AN *IN VIVO-IN VITRO* COMPARISON OF THE 2-AND 3-PHOSPHATE ESTERS OF CLINDAMYCIN

T. F. BRODASKY and C. LEWIS

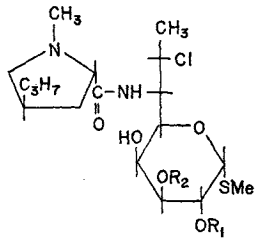
Research Laboratories, the Upjohn Company
Kalamazoo, Michigan 49001, U.S.A.

(Received for publication December 21, 1971)

Culture *Streptomyces coelicolor* MÜLLER NRRL 3532 deactivates clindamycin. The inactive compound identified as clindamycin-3-phosphate on treatment with dephosphorylating enzymes affords the active compound, clindamycin. In as much as the 2-phosphate ester of clindamycin is being clinically evaluated as an injectable form of clindamycin, it was of interest to compare its *in vitro* and *in vivo* characteristics with the 3-phosphate ester. Analysis of the kinetics of hydrolysis of the esters in three *in vitro* enzyme systems indicate marked differences, with the 3-ester being hydrolyzed much more slowly and much less extensively than the 2-ester. An *in vivo* study in rats revealed lower blood levels for the 3-phosphate when compared with the 2-phosphate and may be related to the rates of hydrolysis. Metabolism studies indicate that the esters are metabolized in a similar manner to the parent clindamycin.

The antibiotic clindamycin* (I) is 7-deoxy-7(S)-chlorolincomycin[7], for the structure see Fig. 1.

Fig. 1.



	R_1	R_2	
I	H	H	*Clindamycin*
II	H_2PO_3	H	*Clindamycin-2-phosphate*
III	H	H_2PO_3	*Clindamycin-3-phosphate*

Studies of the biomodification of clindamycin revealed that actively growing cultures of *S. coelicolor* MÜLLER NRRL 3532 deactivated clindamycin (*in vitro*). The inactive compound on treatment with alkaline phosphatase afforded clindamycin[3]. The proposed structure III is shown in Fig. 1.

Since clindamycin-2-phosphate (II)[9] is currently under clinical investigation as an injectable form of clindamycin, it was of interest to compare the *in vitro* and *in vivo* biological properties of the two esters.

The kinetics of hydrolysis of both the 2- and 3-phosphate esters of clindamycin were studied in three *in vitro* enzyme systems. Determination of K_m values allowed certain conclusions to be made concerning the biological activity and structural

* The registered trade mark of the Upjohn Company for clindamycin is Cleocin.

relationships of the two esters. *In vivo* investigations included comparison of the T_{50} and areas under blood level curves, mouse protection studies and metabolism.

Experimental Section

In vitro Studies

All hydrolyses, including those for the LINEWEAVER-BURK plot were carried out in sealed vials at 37°C in a Lauda constant temperature water bath. For each enzyme system, duplicate sets of reactions were run covering time periods from 0 to 72 hours. Each sample on removal from the bath, was prepared for storage at −10°C. This procedure was followed until all samples samples were collected for simultaneous quantitation. The following special procedures were used for individual enzyme systems.

Human Plasma

Fresh human blood, 250 ml, was treated with 2,000 units of heparin. After standing 30 minutes, the blood was centrifuged for 15 minutes at 5,000 rpm in a refrigerated centrifuge. Two-ml aliquots of the resulting plasma were spiked with 5 mcg/ml of either clindamycin-2-phosphate or clidamycin-3-phosphate from aqueous stock solutions (1 mg/ml) of each. One sample was immediately frozen as the 0-hour control and the remainder were then incubated for $^1/_2$, 1, 2, 4, 8, 16, 24 and 48 hours. As each sample was removed from the bath it was immediately frozen for subsequent quantitation.

Alkaline Phosphatase

A 5 mcg/ml solution of Cal-Biochem, calf intestine, B grade alkaline phosphatase (approximately 8 PNPP units) in 0.5 M, pH 9.0 tris buffer was prepared. Five mcg/ml of either clindamycin-2- or 3-phosphate were added to 2 ml aliquots of the enzyme solution. The enzyme solution and spiked samples were held in an ice bath prior to immersion in the 37°C water bath. The samples were incubated for 10, 20, 30, 40 and 50 minutes, 1, 2, 4 and 8 hours for the 2-phosphate and $^1/_2$, 1, 2, 4, 8, 16, 28 and 48 hours for the 3-phosphate. A zero-hour sample was prepared by spiking a 2-ml aliquot of enzyme solution with 5 mcg/ml of either 2- or 3-phosphate and immediately adding 1 volume of acetone. After agitation and filtration, the filtrate was reduced in volume and subsequently brought to the original 2 ml volume with tris buffer. All hydrolysis samples were treated in a like manner as they were removed from the bath. The resulting solutions were frozen subsequent to quantitation. To generate data for a LINEWEAVER-BURK plot, the same procedure was followed using alkaline phosphatase; however, all samples were incubated for 6 hours.

Rat Liver Homogenate

A 20/1 dilution of stock rat liver homogenate in 0.5 M pH 8.0 tris buffer was prepared. Two-ml aliquots were spiked with 5 mcg/ml of clindamycin-2- or 3-phosphate. The samples were treated as described above except that a 72-hour sample was included for the 3-phosphate.

Quantitation

The disc plate technique was used for quantitating the hydrolysis samples. Clindamycin ·HCl was the primary standard and serial dilutions (in plasma or tris buffer) of 5～0.3125 mcg/ml were used. Two sets of standard discs (20 mcl solution on 6.35 mm S & S paper discs) were plated on each tray with a complete set of 2- and 3-phosphate hydrolysis samples. Lincomycin sensitive *Sarcina lutea* was used as the detecting organism and all trays were incubated at 34°C for 16 hours[1]. Zone diameters were read to the nearest 0.5 mm (about 5 % average error for assay values falling within the limits of the regression line).

In vivo Studies

For blood level determinations, the clindamycin equivalent *in vitro* bioactivity of the hydrolyzed phosphate esters was measured by the standard-curve plate bioassay described by HANKA[5]. The technique used *Sarcina lutea* (UC-130) as the assay organism and had

a sensitivity of 0.16 mcg/mg. The free base has an assigned potency of 1,000 mcg/mg and the activities of the esters were calculated and reported as the free base equivalents per mg of antibiotic.

Protection studies were performed in 10-mouse groups of CF-1 male albino mice averaging 18~20 g. The animals were selected randomly from pools and infected experimentally by injecting intraperitoneally approximately 100 median lethal doses (LD_{50}'s) of a standardized suspension of *Staphylococcus aureus* UC-76. Maintenance of the culture and the method of inducing infections have been described in detail previously[6]. Subcutaneous treatment of the infected mice with the esters was initiated immediately with incremental doses of the antibiotic contained in 0.2 ml of water. The mice were treated once daily for four days. Evaluation of antibiotic activity was based, after 7 days, on the median protective dose (CD_{50}) of the ester compared to the CD_{50} calculated for a clindamycin-treated control group. Calculations were made with an IBM 360 computer using the method of SPEARMAN and KARBER[13].

Antibiotic concentrations, including clindamycin and active metabolic products, found in whole blood were determined in 150 g Sprague-Dawley rats*. Clindamycin or its phosphate monoesters were dissolved in 0.05 M PO_4 buffer, pH 7.0 at 3 mg/ml and three-rat groups dosed subcutaneously with 0.5 ml/rat (10 mg/kg). At specified times, free flowing whole blood sufficient to saturate 1/4" S & S paper discs (0.02 ml/disc) was collected from the rats by clipping portions of their tails. The saturated discs were placed on *Sarcina lutea* seeded medium, pH 8.0 in assay trays and incubated at 32°C for 16~18 hours. A standard assay curve was determined on each tray using serially diluted clindamycin solutions of 5~0.156 mcg/ml. Antibiotic cncentrations in whole blood were estimated from the standard curve. Evaluation of absorption was based on a comparison of the amounts of antibiotic in the blood as measured by the area under the time *vs.* concentration curves, time at which 50 % of the area (T_{50}) was under the curve and the time of maximum concentrations. Results were calculated using an IBM 360 computer.

Results and Discussion

In vitro Studies

The quantitative results for the various *in vitro* enzyme systems are given in Tables 1, 2 and 3. Factors accoung for the theoretical value of clindamycin which could be generated from the amount of clindamycin phosphate used, and the quantitation of clindamycin free base (present in the hydrolysis solutions at pH 8) using clindamycin ·HCl as a standard, were applied to the calculation of clindamycin phosphate concentrations.

Since the quantitations were carried out using duplicate experiments spotted on two bacteria seeded trays, the four standard regression lines (2 per tray) were analyzed by an ANOCV program with a Hewlett-Packard 9100 A comduter for significance of the regression coefficients a and b in the least squares line.

$$Y = a + b \log x :$$

Based on these calculations the data were pooled to form a single standard curve for quantitation. Regression statistics (r, $S_{y \cdot x}$)[12] for these lines appear in Tables 1, 2 and 3. Once the clindamycin phosphate concentration had been calculated, a plot of $[ClPO_4]_t$ *vs.* time was constructed to determine the asymptotic phosphate concentration,

* Rat strain-Upj : TUC(SD) spf. Animals used in the research described in this paper were maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

C_∞. This value was then used in the rate equation :

$$\ln (C_t - C_\infty) = -kt + \ln (C_0 - C_\infty)$$

to test for first order kinetics[1]. The kinetic values are given in Table 4. Examination of the PEARSON's correlation coefficients reveals a very high degree of linear correlation and indicates a first order model is applicable. However, using the data from various hydrolysis reactions, tests for other kinetic models were made. Using the method of MULLET and NODDINGS[10] all reactions proved to be first order or between first order and three halves order. Applying least squares regression analysis to the data to obtain second order reaction plots and examination of the PEARSON's correlation coefficients for these lines revealed a higher degree of linear correlation for first order than for second order plots. Other methods as well support a first order model and for comparative purposes this appears suitable. A plot of percent conversion of phosphate ester *vs.* time is shown in Fig. 2. The curves reflect

Table 1. Hydrolysis of clindamycin-2-and 3-phosphate-human plasma

2-Phosphate

Hrs.	$[Cl]_t$ Corr.[a]	% Conv.	$[ClPO_4]_t$	$[ClPO_4]_t$ Corr.[b]
0	0.00	0.0	5.00	3.30
0.5	0.35	8.4	4.58	2.88
1	0.60	14.3	4.29	2.59
2	0.63	15.0	4.25	2.55
4	0.81	19.2	4.04	2.34
8	1.31	31.2	3.44	1.74
16	1.76	41.8	2.91	1.21
24	2.35	55.9	2.21	0.51
48	2.72	64.7	1.77	0.07

$C_\infty = 1.70$

3-Phosphate quantitation : No observable hydrolysis

Regression Statistics : $r = 0.9961$. $S_{y.x} = 0.4703$.

a) Corrected for quantitating clindamycin free base using clindamycin ·HCl ; all conc. are in mcg/ml.

b) Corrected for C_∞.

Table 2. Hydrolysis of clindamycin-2- and 3-phosphate-alkaline phosphatase

2-Phosphate

Hrs.	$[Cl]_t$ Corr.[a]	% Conv.	$[ClPO_4]_t$	$[ClPO_4]_t$ Corr.[b]
0	1.51	35.9	3.21	2.76
0.17	1.67	39.6	3.02	2.57
0.33	1.99	47.1	2.64	2.19
0.50	2.21	52.4	2.54	2.09
0.67	2.28	54.0	2.46	2.01
0.83	2.35	55.8	2.37	1.92
1	2.43	57.8	2.11	1.66
2	2.79	66.3	1.69	1.24
4	3.09	73.4	1.33	0.88
8	3.67	87.1	0.65	0.20

$C_\infty = 0.45$

3-Phosphate

Hrs.	$[Cl]_t$ Corr.[a]	% Conv.	$[ClPO_4]_t$	$[ClPO_4]_t$ Corr.[b]
0	0	0	5	4.05
2	0.58	13.8	4.31	3.36
4	0.79	18.8	4.06	3.11
8	1.22	29.1	3.55	2.60
16	1.72	40.9	2.95	2.00
24	2.05	48.6	2.57	1.62
48	3.04	72.2	1.39	0.44

$C_\infty = 0.95$

Regression statistics : $r = 0.9967$. $S_{y.x} = 0.4914$.

a) See Table 1. b) See Table 1.

Table 3. Hydrolysis of clindamycin-2- and 3-phosphate-rat liver homogenate

2P-hosphate

Hrs.	$[Cl]_t$ Corr[a].	% Conv.	$[ClPO_4]_t$	$[ClPO_4]_t$ Corr.[b]
0	0	0	5.00	3.62
0.17	0.42	10.1	4.50	3.12
0.33	0.74	17.7	4.11	2.73
0.50	1.02	24.3	3.79	2.41
0.67	1.18	28.0	3.60	2.22
0.83	1.40	33.3	3.34	1.96
1	1.50	35.7	3.22	1.84
2	2.29	54.5	2.28	0.90
4	2.84	67.4	1.63	0.25
8	3.05	72.4	1.38	—
16	3.05	72.4	1.38	—

$C_\infty = 1.38$

3-Phosphate

Hrs.	$[Cl]_t$ Corr[a].	% Conv.	$[ClPO_4]_t$	$[ClPO_4]_t$ Corr.[b]
0	0	0	5	1.01
4	0.23	5.6	4.72	0.73
8	0.31	7.3	4.63	0.64
16	0.44	10.4	4.48	0.49
24	0.56	13.3	4.33	0.34
48	0.85	20.2	3.99	—
72	0.85	20.2	3.99	—

$C_\infty = 3.99$

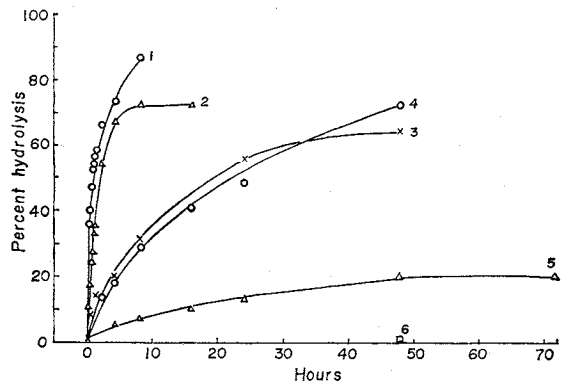
Regression statistics : $r = 0.9958$. $S_{y.x} = 0.6767$.

a) See Table 1. b) See Table 1.

Table 4. First order kinetic values for enzymatic hydrolysis of clindamycin-2- and 3-PO_4

Regression values	Human plasma		Alkaline phosphatase		Rat liver homogenate	
	2-PO_4	3-PO_4	2-PO_4	3-PO_4	2-PO_4	3-PO_4
$-b(k)$	0.0774	—	0.3100	0.0437	0.6607	0.0423
a	1.16	—	0.9240	1.3592	1.2415	0.0692
r	−0.9955	—	−0.9932	−0.9928	−0.9995	0.9891
r^2	0.9910	—	0.9864	0.9857	0.9990	0.9783
$t\ ^1/_2$ (hrs)	8.6	—	2.24	15.85	1.05	16.40

Fig. 2. Percent hydrolysis of clindamycin phosphate esters in enzyme systems
1. Clindamycin-2-PO_4 in alkaline phosphatase
2. Clindamycin-2-PO_4 in rat liver homogenate
3. Clindamycin-2-PO_4 in human plasma
4. Clindamycin-3-PO_4 in alkaline phosphatase
5. Clindamycin-3-PO_4 in rat liver homogenate
6. Clindamycin-3-PO_4 in human plasma



the significant differences in the hydrolysis kinetics. It should be noted that in none of the systems did the reaction reach completion (especially for the 3-phosphate) and only in alkaline phosphatase did hydrolysis exceed 75% of the starting concentration of the 2-phosphate. Plasma was least effective in hydrolyzing either ester particularly the 3-phosphate for which no reaction was observed after 48 hours. This could be accounted for, in part, in the alkaline phosphatase and rat liver homogenate by thermal and product inactivation of the enzyme system. The rather poor hydrolysis of 2-phosphate and lack of observable reaction for 3-phosphate in plasma may reflect a low specific activity, for these substrates, of serum phosphatases. It has been demonstrated[11] that certain lincomycin-3 esters migrate readily to the 4-position markedly reducing the ease with which they are hydrolyzed. There is some TLC evidence that clindamycin-3-phosphate undergoes a similar reaction and may be removed from the reaction pool. This could also contribute to the low extent of hydrolysis for this particular ester in the three systems studied.

In an attempt to elucidate the nature of the difference in the hydrolysis rates of the two esters, alkaline phosphatase reactions were run in pH 6 tris buffer. Although the reaction appeared to be somewhat faster at this pH (based only on the size of the inhibition zone), the relative reaction rates of the 2- and 3-phosphates at pH 6 were not significantly different from those at pH 8. At this point it seemed likely that determination of K_m values would indicate some basis for the differences in the hydrolyses. Reaction velocities were determined at various substrate concentrations using 6-hour hydrolysis. The resulting data and regression statistics for the quantitations appear in Table 5, and the LINEWEAVER-BURK plot constructed from these data is shown in Fig. 3[8]. Using the reverse-estimator[14]:

$$\frac{R \pm t/b\,\{S_a^2 + R^2 S_b^2 - (t^2/b^2) S_a S_b\}^{1/2}}{1-(t^2/b^2) S_a S_b}$$

Table 5. Data for LINEWEAVER-BURK plot and calculation of K_m in alkaline phosphatase

	1/[S] (ml/mcg)	1/ν ((hr ml/mcg)[a)]	Regression values
2-Phosphate	2	8.32	b=3.7499
	1	4.76	a=0.8858
	0.5	2.78	r=0.9994
	0.25	1.88	$S_{y\cdot x}$=0.1175
	0.125	1.22	
3-Phosphate	1[b]	11.35	b=8.7506
	0.5	7.18	a=2.7157
	0.25	5.45	r=0.9915
	0.125	3.29	$S_{y\cdot x}$=0.5432

[a)] Means of 4 experiments.
[b)] No measurable response for 3-PO_4 at 1/[S]=2.

	95 % LL	$K_m\times10^{-5}$ (M^{-1})	95 % UL
2-Phosphate	1.520	1.186	0.865
3-Phosphate	1.870	1.440	1.040

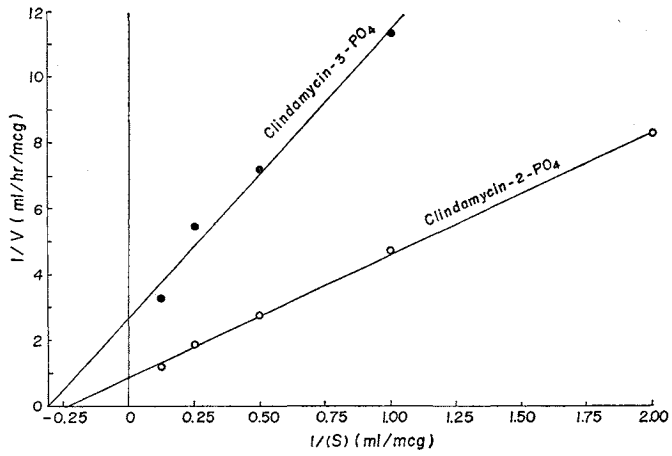
Table 6. Comparative activities of clindamycin, clindamycin-2-PO_4 and clindamycin-3-PO_4. When administered subcutaneously to *Staphylococcus aureus* infected mice.

Antibiotic	Median protective dose (mg/kg)[a)]	Relative activity[b)]
Clindamycin	4.9(2.1～ 7.7)	1.00
Clindamycin-2-PO_4	6.6(4.3～ 9.7)	0.74
Clindamycin-3-PO_4	12.0(7.7～19.)	0.42

[a)] Median protective dose (CD_{50}) with 95% confidence interval. Values represent the mean of three determinations. Values expressed in mg/kg/day as molecular equivalents of clindamycin base.
[b)] Relative activity=Activity relative to that of clidamycin. Clindamycin=1.00.

applied to the LINEWEAVER-BURK plot, the K_m values and corresponding 95 % confidence limits were calculated (Table 5). In as much as the K_m for each ester is contained within the 95 % confidence interval of the other isomer, the difference in the K_m values is not statistically significant. We can assume from these results that the affinity of the enzyme for the individul esters is, within statistical limits, equal. However, the catalytic activity leading to products is significantly different. The conformation of the phosphate group at either the 2- or 3-position is the same (equatorial) for both esters, but the probable difference in the configuration of the two esters relative to the enzyme and their proximity to carbon atoms bearing markedly different functional group (see Fig. 1) must be sufficient to acccount for the difference in the hydrolysis of these compounds.

Fig. 3. LINWEAVER-BURK plot for clindamycin phosphate esters in alkaline phosphatase



In vivo Studies

The bioactivity of the mono-phosphate esters of clindamycin as measured by *in vitro* methods was of a low order, *i.e.* <10 mcg/mg and this small amount of activity could be accounted for as clindamycin base released by hydrolysis of the esters by the assay organism.

Data summarizing the relative abilities of clindamycin and the two phosphate esters to protect mice from a standardized *S.aureus* infection are summarized in Table 6. Both

Table 7. Comparative bioactivities in whole blood of rats after a single subcutaneous dose of clindamycin, clindamycin-2-PO_4 or clindamycin-3-PO_4 at 10 mg/kg[a].

Antibiotic	Area under curve ±2 SE[b]	Relative area of curves[c]	Mean time of 50 % area ±2 SE[d]	Max. conc. (mcg/ml) at time *t*
Clindamycin	177±30	1.00	72±10	2.25 mcg/ml at 30 min.
Clindamycin-2-PO_4	136±32	0.76	74±5	1.23 mcg/ml at 45 min.
Clindamycin-3-PO_4	38±7	0.21	62±4	0.52 mcg/ml at 30 min.

[a] Calculated as molecular equivalents of clindamycin base.
[b] Area under time : concentration curve±standard errors. Values represent mean of three determinations with 3 rats per determination.
[c] Area values relative to that of clindamycin. Clindamycin=1.00.
[d] Time (min) at which 50 % of area is under the time : concentration curve.

clindamycin-2-phosphate and clindamycin-3-phosphate were effective in protecting mice against lethal *S. aureus* challenges. The therapeutic efficacy of the 2-phosphate was statistically equal (P=0.05) to that of clindamycin. The 3-phosphate ester, however, displayed only 42 % of the protective ability of clindamycin in the mouse protection system, which was statistically different (P=0.05).

Results of studies designed to measure clindamycin levels in the whole blood of normal rats following subcutaneous administration (10 mg/kg clindamycin molar equivalents) are summarized in Table 7. Maximum concentrations (clindamycin-2-PO_4=1,2,3 mcg/ml ; clindamycin-3-PO_4=0.52 mcg/ml) and total areas under the time *vs.* concentration curves (clindamycin-2-PO_4=136±32 ; clindamycin-3-PO_4=38±7) reveal that a significantly higher clindamycin level was achieved with the 2-phosphate than with the 3-phosphate. Since the mean times of 50 % area under the curves were the same for clindamycin-2-phosphate, clindamycin and clindamycin-3-phosphate, the observed differences in total area most probably reflected differences in the rates of hydrolysis rather than failure to be absorbed. The relative activities of phosphate esters of clindamycin are predictable from the *in vitro* hydrolysis data.

Metabolism

Since the phosphate esters are hydrolyzed to clindamycin *in vitro* and *in vivo*, it was expected that the metabolic fate of these compounds would parallel clindamycin except as modified by the presence of the phosphate groups. Because the esters are hydrolyzed before absorption it was believed that only rates of appearance of metabolites might be affected. Limited studies of the metabolites of the phosphates in urine and serum from rats and dogs (unpublishied data) confirm the similarity between clindamycin in humans[2] and the esters in animals. A TLC bioautogram of the urine and serum from dogs which had received 10 mg/kg of clindamycin-2-phosphate

Fig. 4. TLC bioautogram of dog urine and serum after treatment with clindamycin-2-PO_4

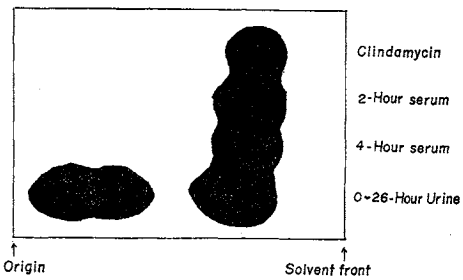
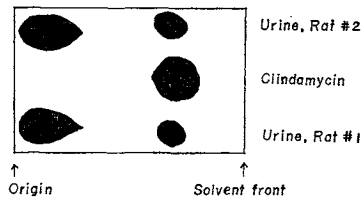


Fig. 5. TLC bioautogram of rat urine after treatment with clindamycin-3-PO_4



(calcium salt) intramuscularly is shown in Fig. 4. The serum is typical in that only clindamycin is present. However, in the urine two slow moving zones are observed. The slowest, Rf 0.14, is consistent with the 1′-demethyl analog of clindamycin while the zone at Rf 0.32 is most probably the sulfoxide of clindamycin. The latter has been observed in a number of studies involving dogs and rats using clindamycin. A TLC bioautogram of rat urine from animals given the 3-ester (15 mg/animal) is shown in Fig. 5. This is typical of clindamycin metabolism in rats in which the slower moving 1′-demethyl analog is the principal metabolite and little if any sulfoxide is observed.

List of Symbols

y=size of inhibition zones on bacterial seeded trays.
x=initial concentration of antibiotic on paper disc.
a=intercept of regression line.
b=slope of regression line.
r=PEARSON correlation coefficient r=1,100 % linear correlation; r=0, no linear correlation.
r^2=Coefficient of determination, measures the % variance accounted for by correlation of two variables.
$S_{y\cdot x}$=Standard error of estimate of regression line.
C_t=antibiotic concentration in hydrolysis reaction solution at time (t).
C_0=antibiotic concentration in hydrolysis reaction solution at time (0).
C_∞=antibiotic concentration in hydrolysis reaction solution at infinite time (termination of reaction).
k=slope (1st order rate constant) of 1st order rate equation.
$R=-a/b$
t="t" statistic
S_a=standard deviation of intercept of regression line.
S_b=standard deviation of slope of regression line.
p=statistical probability (significance levels).
$[S]$=substrate concentration.
v=specific hydrolysis rate.
K_m=MICHAELIS-MENTEN constant.
LL=lower 95 % confidence limit.
UL=upper 95 % confidence limit.
SE=standard error of the mean $s/\sqrt{n}$.

Acknowledgments

The authors express appreciation to B. CZUK for compiling the raw experimental data, to K. STERN for supplying us with urines and plasma from test animals and our collegues in the Infectious Diseases Unit for their helpful suggestions. We thank C. METZLER for his help with some of the statistical problems.

References

1) BRODASKY, T. F. & W. L. LUMMIS: Quantitative thin-layer chromatographic assay for lincomycin and related antibiotics. Antimicr. Agents & Chemoth.-1964: 18~23, 1965
2) BRODASKY, T. F.; A. D. ARGOUDELIS & T. E. EBLE: The characterization and thin-layer chromatographic quantitation of the human metabolite of 7-deoxy-7(*S*) chlorolincomycin (U-21, 251 F). J. Antibiotics 21: 327~333, 1968
3) COATS, J. H. & A. D. ARGOUDELIS: Microbial transformation of antibiotics: Phosphorylation of clindamycin by *Streptomyces coelicolor* MÜLLER. J. Bact. 108: 459~464, 1971
4) EBLE, T. E. & E. R. GARRETT: Studies on the stability of fumagillin. II. Photolytic degradation of crystalline fumagillin. J. Am. Pharm. Assoc. 43: 536~538, 1954
5) HANKA, L. J.; D. J. MASON, M. R. BURCH & R. W. TREICK: Lincomycin, a new antibiotic. III.

Microbiological assay. Antimicr. Agents & Chemoth.-1962 : 565~569, 1963
6) Lewis, C.; H. W. Clapp & J. E. Grady : *In vitro* and *in vivo* evaluation of lincomycin, a new antibiotic. Antimicr. Agents & Chemoth.-1962 : 570~582, 1963
7) Magerlein, B. J.; R. D. Birkenmeyer & F. Kagan : Chemical modification of lincomycin. Antimicr. Agents & Chemoth.-1966 : 727~736, 1967
8) Mahler, H. R. & E. H. Cordes : Biological Chemistry. (Chapter 6) Harper & Row, New York, 1966
9) Morozowich, W.: Clindamycin-2-phosphate, an injectable derivative of clindamycin with improved muscle tolerance. Abstract of 117 th Amer. Pharm. Assoc., Academy of Pharm. Sci., Washington, D.C., 1970
10) Mullet, G. M. & C. R. Noddings : Plotting kinetic data to determine reaction order and specific rate constant. Science 138 : 1346, 1962
11) Oesterling, T. O.: Acidic and alkaline isomerization and hydrolysis of lincomycin monoesters. Carbohydrate Research 15 : 285~290, 1970
12) Snedecor, G. W.: Statistical methods. (Chapters 6 and 7) 5 th ed. Iowa State College Press, Iowa 1957
13) Spearman, C. & G. Karber : Statistical methods in biological assays. pp. 523~530, 2 nd ed. Hafner, New York 1964
14) Williams, E. J.: Regression analysis. (Chapter 6) John Wiley & Sons, 1959