
Plasma	11·6	11·9	102
Oxalated blood	11·6	11·1	96
Hemolyzed blood	11·6	10·9	94

Such low recovery in the blood might be explained by (1) rapid degradation of pactamycin or its binding by blood or tissues, or (2) rapid excretion of pactamycin.

Pactamycin incubated for 30 min with whole blood *in vitro* was recovered in good yields (94 to 113%) from serum, plasma, oxalated blood, and hemolyzed blood (Table 2). The blood was collected in heparinized tubes for preparation of plasma. Rapid freezing and thawing were used to obtain hemolyzed blood. Since good recovery (94 to 113%) was obtained in all cases, pactamycin is not bound to blood constituents. Pactamycin also was stable in blood, *in vitro*, for 2 hr at 37°.

Serum levels and renal excretion in dogs

The alternative possibility, that pactamycin was rapidly excreted, was investigated with dogs. The results given in Table 3 indicate that of a total of 5 mg of pactamycin injected, less than 1 per cent could be recovered from the urine, feces, and vomitus. Bioautographic examination of the serum and urine samples showed that pactamycin was the only bioactive component present.

The level of pactamycin in dog serum also was determined by the activity of the agent on KB cells growing in tissue culture; the levels determined in this manner correlated well with the results obtained with the *B. subtilis* bioassay. Accordingly, on the basis of its chromatographic characteristics, its effect on *B. subtilis* and its ability

to inhibit KB cells in tissue culture, the antibiotic present in serum and urine was characterized as pactamycin.

The low levels of pactamycin obtained in blood immediately after injection and the low recovery from urine suggested that pactamycin either was inactivated in other tissues or was concentrated and stored. The latter alternative was tested by preparing

TABLE 3. BLOOD LEVELS AND EXCRETION OF PACTAMYCIN IN THE DOG*

Sampling time	Pactamycin in serum (μg/ml)	Pactamycin recovered (μg)		
		Urine	Feces	Vomit
1 min	0·24			
5 min	0·15			
10 min	<0·1			
30 min	trace	57		
1 hr	0	10		
2 hr		10		
6 hr		0		
12 hr		0	0	5
24 hr		0	0	0

* The dogs were injected intravenously with 5 mg pactamycin.

20 per cent tissue homogenates (liver, gastrocnemius muscle, kidney, and spleen) in isotonic saline 10 and 30 min after an intravenous injection of 0·5 mg/kg to a dog. Pactamycin activity was not detected in the liver and muscle homogenate, and only 0·1 μg/ml was found in the kidney and spleen homogenates. These results indicated that pactamycin was not bound to an appreciable extent, at least not in an easily extractable form, by the tissues.

Pactamycin inactivation by tissues

Pactamycin metabolism by 10 per cent (wet weight per volume) homogenates of various tissues (liver, kidney, spleen, and heart) prepared in isotonic saline was studied. No degradation of pactamycin by such homogenates was observed after incubation for 1 hr at 37°. Different suspending media, supplemented with various metabolites, were used to maintain enzymatic activity in tissue homogenates;[6] therefore, degradation of pactamycin by liver homogenates was studied in different suspending media, supplemented with various metabolites, and under anaerobic and aerobic conditions. The results presented in Table 4 show that greater degradation of the antibiotic was obtained with Krebs–Ringer phosphate than with either isotonic saline or sucrose solutions as the suspending medium. Under nitrogen, antibiotic degradation was lower than in an aerobic atmosphere. The greatest inactivation of pactamycin occurred when Krebs–Ringer phosphate was supplemented with various metabolites, ATP, NAD, and NADP; similar results were obtained with homogenates of kidney, spleen, or muscle.

Since the number of physically damaged cells is much smaller in slices than in minces or homogenates of tissues, it is reasonable to assume that slices are nearer to the natural state of the tissue than homogenates; therefore, the metabolism of pactamycin *in vitro* by tissue slices was studied, and the results obtained with liver

slices are given in Table 5. The rate of pactamycin degradation was higher in liver slices suspended in Krebs–Ringer phosphate supplemented with various metabolites than in saline; similarly, liver slices suspended in serum showed a much higher rate of degradation when supplemented with metabolites. The antibiotic left at the end of the period of incubation with liver slices was characterized as pactamycin by bioautography. Under similar conditions, kidney, heart, and spleen slices (1 g) degraded approximately 150 μg of the added pactamycin in 1 hr.

TABLE 4. EFFECT OF MEDIUM ON THE DEGRADATION OF PACTAMYCIN BY DOG LIVER HOMOGENATE*

Suspending medium	Atmosphere	Total pactamycin degraded (μg/hr/g liver)
Saline (0·9%)	air	0
Sucrose (8·55%)	air	17
Krebs-Ringer phosphate	air	123
K-R phosphate	N_2	53
K-R phosphate†	air	137
K-R phosphate‡	air	173

* One gram of homogenized liver was mixed with 1 mg of pactamycin and incubated for 1 hr at 37°. The reaction was stopped by pipetting the sample into 50% alcohol.

† Krebs–Ringer phosphate supplemented with glucose, glutamate, fumarate, and pyruvate.

‡ Krebs–Ringer phsophate supplemented with above metabolites and ATP, NAD and NADP.

TABLE 5. DEGRADATION OF PACTAMYCIN BY SLICES OF DOG LIVER*

Suspending medium	Atmosphere	Pactamycin degraded (μg/hr/g liver slice)
Saline (0·9%)	air	320
Krebs-Ringer phosphate	air	487
K-R phosphate†	air	440
K-R phosphate‡	air	393
K-R phosphate‡	N_2	355
Serum	air	133
Serum§	air	355

* One gram of liver slices was mixed with 500 μg pactamycin and incubated at 37°. The reaction was stopped by pipetting the sample into 50% ethanol.

† Supplemented with glucose, glutamate, fumarate, and pyruvate.

‡ Supplemented with glucose, glutamate, fumarate, pyruvate, ATP, NAD, and NADP.

§ Supplemented with glucose, fumarate, pyruvate, and ATP.

Since 1 g of liver slices degrades 487 μg of added pactamycin in 1 hr, a dog with a 500-g liver could inactivate approximately 250 mg pactamycin/hr or 4 mg pactamycin/min. If the counterpart of the *in vitro*-system operates *in vivo*, then the rapid inactivation of pactamycin and low recoveries *in vivo* can be explained.

The degradation of pactamycin by tissue slices could be stopped by inactivating the liver proteins with 50 to 75% ethanol.

*Serum and urine levels in cancer patients**

The pactamycin levels obtained are shown in Table 6; BJ-47-F, LC-52-F, and WM-67-M were given 20 μg/kg intravenously; the remaining subjects received 80 μg/kg. The results indicate that pactamycin rapidly disappears from blood in man, as had been observed previously with other animals; less than 5 per cent of the total pactamycin injected was recovered in the urine. Although WR-47-M was given the same dose as were KH-66-F and BV-44-F, no pactamycin was present in his urine; this might be attributable to the efficient degradation of the antibiotic by the liver. KH-66-F and BV-44-F suffered from extensive liver metastases, and this could account for lesser degradation and greater excretion of the antibiotic. The inability of the liver to cope with the pactamycin may account for the somewhat more violent reaction experienced by the patients with hepatic impairment.

The antibiotic in the serum and urine was identified as pactamycin by bioautography.

DISCUSSION

Within 1 min after intravenous injection of pactamycin into animals, the concentration in blood was less than 10 per cent of the value anticipated by assuming even distribution throughout the vascular system. Since only approximately 1 per cent of the antibiotic was recovered in the urine and feces, rapid excretion would not account for the low levels in blood. Quantitative recovery of pactamycin from different blood fractions indicated that the antibiotic was neither irreversibly bound nor inactivated by any of the blood consituents. Since none of the organ homogenates contained any antibiotic, it was assumed that the antibiotic was not concentrated by organs; however, it is possible that pactamycin might be irreversibly bound by the tissues. The concentration of pactamycin in the suspending medium decreased rapidly when the antibiotic was incubated with tissue homogenized in Krebs–Ringer phosphate; no such loss was observed when isotonic saline was used as the suspending medium. The loss of pactamycin could be attributed to either inactivation by tissue enzymes or irreversible binding by tissue proteins. When liver slices were used, and the suspending medium was supplemented with metabolites, pactamycin was lost at a rate much greater than that obtained with homogenates. This increase in the rate of loss of pactamycin, when more nearly physiological conditions were used, would indicate possible metabolic destruction of pactamycin rather than irreversible binding by tissues. Radioactive pactamycin will be used in future studies more definitely to clarify this point. Similar degradation of mitomycin by liver, under anaerobic conditions, has been oberserved.[7] The degradation was stimulated by the addition of nicotinamide, adenosine triphosphate, and phosphopyridine nucleotides.

Results similar to those obtained with pactamycin have been observed with alkylating agents. In mice, 90 per cent of the radioactivity of intravenously injected ^{14}C-labeled nitrogen mustard disappeared from the blood within 30 sec.[8] Although no tissue localization was observed, the rapid interaction of the alkylating agent with tissues could account for their disappearance from blood.

* These patients were treated by Dr. Donald Korst of the St. Joseph Mercy Hospital, Ann Arbor, Mich., and by Dr. Bertha Isacs, Wesley Memorial Hospital, Chicago, ill. The blood and urine samples were obtained through their cooperation.

TABLE 6. LEVELS OF PACTAMYCIN IN THE SERUM AND URINE OF CANCER PATIENTS

Patients*	Cancer	Injected (mg)	Serum level (μg/ml)			Pactamycin in urine (μg)			Total urinary recovery (μg)
			1 min	5 min	15 min	2 hr	4 hr	8 hr	
BJ-47-F	Bronchial carcinoma	1·10	0·14	0	0	24	9·4	0	33·4
LC-52-F	Glioblastoma	1·4		0					
WM-67-M	Carcinoma of penile urethra with metastases	1·82	0·105	0	0	6·6	6·6	0	13·2
KH-66-F	Colon carcinoma and liver metastases	3·5	1·05	0·43	0	34	7·5	13·8	55·3
SM-67-F	Carcinomatoses	3·64		0·3					
WR-47-M	Bladder carcinoma	4·4	0·88	0·26	0	0	0	0	0
BV-44-F†	Breast carcinoma with liver metastases	5·0	1·17	0·38	0·21	51·6	64	22·5	138·1
SS-56-M	Scirrhous adenocarcinoma of lung	5·76		0					
RW-32-F	Melanoma	7.12		0.3					

* The patients' initials, age, and sex are indicated.

† BV-44-F had no antibiotic activity in 30% homogenates of brain tumor, liver tumor, spleen, kidney, muscle, or liver at autopsy 48 hr after last intravenous injection of pactamycin.

Pactamycin caused approximately 90 per cent inhibition of the growth of Walker 256 tumor in rats and of human choriocarcinoma in hamsters.[1] In view of the low blood levels of the antibiotic, its antitumor activity is surprising. Studies with radioactive pactamycin might indicate a specific localization of the drug in the tumor or conversion of the drug to a substance inactive in the *B. subtilis* assay, but with antitumor activity *in vivo*.

Rapid disappearance of pactamycin from blood also was observed in cancer patients. Those with extensive hepatic metastases excreted more pactamycin than did patients without involvement of the liver; this could be attributed to the inability of the metastatically involved liver to degrade the antibiotic and could account for the somewhat more violent reactions of these patients to the drug.

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