Relative Enzymatic Hydrolysis Rates of Lincomycin Esters I: Soluble Esters

MILDRED J. TARASZKA

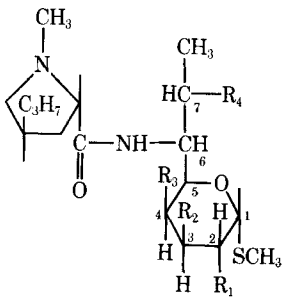
Abstract □ Enzymatic hydrolysis rates were determined for different positional and structural esters of lincomycin in dog serum and simulated intestinal fluid USP. In general, the hydrolysis rates were faster in simulated intestinal fluid than in dog serum, indicating a higher esterase activity in simulated intestinal fluid. The order of hydrolysis rates for positional lincomycin monohexanoate esters was $2 \gg 3 > 4 \sim 7$ in simulated intestinal fluid and $2 > 3 \sim 4 > 7$ in dog serum. The 2-propionate ester of lincomycin was hydrolyzed slower than the longer chain 2-hexanoate ester, with the greatest difference in rates occurring in simulated intestinal fluid. Sterically hindered esters, *e.g.*, lincomycin-2-pivalate and lincomycin-2-(3,3-dimethyl)butyrate, were hydrolyzed at extremely slow rates. The significance of the relative hydrolysis rates when compared to the *in vivo* activity of the esters is discussed.

Keyphrases □ Lincomycin esters, dog serum, simulated intestinal fluid—enzymatic hydrolysis rates □ Hydrolysis rates, *in vivo* enzymatic—lincomycin esters

Monoesters of the antibiotic lincomycin were synthesized to improve the pharmaceutical (taste, solubility, *etc.*) and biological (absorption, *etc.*) properties of lincomycin (1–4). Since most monoesters showed poor *in vitro* bioactivity on the standard curve plate bioassay (4, 5), it is conceivable that hydrolysis of these compounds in the body to lincomycin may be essential for *in vivo* antibacterial activity. The hydrolyses of several monoesters of lincomycin in fresh dog serum and simulated intestinal fluid were studied to determine the effects of: (*a*) derivative position and structure, and (*b*) differences in body fluid esterase specificity on the *in vitro* enzymatic hydrolysis rates of the esters.

Fresh dog intestinal fluid was unavailable so simulated intestinal fluid was used to approximate the relative intestinal fluid enzymatic hydrolysis rates. If there are appreciable differences in esterase specificity between dog serum and simulated intestinal fluid, it might be possible to design a reversible drug derivative which would be inactive *in vitro* and also tasteless, better absorbed, *etc.* However, once the drug derivative is absorbed and/or transported to its receptor site, it would be enzymatically converted to the active form of the drug. From the monoesters studied, it appears that the faster hydrolyzing monoesters have greater *in vivo* antibacterial activity and that no gross differences in esterase specificity exist between dog serum and simulated intestinal fluid.

The structures of lincomycin and its monoesters studied are given here.



Compound	R_1	R_2	R_3	R_4
Lincomycin	—OH	—OH	—OH	—OH
Lincomycin-2-hexanoate	—O—C(=O)—C_5H_{11}	—OH	—OH	—OH
Lincomycin-3-hexanoate	—OH	—O—C(=O)—C_5H_{11}	—OH	—OH
Lincomycin-4-hexanoate	—OH	—OH	—O—C(=O)—C_5H_{11}	—OH
Lincomycin-7-hexanoate	—OH	—OH	—OH	—O—C(=O)—C_5H_{11}
Lincomycin-2-propionate	—O—C(=O)—C_2H_5	—OH	—OH	—OH
Lincomycin-2-pivalate	—O—C(=O)—C(CH_3)$_2$—CH_3	—OH	—OH	—OH
Lincomycin-2-(3,3-dimethyl)butyrate	—O—C(=O)—CH_2—C(CH_3)$_2$—CH_3	—OH	—OH	—OH

structures of lincomycin and its monoesters

EXPERIMENTAL

Enzymatic Hydrolysis—The pH of fresh-pooled dog serum at 37° was checked and adjusted to pH 7.4, if needed, at the start of each experiment. At the end of the experiment, the pH was in the range 7.4–8.0. One milliliter of a concentrated lincomycin ester solution was added to 15 ml. of the pH-adjusted dog serum to give an initial

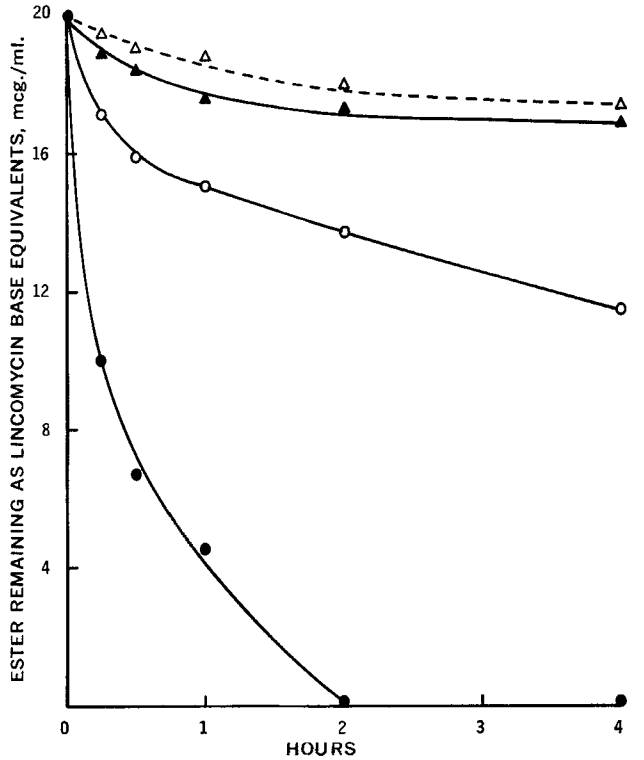


Figure 1—*Simulated intestinal fluid hydrolysis at pH 7.4 and 37° of lincomycin monoesters. Key: 2-hexanoate, ●; 3-hexanoate, ○; 4-hexanoate, △; and 7-hexanoate, ▲.*

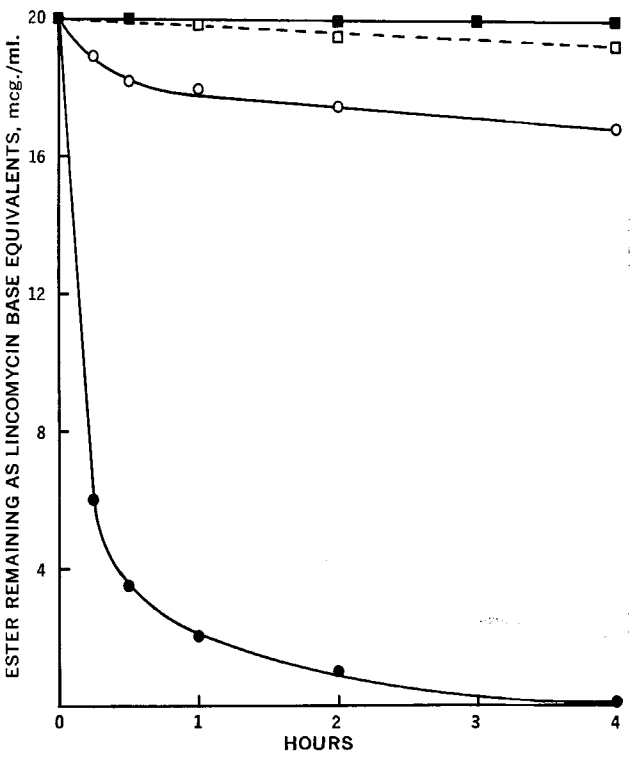


Figure 2—*Simulated intestinal fluid hydrolysis at pH 7.4 and 37° of lincomycin monoesters. Key: 2-hexanoate, ●; 2-propionate, ○; 2-pivalate, □; and 2-(3,3-dimethyl)butyrate, ■.*

ester concentration of 20 mcg. of lincomycin base/ml. At given time intervals, samples of the reaction mixture were assayed for the amount of lincomycin present. All dog serum hydrolysis experiments were started within 2.0–2.5 hr. after the blood was withdrawn from the dogs.

Simulated intestinal fluid USP was prepared from analytical grade buffer components and USP grade pancreatin[1]. Pancreatin was added to the buffer solution 1 hr. before the beginning of the hydrolysis experiment. The same procedure was used as for dog serum. Due to the better buffering capacity of simulated intestinal fluid, the pH of the solution was 7.4 ± 0.1 throughout the experiment.

Since enzyme concentration or degradation could not be controlled, lincomycin-2-hexanoate was chosen as the standard to measure the enzyme activity of dog serum and simulated intestinal fluid on different days. All enzymatic hydrolysis experiments were done at 37°.

Lincomycin Assay—To use the standard curve plate bioassay (6) for lincomycin in the reaction solutions, the esters of lincomycin first had to be extracted since they also showed some bioactivity. Two milliliters of the reaction solution was added to 2 ml. of water-saturated ether in a 15-ml. stoppered centrifuge tube. The tube was shaken vigorously and centrifuged, and the ether layer was discarded. The ether extraction process was repeated a total of three times immediately after sampling the reaction solution. After the last centrifugation, 1–1.5 ml. of the aqueous layer was transferred to a 2-ml. volumetric flask. The dissolved ether in the reaction mixture was evaporated by placing the samples under house vacuum for 1 hr. The samples were held in a frozen state and assayed collectively by spotting 0.08 ml. on 1.27-cm. (0.5-in.) diameter paper disks[2], using *Sarcina lutea* (ATCC-9341) inoculated agar (6). The assay values were determined graphically with 10 standard points for each set of unknowns.

The ether extractions did not interfere with the standard curve plate bioassay for lincomycin, since the same inhibition zone diameters were obtained when lincomycin solutions were spotted before and after the three ether extractions. Ester bioactivity was completely removed with the three ether extractions in the plate bioassay for lincomycin-2-hexanoate, lincomycin-3-hexanoate, lincomycin-2-pivalate, lincomycin-4-hexanoate, and lincomycin-2-(3,3-dimethyl)butyrate at concentrations up to 50 mcg./ml. The ether extractions, however, did not completely remove the bio-

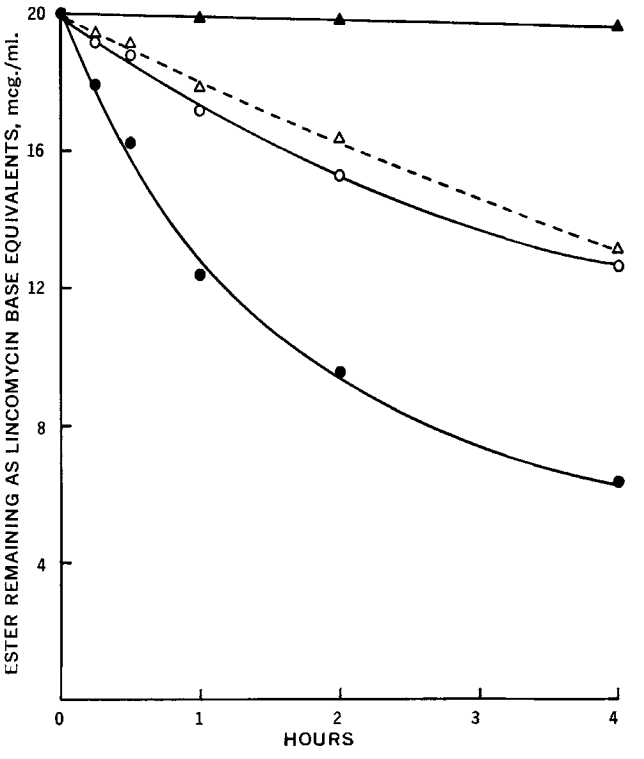


Figure 3—*Fresh dog serum hydrolysis at pH 7.4 and 37° of lincomycin monesters. Key: 2-hexanoate, ●; 3-hexanoate, ○; 4-hexanoate, △; and 7-hexanoate, ▲.*

[1] Nutritional Biochemicals Corp.
[2] Schleicher and Schuell.

Table I—Antibacterial Activity and Relative Enzymatic Hydrolysis Rates of Lincomycin Monoesters

Lincomycin Esters	Antibacterial Activity: *In Vitro* Activity, mcg./mg.[a]	Antibacterial Activity: Relative *In Vivo* Activity[b]: Subcutaneous Route	Antibacterial Activity: Relative *In Vivo* Activity[b]: Oral Route	Enzymatic Rates[c]: Dog Serum	Enzymatic Rates[c]: Simulated Intestinal Fluid
4-Hexanoate	86	0.28 (0.13–0.45)	0.15 (0.09–0.21)	0.33 (0.22–0.50)	0.07 (0.05–0.10)
7-Hexanoate	59	0.34 (0.17–0.55)	0.33 (0.18–0.51)	<0.02	0.13 (0.11–0.15)
3-Hexanoate	490	0.50 (0.28–0.78)	1.27 (0.81–2.10)	0.42 (0.34–0.53)	0.31 (0.28–0.32)
2-Hexanoate	1050	0.70 (0.34–1.29)	1.37 (0.86–2.34)	1.00	1.0
2-(3,3-Dimethyl)-butyrate	16	0.45 (0.20–0.78)	0.86 (0.59–1.22)	0.06 (0.03–0.09)	<0.02
2-Pivalate	<4	0.68 (0.35–1.13)	1.23 (0.78–1.94)	0.16 (0.14–0.18)	0.03 (0.01–0.04)
2-Propionate	245	0.76 (0.37–1.35)	1.21 (0.75–2.02)	0.61 (0.54–0.70)	0.13 (0.08–0.16)

[a] As measured on a standard curve agar assay *versus Sarcina lutea*. Results expressed as micrograms of lincomycin base per milligrams of ester (4, 5). [b] Median protective dose or CD_{50} relative to that of lincomycin as 1. The numbers in parentheses are the 95% confidence intervals (4, 5). [c] Enzymatic hydrolysis rates relative to that of lincomycin-2-hexanoate as 1. The numbers in parentheses are the ranges.

activity due to lincomycin-7-hexanoate and lincomycin-2-propionate. The error due to the incomplete extraction of these two esters was, therefore, corrected by using the equation:

$$X(20) + (1 - X)1 = A \quad \text{when } A \leq 20 \text{ mcg./ml.} \quad \text{(Eq. 1)}$$

$$\begin{pmatrix}\text{contribution due}\\ \text{lincomycin}\end{pmatrix} + \begin{pmatrix}\text{contribution due to}\\ \text{lincomycin ester}\end{pmatrix} = \text{total value of lincomycin obtained in bioassay (mcg./ml.)}$$

where X is the fraction present as lincomycin.

RESULTS AND DISCUSSION

The amount of monoester remaining *versus* time is plotted in Figs. 1 and 2 for simulated intestinal fluid hydrolysis and in Figs. 3 and 4 for fresh dog serum hydrolysis. No attempt was made to analyze these plots into kinetic hydrolysis mechanisms since no correction was made to account for any loss of esterase activity during the hydrolysis runs.

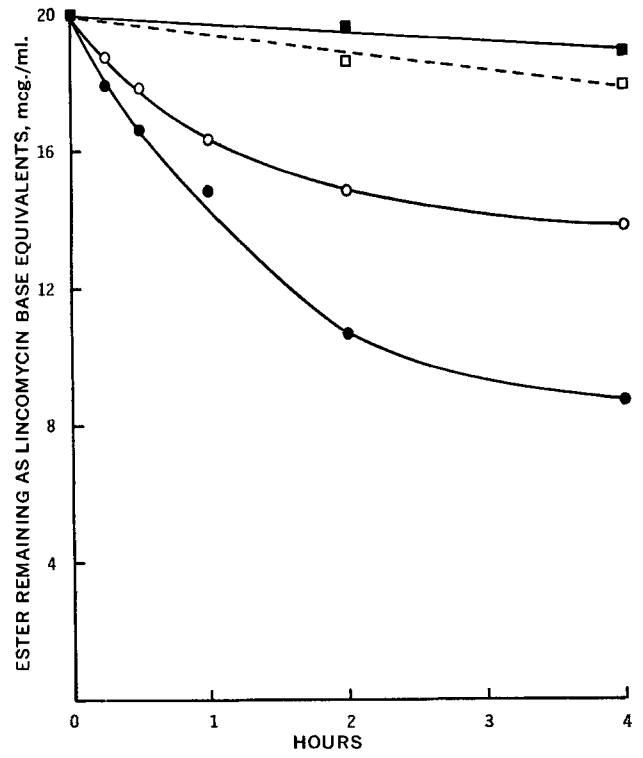


Figure 4—*Fresh dog serum hydrolysis at pH 7.4 and 37° of lincomycin monoesters. Key: 2-hexanoate, ●; 2-propionate, ○; 2-pivalate, □; and 2-(3,3-dimethyl)butyrate, ■.*

Positionally Isomeric Esters—The four positionally isomeric hexanoate esters of lincomycin were enzymatically hydrolyzed in the order $2 \gg 3 > 4 \sim 7$ in simulated intestinal fluid (Fig. 1) and $2 > 3 \sim 4 > 7$ in fresh dog serum (Fig. 3). The difference in hydrolysis rates of corresponding esters in simulated intestinal fluid and dog serum was probably due to a difference in concentration or activity of specific esterases in these two media.

Molecular models show that the four hydroxyl groups in lincomycin have different steric and electronic environments. These nonsymmetrical environments probably contribute to the rather large differences observed in enzymatic hydrolysis rates for the positionally isomeric esters. The large differences in hydrolysis rates were not observed for the nonenzymatic hydrolyses of the isomeric lincomycin hexanoate esters, where the order was $2 \sim 3 \sim 4 > 7$ (7, 8). The steric and electronic differences appear to have a larger effect on the enzymatic rates than on the nonenzymatic rates of hydrolysis. This would be anticipated since the steric requirements for enzymatic reactions are usually more stringent than for hydrolytic reactions.

The *in vitro* and *in vivo* bioactivities of the isomeric hexanoate esters of lincomycin are reproduced in Table I (4), along with the relative enzymatic hydrolysis rates. While the error is rather large, there still is some indication of a correlation between the bioactivity and the enzymatic hydrolysis rate for the hexanoate positional isomers, with the faster hydrolyzing positional isomers having the larger *in vivo* antibacterial activity.

Sterically Hindered Esters—Although lincomycin-2-hexanoate and lincomycin-2-(3,3-dimethyl)butyrate have the same number of carbon atoms in the acid moiety, the sterically hindered 2-(3,3-dimethyl)butyrate ester was enzymatically hydrolyzed much slower than the 2-hexanoate ester (Figs. 2 and 4). The sterically hindered 2-pivalate ester was also hydrolyzed extremely slowly. These results are in agreement with literature data on crude enzyme mixtures (9).

Lincomycin-2-propionate was enzymatically hydrolyzed slower than lincomycin-2-hexanoate, with the greatest difference in rates occurring in simulated intestinal fluid (Figs. 2 and 4). The dependence of enzymatic hydrolysis rate on chain length of the acid moiety is in agreement with the literature (10). The greatest rate for the longer chain ester (hexanoate) might be expected for simulated intestinal fluid, since it contains digestive enzymes which are responsible for the hydrolysis of fats (glyceryl esters of long-chain fatty acids).

The correlation between bioactivity and enzymatic hydrolysis rate for the sterically hindered esters is not as good as for the positionally isomeric esters of lincomycin (Table I). Possible reasons for this lack of correlation are: (*a*) the crude enzyme mixture hydrolysis rates are not representative of the *in vivo* fluid enzymatic hydrolysis rates; and (*b*) other factors such as solubility and absorption, as well as enzymatic rates, are important for *in vivo* antibacterial activity. For the monoesters of lincomycin studied, the enzymatic rates of hydrolysis were generally faster in simulated intestinal fluid than in dog serum. This finding indicates that if an ester derivative is resistant to enzymatic hydrolysis in the intestinal tract and absorbed intact, it probably will be resistant to hydrolysis in the blood. However, with the large variety and high concentration of enzymes in the liver, the hydrolysis in the total vascular system may be sufficiently rapid to yield activity comparable to the parent

drug. Possibly this is the explanation for the good *in vivo* antibacterial activity of the 2-propionate, 2-pivalate, and 2-(3,3-dimethyl)-butyrate monoesters of lincomycin, even though they are enzymatically hydrolyzed *in vitro* relatively slowly.

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Free and Bound Water in Crude Drugs: Effect of Extraction Method on Subsequent Analysis by GLC

NOURI Y. MARY

Abstract □ The effect of the extraction procedure on the recovery of water from crude drugs was related with its quantitative analysis by GLC. Some crude drugs seem to retain part of their water in a bound form, which is not available for extraction with methanol by disintegration in a blender but can be readily removed by boiling the product with the solvent under reflux for 1 hr. A number of crude drugs, representing a variety of plant parts and products, were extracted with methanol by the two procedures; the extracts were subsequently analyzed for their total water by GLC. Reflux extraction is the method of choice for the preparation of extracts of crude drugs that contain bound water prior to their analysis by this technique.

Keyphrases □ Water, recovery from crude drugs—effect and comparison of extraction procedures □ Reflux extraction—preparation of crude drug extracts □ Crude drugs—extraction procedures, effect on GLC analysis □ GLC—analysis of crude drugs, effect of extraction procedures

A previously published paper from this laboratory (1) described a GLC method for the quantitative determination of water in natural products by reaction with 2,2-dimethoxypropane. In the course of analysis by this method, it was observed that the extraction procedure normally used to remove water from these products (disintegration with methanol in a blender) gave, in one instance (pectin), results that markedly deviated from the values given by the official methods. Pectin seems to retain part of its water in a bound form which does not lend itself readily to extraction by this procedure.

Further studies on this problem showed that some degree of water binding also exists in other crude drugs and natural products besides pectin. For accurate quantitative analysis, water in this form must be completely extracted from such products prior to determination by GLC. The purposes of this paper are to record the effect of extraction on water recovery from crude drugs and to relate this effect with quantitative analysis by this technique.

EXPERIMENTAL

The plant materials used in this investigation were obtained, in powdered form, from a commercial source[1].

Water Determination by Direct GLC

Extraction and Sample Preparation—*Procedure A: Blender Extraction*—In a typical analysis, 10.00 g. of the crude drug was placed in a blender jar[2] containing 100.00 ml. of anhydrous methanol[3] and 3.00 ml. of *n*-propanol[4] as the internal standard. After blending for 5 min., the mixture was allowed to settle; then a sample of the clear supernatant was drawn into a vial, and 4.00 μl. was injected with a microliter syringe[5] into a gas chromatograph[6] equipped with thermistor detector.

Procedure B: Reflux Extraction—A 10.00-g. sample of the plant material was heated for 1 hr. under reflux with 100.00 ml. of anhydrous methanol and 3.00 ml. of *n*-propanol as the internal standard. After cooling the mixture, a sample of the clear supernatant was transferred into a vial, and 4.00 μl. was injected into a gas chromatograph equipped with thermistor detector.

Calculations—The percent water in the original sample of the crude drug was determined by computing the ratio of peak height of water to *n*-propanol from the chromatogram, obtaining the corresponding weight ratio of water to *n*-propanol from a standard curve prepared by chromatographing samples containing various amounts of water in mixtures of 100.00 ml. anhydrous methanol and 3.00 ml. *n*-propanol, and multiplying by the weight of *n*-propanol[7].

[1] S. B. Penick and Co., New York, N. Y.
[2] Waring Products Co., Winsted, Conn.
[3] Reagent grade, Merck and Co., Inc., Rahway, N. J.
[4] Matheson, Coleman and Bell, East Rutherford, N. J.
[5] Hamilton No. 701, Hamilton Co., Whittier, Calif.
[6] Perkin-Elmer model 154 vapor fractometer, Norwalk, Conn.
[7] Water and *n*-propanol were measured by volume and converted to weight using specific gravity calculations.