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BIOAUTOGRAPHIC THIN-LAYER CHROMATOGRAPHIC ANALYSIS OF ANTIBIOTICS AND THEIR METABOLITES IN THE WHOLE ANIMAL

I. CLINDAMYCIN IN THE RAT

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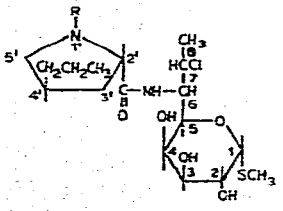
SUMMARY

The results of this investigation on clindamycin and its metabolites support the use of bioautographic thin-layer chromatography (TLC) in metabolite distribution studies. In addition, the use of this technique has established that N-demethylclindamycin contributes significantly to the biological activity of clindamycin *in vivo*. In spite of the biochemical nature of the results of this study, it should be pointed out that TLC, even in the face of the more sophisticated techniques of gas–liquid chromatography, gas–liquid chromatography–mass spectrometry, and high-pressure liquid chromatography, can still prove to be of considerable value in the solution of certain problems. It is a quick and sensitive method not hampered by the difficulties encountered with the instrumental techniques of separation in terms of interfering substances, particularly when a specific detection system such as bioautography is used. In this light, TLC should still be considered among the more valued analytical techniques.

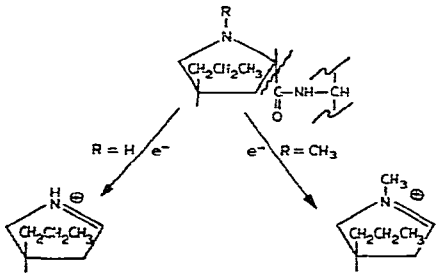
INTRODUCTION

Clindamycin (I) an analog of lincomycin was synthesized in 1965[1]. It is a clinically useful antibiotic in the treatment of both Gram-positive aerobic bacterial infections and anaerobic infections.



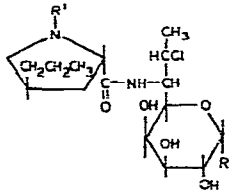
I R = CH_3, clindamycin
II R = H, N-demethylclindamycin

During early clinical trials of clindamycin, chromatographic analysis of urine and serum samples from treated human subjects revealed the presence of an unidentified metabolite. In 1968 Brodasky *et al.*[2] reported the isolation of this metabolite from human urine. It was characterized by IR and mass spectroscopy as N-demethylclindamycin (II). The presence of the ion III, with a mass of 112 instead of the *m/e* 126 peak (ion IV) observed for clindamycin, in the mass spectrum identified this compound as the N-demethyl analog.



III (*m/e* 112) IV (*m/e* 126)

Three other human metabolites of clindamycin were subsequently discovered and characterized in this laboratory[3]. These were clindamycin 1-sulfoxide (V), N-demethylclindamycin 1-sulfoxide (VI), and clindamycose (VII). The *in vitro* antimicrobial activity of these metabolites against *Sarcina lutea*[4] decreases in the order N-demethylclindamycin > clindamycin > clindamycin sulfoxide > N-demethylclindamycin sulfoxide ≫ clindamycose.



V R′ = CH_3, R = $-\overset{\overset{O}{\uparrow}}{S}-CH_3$, clindamycin sulfoxide

VI R′ = H, R = $-\overset{\overset{O}{\uparrow}}{S}-CH_3$, N-demethylclindamycin sulfoxide

VII R′ = CH_3, R = –OH, clindamycose (anomeric mixture)

In view of the number of metabolites produced *in vivo* and because clindamycin was increasing in clinical importance, we considered it imperative to acquire knowledge concerning the time of appearance and eventual distribution of the metabolic products in the body. This could not be achieved by examining urine, serum, and feces from

human subjects. Therefore, a suitable animal system was sought in order to allow study of major internal organs. The rat was chosen because previous studies had indicated that it metabolized clindamycin, at least qualitatively, to the same end products as humans.

Once the animal species had been selected, it was necessary to design an analytical technique which was sufficiently precise and accurate and yet rapid enough to permit a facile study of clindamycin metabolism. Such a technique would also be useful in studying the metabolism of numerous candidates from our analog program. Normally with drugs not metabolized, bioactivity studies of this type are conducted using a disc plate quantitation[4], a modification of the cup plate method[5]. However, as several compounds were to be quantitated in the same samples, a chromatographic procedure was necessary for their separation prior to quantitation. In preliminary studies we found that clindamycin and N-demethylclindamycin in biological samples could be separated by paper chromatography (PC) or thin-layer chromatography (TLC), using bioautographic detection. This is a somewhat lengthy procedure, particularly in the case of PC, which requires 16 h of irrigation to effect the separation in addition to the 16 h required to incubate the detecting organism. In view of this, alternative methods of analysis —gas chromatography (GC) and high-pressure liquid chromatography (HPLC)— were investigated. Antibiotics belonging to the clindamycin family are not amenable to direct GC[6] due to their lack of volatility. The hydroxyl groups must be derivatized to reduce polarity and impart sufficient volatility to the molecule. For GC analysis of clindamycin or its metabolites in biological fluids, derivatization requires extraction of the compounds in question from the samples[7]. At the concentrations usually encountered in biological fluids and tissues, this is at best difficult and would add substantially to the complexity and extent of the analytical procedure. Although HPLC is a rapid and powerful chromatographic procedure, the absence of UV absorption by clindamycin and its metabolites precludes the use of the highly sensitive UV detectors. It is possible to derivatize these compounds with reagents containing groups with high extinction coefficients but this would require extraction of the antibiotics to reduce interference by undesired substances. Differential refractometry (RI) could be used for detection without derivatization but RI detectors lack the necessary sensitivity. In either case derivatization renders clindamycin and its metabolites biologically inactive and as we are interested in assessing the presence of antibacterially active metabolites in the body organs, regeneration of the original compounds would be necessary. This would add further complications and possible sources of error.

These considerations led us to adopt bioautographic (TLC) as the analytical method for this study. By comparison with the instrument techniques, it has advantages of minimal sample preparation, absence of interference by biologically inactive compounds, and sensitivity, in some cases down to 15 ng, of clindamycin and its metabolites.

This report describes a study to determine the feasibility of using bioautographic TLC to quantitate antibiotic levels in animal organs. A systems approach, applying this method, is used to evaluate the contribution of clindamycin and its metabolites to clindamycin *in vivo* bioactivity and to determine the distribution of these antibiotics in the major organs of the rat. This method has also proven useful in assessing the metabolic consequences of the chemical modification of clindamycin.

EXPERIMENTAL

Animals

The Upjohn strain of Sprague-Dawley white male rats (UPJ:TUC[S-D]SPF), averaging approximately 300 g, were used in this study. The animals were fasted for 16–18 h prior to dosing; however, water was always available.

Drug dosage administration

Clindamycin hydrochloride was given orally at a rate of 82.5 mg/kg (approximately 25 mg/rat). The drug, dissolved in triply distilled water at 50 mg/ml, was administered by oral gavage using a hypodermic syringe fitted with a 1½-in. No. 18 ball-tipped needle.

Collection and preparation of tissue samples

Three rats comprising each study group were exsanguinated under cyclopal anesthesia, at specified times (0.5, 1, 2, 4, and 8 h), by bleeding from the descending aorta using a heparinized hypodermic syringe. After the death of each of the animals, the kidneys, liver and gastrointestinal tract, comprising the stomach and the small and large intestines, were stripped from the carcass. The head, tail and feet were severed from the carcass and discarded. The remaining portion of the carcass was skinned, and the skin was discarded.

TLC analysis

The homogenates of the organs of the treated animals were thawed and subsequently centrifuged for 15 min at 1610 *g*. The supernatants were collected and filtered through a medium-pore glass frit. The whole blood was filtered through a glass frit and the entire plasma sample thus collected was processed for analysis. Aliquots of the homogenates were also processed for quantitative analysis. To each of these samples 4–5 volumes of acetone were added. After agitation, the acetonates were filtered and the solvent was evaporated from the filtrates. The resulting aqueous solutions were diluted to the original volume using phosphate buffer. Preliminary chromatograms were run to establish application levels. Suitable volumes of the treated samples were then chromatographed on Eastman silica gel 6061 sheets using acetone–methyl ethyl ketone–water (20.1:72.1:7.8) as solvent. After drying, the chromatographic sheets were applied, silica gel side down, to the surface of trays containing agar seeded with lincomycin-sensitive *S. lutea* and left in contact for 20 min. The sheets were removed and the trays were incubated for 16 h at 32°. The zones of inhibition were then read to the nearest millimeter and these data were used in a least squares regression program with a Hewlett-Packard 9100A computer to estimate antibiotic concentrations.

Standards for quantitation

Using concentrated aqueous stock solutions (which were stored at −10°) serial dilutions were prepared in the menstruum applicable to a particular organ at the following concentration:

clindamycin 10–0.625 or 20–1.25 μg/ml
clindamycin sulfoxide 100–6.25 or 200–12.5 μg/ml

N-demethylclindamycin 10–0.625 or 20–1.25 μg/ml
N-demethylclindamycin sulfoxide 200–12.5 μg/ml

Filtrates of organs from untreated animals were processed as described for the treated samples and used for dilution of standards and samples.

The range of standards for quantitative chromatography was determined by the response observed for the samples to be assayed. Standards and samples were intermixed on the chromatography sheets. Zone diameters were read to the nearest millimeter and these values were used to construct the regression lines for estimation of antibiotic concentrations. Appropriate controls were included in these runs.

RESULTS AND DISCUSSION

Betina[8] has recently reviewed quantitative TLC procedures and their limitations. In our study, two major potential problems had to be resolved before applying bioautographic TLC to the clindamycin metabolism study. The first involved the possible interference in the chromatographic separation of clindamycin and its metabolites by antibacterially inactive substances in the filtrates of organ homogenates. The lack of such interference was demonstrated by adding mixtures of the compounds in question to homogenates of freshly excised organs, processing the samples and separating them as described. These chromatograms were compared with those from organ homogenates of treated animals and they revealed excellent resolution of clindamycin and its metabolites. The TLC bioautograms of typical organ homogenates in Fig. 1 are examples of the results of these experiments.

Secondly, it was necessary to determine whether the nature of the diffusion of antibiotics from the TLC matrix into agar would preclude the use of mathematical

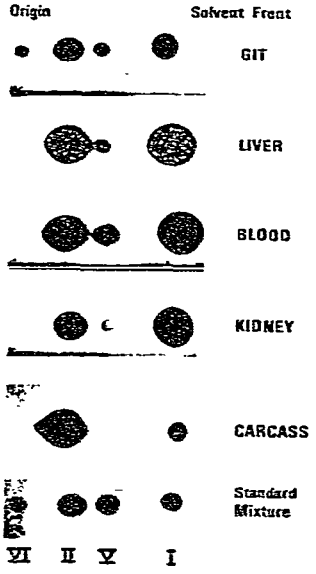


Fig. 1. Typical bioautograms obtained from the rat. See text for details on the components of the standard mixture.

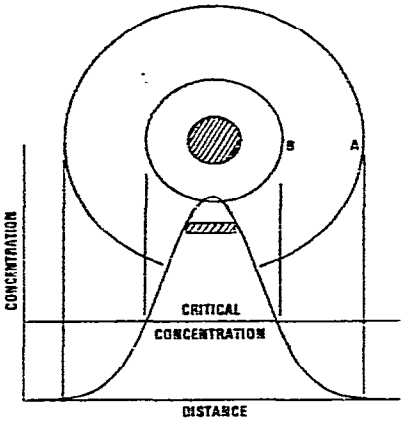


Fig. 2. Schematic representation of antibiotic diffusion on an agar surface (see text for details).

relationships, and existing computer software established for the disc-plate technique. In this method, a paper disc saturated with a solution containing a given concentration of antibiotic is applied to an agar surface. The antibiotic diffuses according to Fick's law[9] and its concentration as a function of the distance from the disc may be represented by the curve shown in Fig. 2. If it were possible to detect the substance at its lowest concentration, zone A would be observed. However, bioautographic detection limits the zone to B, in Fig. 2, since only that concentration of antibiotic above the critical concentration[9] will produce antibiosis. We have determined that for this situation the biological response as a function of initial concentration obeys the linear relationship shown in the following equation. (See Appendix for a description of terms.)

$$R = I + S \log C_0$$

When a solute is chromatographed on a TLC matrix, it undergoes both lateral and longitudinal diffusion, producing the concentration effect depicted in Fig. 3[10]. When the matrix is applied to an agar surface, the solute whose concentration is de-

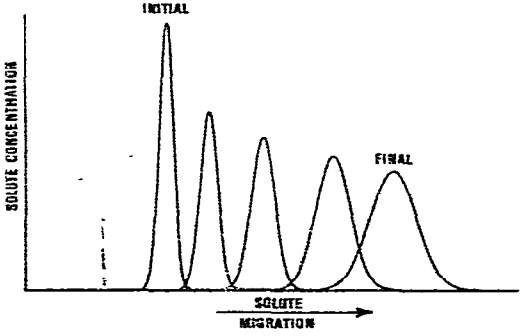


Fig. 3. Change of zone geometry (depicted schematically) during chromatographic development.

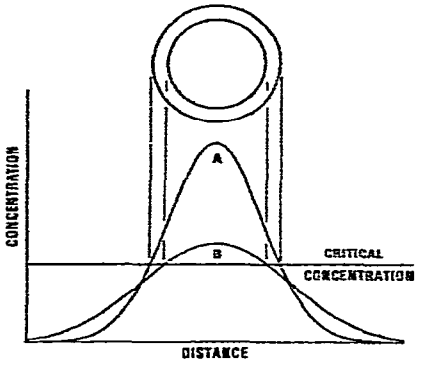


Fig. 4. Schematic representation of the absorption of a chromatographically separated antibiotic onto an agar surface.

picted by curve A in Fig. 4 is desorbed from the matrix and undergoes further diffusion into the agar as represented by curve B in Fig. 4. The effects of the critical concentration again result in a zone diameter somewhat smaller than expected had the original concentration of solute not been changed by diffusion into the agar. To determine whether or not the above equation would apply in this case, zone diameters and initial concentrations from several TLC experiments using serial dilutions of clindamycin and its metabolites in organ homogenates were analyzed by least squares regression of the equation[11]. The statistical values obtained (see Appendix for description of terms) are given in Table I. A high degree of correlation, and coefficients of determination in excess of 99% are observed, both of which support the use of the above equation to represent the relationship of zone diameter and initial antibiotic concentration in bioautographic TLC. Although the slopes (S) are similar, there is a significant difference in the values of the intercepts I, which is a reflection of the intrinsic activity of the individual antibiotics.

TABLE I

REGRESSION STATISTICS FOR COMPOSITE STANDARD CURVES

VI = N-Demethylclindamycin sulfoxide; II = N-demethylclindamycin; V = clindamycin sulfoxide; I = clindamycin.

	*VI**	*II***	*V***	*I***
S	14.4504	13.0109	15.2809	13.9521
$(sd)_S$	0.5989	0.2096	0.3383	0.4826
$t \times (sd)_S$	1.8822	0.6588	1.0632	1.5169
I	−10.9508	20.7891	2.1716	19.0146
$(sd)_I$	1.0489	0.1222	0.4943	0.2812
$t \times (sd)_I$	3.2967	0.3839	1.5537	0.8840
$S_{y \cdot x}$	0.5701	0.1995	0.3220	0.4594
r	0.9974	0.9996	0.9993	0.9982
r^2	0.9949	0.9992	0.9985	0.9964

* Based on the mean of two experiments.

** Based on the mean of six values obtained for blood, carcass, and three organ curves.

Since we will establish, in this study, the contribution of clindamycin (I), N-demethylclindamycin (II), clindamycin sulfoxide (V) and N-demethylclindamycin sulfoxide (VI) to total *in vivo* antibacterial activity, it is important to compare the intrinsic activity of these antibiotics in terms of their relative antibacterial inhibition on agar. Clindamycose has not been observed in any of the animal samples and therefore has not been included. The relative activities reported here were measured as inhibition of *S. lutea*. To estimate the relative biological activity of the antibiotics, zone diameters and initial concentration of standard samples used in the analysis of each organ were averaged to compute single regression lines as shown in Fig. 5. The coefficient of variation of the slopes of these lines is less than 7%, therefore it may be assumed that the lines are parallel. Using 20 mm (see Fig. 5) as an arbitrary response value, the amount of antibiotic necessary to give this response was estimated and the relative potencies (II = 1.4, I = 1, V = 0.08, and VI = 0.009) were calculated. These data are in general agreement with both *in vitro* broth dilution values and *in vivo* CD_{50} values of the various compounds, thus allowing us to assess the contributions of each component to the *in vivo* biological activity of clindamycin.

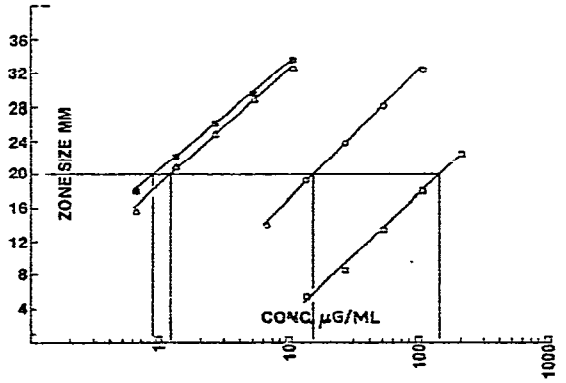


Fig. 5. Relationship between zone size and concentration for clindamycin and some of its metabolites. △, I; ▲, II; ○, V; □, VI.

Having established the feasibility of applying bioautographic TLC to the metabolism study we have described, a detailed investigation of clindamycin in the rat was completed. In the interest of brevity, we will present here only data related to the liver as representative of the results of this study. Complete results will be published elsewhere. The data which follow demonstrate the computational approach used in our evaluation. The results of quantitative analysis of liver homogenates are given in Table II. Included in these tables are statistical data to indicate the quality of the regression lines from which these estimates were made. Based on the volumes of homogenates and the molecular weights of the individual compounds, the data in this table were converted to µmoles as given in Table III. The micromolar values of the individual metabolites were corrected for the weight of organs and converted to nmoles/g in order to plot the metabolic profiles, of the various organs. The total nmoles/g recovered in all organs over any given time period was used to calculate

TABLE II

METABOLITES RECOVERED FROM LIVER

	Recovered (μg/ml)			
	VI	*II*	*V*	*I*
r	0.9871	0.9932	0.9942	0.9905
r^2	0.9744	0.9864	0.9884	0.9811
$S_{y \cdot x}$	1.1152	0.7246	0.7458	0.9220
Time (h)				
0.5	—	6.8	3.4	10.5
1	17.5	7.4	2.3	3.9
2	12.7	4.0	1.2	0.64
4	—	2.5	—	0.12
8	—	1.3	—	—
Control A		4.7	55	5.9
Control B		1.0	10	1.2
Controls VI	A		B	
II	A 5 μg/ml		B 1 μg/ml	
V	A 50 μg/ml		B 10 μg/ml	
I	A 5 μg/ml		B 1 μg/ml	

TABLE III

TOTAL AMOUNT OF METABOLITES RECOVERED FROM LIVER

Time (h)	*Amount (μmoles)*				
	VI	*II*	*V*	*I*	*Total*
0.5	0	3.6	1.7	5.4	10.70
1	8.1	3.6	1.0	1.8	14.50
2	6.2	2.0	0.57	0.32	9.09
4	0	1.2	0	0.05	1.25
8	0	0.60	0	0	0.60

material balances. The liver profile is shown in Fig. 6. These values were also used to compute the area under response curves and the T_{50} (see Appendix) associated with each metabolite as given in Table IV. In order to establish the contribution of each metabolite to antibacterial activity recovered from the various biological compartments, the corrected molar quantities were converted to percentages of the total weight of material found in each organ and these values were corrected on the basis of the relative potency of each metabolite. These values are given in Table V. Using only suitable points after the maximum concentration had been reached in each profile, the first order rate constants for clearance and half-lives for the individual metabolites in the various organs were estimated as shown in Table IV. These values are not meant to represent model dependent pharmacokinetic parameters but only a quantitative means of comparing clearance of the metabolites from the various organs.

Examination of these data led us to several important observations. After 30 min, the first post-treatment sample, 58% of the administered dose of antibiotic

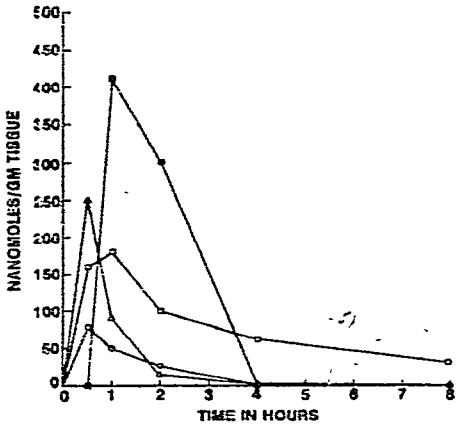


Fig. 6. Concentration profiles of clindamycin and some of its metabolites in liver. △, I; □, II; ○, V; ▣, VI.

TABLE IV

KINETIC PARAMETERS FOR CLINDAMYCIN AND SOME OF ITS METABOLITES IN LIVER

The area units are presented in Fig. 6.

	VI	*II*	*V*	*I*
Liver area	758	615	117	224
T_{50} (h)	1.3	2.5	1.2	0.79
r^2	0.9125	0.9366	0.9906	0.9600
K	2.9487	0.2288	0.6942	1.2758
$t_{\frac{1}{2}}$(h)	0.24	3.0	1.0	0.54

TABLE V

PERCENTAGE OF METABOLITES RECOVERED FROM LIVER

The values in parentheses represent the percentage contribution of each component to the total antibacterial activity in the compartment.

Time (h)	*VI*	*II*	*V*	*I*
0.5	0 (0)	33 (47)	16 (1)	51 (52)
1	56 (1)	25 (73)	7 (1)	12 (25)
2	68 (2)	23 (89)	6 (1)	3 (8)
4		96 (97)	0 (0)	4 (3)
8		100 (100)	0 (0)	0 (0)

is unaccounted for (Fig. 7). This experiment, therefore describes the distribution of the remaining 42%, of this 33% is clindamycin. After 8 h only 2% of clindamycin was found but 22% of the administered dose was recovered as bioactive metabolites.

It was unexpected that 58% of the antibiotic would be unaccounted for since Sun[12,13] reported that 95% of a dose of radioactive clindamycin could be recovered from rats as clindamycin and its metabolites. It is conceivable that in the present study glucuronidation, acetylation or phosphorylation of clindamycin and its metabolites

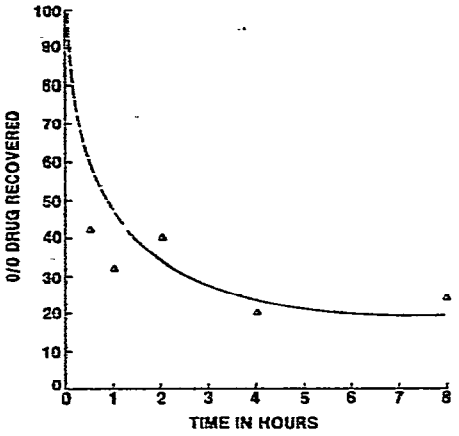


Fig. 7. Recovery of clindamycin and its metabolites following oral administration of clindamycin.

to yield biologically inactive products may have occurred. Future experiments will be designed to examine the distribution of metabolites at time periods prior to 30 min.

The profiles for clindamycin are unspectacular in that it is distributed throughout all organs and in each case exhibits a maximum concentration at 30 min, subsequently it displays the highest clearance rate of all components (see Table IV). Although clindamycin is associated with some of the highest concentration curve areas, this results from high levels in the early samples. It therefore contributes the majority of biological activity only at these early sampling periods (see Table V).

The metabolites V and VI appear at their highest concentration in the gastrointestinel tract (GIT) or in blood. There are increasing concentrations of both sulfoxides which plateau in the GIT. The rapid appearance of V in the GIT raises the possibility that sulfoxidation occurs, not only in the endoplasmic reticulum of liver cells[14], but also in the GIT[15]. Substantial molar quantities of both VI and V are associated with only marginal contribution to biological activity.

Metabolite II appears in all body organs and, with the exception of the GIT, exhibits a maximum concentration at 1 h or later. In the GIT its appearance parallels that of V and VI, with an increasing concentration up to 8 h. It does not plateau, however, as do the sulfoxides, and its peak concentration seems to occur somewhere beyond 8 h. Its high concentration in the liver, well above any other organ except GIT at 8 h, attests to the role of the liver in the demethylation of clindamycin. It eventually exceeds clindamycin in the compartments studied as the major contributor of biological activity (see Table V). This is consistent with the slower clearance rates of II (T_{50} and $t_{\frac{1}{2}}$ in Table IV).

APPENDIX

R = Biological response (zone of inhibition in millimeters against *S. lutea*)

C_0 = Concentration of antibiotic (μg/ml)

S = Slope of least squares regression line, $n\Sigma RC_0 - \Sigma R\Sigma C_0/n\Sigma C_0^2 - -(\Sigma C_0)^2$, where n represents the number of points on the line

I = Intercept of regression line, $(\Sigma R - S\Sigma C_0)/n$

$S_{y.x}$ = Standard error of estimate of regression line,

$$\sqrt{\frac{\Sigma R^2 - 2I\Sigma R - 2S\Sigma RC_0 + nI^2 + 2SI\Sigma R + S^2\Sigma R^2}{n-2}}$$

r = Pearson's correlation coefficient (1.000 = straight line),

$$\frac{n\Sigma RC_0 - \Sigma R\Sigma C_0}{\sqrt{[n\Sigma R^2 - (\Sigma R)^2][n\Sigma C_0^2 - (\Sigma C_0)^2]}}$$

r^2 = Coefficient of determination (percentage of variance of data associated with the correlation of two variables)

$(sd)_S$; $(sd)_I$ = Standard deviation of slope or intercept of regression line, f($S_{y.x}$, C_0)

$(CI)_S$; $(CI)_I$ = 95% confidence intervals of slope or intercept, $t(sd)_S$; $t(sd)_I$, where t = f($d.f.$); $d.f. = n - 2$

CD_{50} = Antibiotic dose in mg/kg which results in a cure of 50% of experimentally infected animals

T_{50} = Time required to develope to 50% of the area under a concentration–time curve

$t_{\frac{1}{2}}$ = Kinetic half-life calculated from first-order kinetic plot (log C *vs.* time)

K = 1st order rate constant for clearance

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