(21) A. Cipiciani, P. Linda, G. Savelli, and C. A. Bunton, *J. Org. Chem.*, **46**, 911 (1981).
(22) M. J. Schick, *J. Am. Oil Chem. Soc.*, **43**, 681 (1966).
(23) C. A. Bunton, L. S. Romsted, and L. Sepulveda, *J. Phys. Chem.*, **84**, 2611 (1980) and references cited therein.
(24) V. Gani, G. Lapinte, and P. Viout, *Tetrahedron Lett.*, **1973**, 4435.
(25) C. A. Bunton, A. Kanego, and M. J. Minch, *J. Org. Chem.*, **37**, 1388 (1972).
(26) C. Lapinte and P. Viout, *Tetrahedron Lett.*, **1972**, 4221.
(27) C. A. Bunton, "Nucleophilic Substitution at a Saturated Carbon Atom," Elsevier, New York, N.Y., 1963.
(28) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N.Y., 1968.

ACKNOWLEDGMENTS

Thanks are due the Italian National Research Council for financial support, Prof. G. Savelli for helpful discussion, and the referee for useful suggestions.

Kinetics and Mechanism of Enzymatic Hydrolysis of Pivampicillin Monolayers

O. VALLS, M. ALVAREZ, A. ALSINA, and S. GARCÍA FERNANDEZ ˣ

Received May 19, 1982, from the *Department of Physical-Chemistry, Faculty of Pharmacy, University of Barcelona, Spain.* Accepted for publication October 17, 1983.

Abstract □ A study of the enzymatic hydrolysis of pivampicillin (an insoluble penicillin) extended as a monolayer on the aqueous interface at a constant surface pressure has been performed. Penicillinase promotes intensive hydrolysis of the pivampicillin monolayers, inducing their solubility. However, no action was observed with dog liver esterase. The hydrolytic process, which was dependent on the film surface pressure and on the quantity of the injected enzyme, is of the Michaelis-Menten type in two dimensions.

Keyphrases □ Pivampicillin—enzymatic kinetics, surface monolayers, penicillinase, esterases □ Kinetics—enzymatic, pivampicillin, surface monolayers, Michaelis-Menten

The catalytic action of an enzyme can generally be measured by the quantity of transformed substance or by the product generated per unit of time. The processes of enzymatic kinetics in bulk materials are usually Michaelis-Menten in character (1). Under these conditions, both soluble substrates and enzyme-substrate complexes are in a homogeneous system. However, insoluble substrates are normally in the form of micellar aggregates, which act as the real substrate. In such cases, study of the kinetics is complicated by the difficulty of knowing the concentration of substrate in contact with the enzyme.

This difficulty can be overcome by extending the insoluble substrate as a monolayer film over an aqueous mass which contains the soluble enzyme. Under these conditions, all the molecules of substrate are equally exposed to the action of the enzyme. Thus, it is possible to quantitate the amount of substrate in contact with the enzyme (by controlling its surface concentration in the film) and the amount of enzyme reacting with the surface (by controlling its concentration in the subphase). This system makes it possible to obtain information about action of the enzyme sites in the substrate molecules.

The first studies of enzymatic kinetics using the surface monolayer technique were performed by Hughes (2) in 1935. Measuring the decrease in surface potential during the hydrolysis process, Hughes observed that the hydrolysis rate of lecithin in monomolecular films catalyzed by phospholipase A_2 decreased when the surface concentration of the lecithin molecules in the interface increased. This method is, however, subject to significant errors in interpretation.

Since that time, several authors have used the monolayer technique to measure the activity of lipolytic processes catalyzed by lipases and phospholipases. Short-chain phospholipids and triglycerides were used as substrates so that the reaction products would be soluble and, therefore, diffuse into the aqueous subphase, leaving the monolayer. Garner and Smith (3) performed these determinations in a constant area, measuring the decrease of the monolayer surface pressure as the substrate was dissolved by the lipolytic effect of the enzyme. An inherent difficulty of this method is that the surface concentration of the film molecules vary continually during the process; therefore, the decrease of the surface pressure is linear with respect to time only in the first moment. Lagocki *et al.* (4), Dervichian (5), and Verger and de Haas (6) used methods with constant pressure, measuring the kinetic rate by the decrease of the area covered by the film (directly connected with the number of substrate molecules which escape from the surface).

The method of "zero-order trough" or "two-compartment trough" proposed by Verger and de Haas (6) is particularly interesting. In this method the enzymatic reaction takes place in a different compartment than the one which acts as a substrate reservoir. In this way, the number of the substrate molecules in the reaction compartment can be kept constant during the entire process. The process develops in a pseudo-zero-order manner, *i.e.*, the decrease of the monolayer area is proportional to time, yielding a straight line, the course and slope of which give the rate of the kinetic process.

Kinetic studies have been performed on monolayers of insoluble penicillins (7). The penicillin molecules are transformed by hydrolysis into soluble products that leave the monolayer and dissolve in the aqueous subphase. The number of hydrolyzed molecules can be determined by the monolayer surface decrease, maintaining a constant surface pressure at all times (Fig. 1).

For this type of study, pivampicillin is a particularly interesting test drug. It is a penicillin which is insoluble in neutral solutions and soluble in acidic media. Pivampicillin is activated by esterases, which transform it into ampicillin (8), and it is

0022-3549/84/1200-1694$01.00/0
© 1984, American Pharmaceutical Association

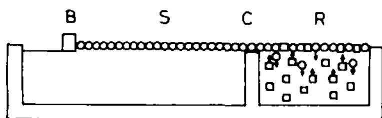


Figure 1—*Schematic of the work principle of the enzymatic hydrolysis process. Key: (B) mobile barrier; (S) reservoir of the substrate (penicillin); (C) communication channel between compartments; (R) reaction compartment.*

quickly inactivated by the action of β-lactamase enzymes secreted by penicillin-resistant microorganisms, *i.e.*, penicillinase (9). A previous study (7) with this antibiotic verified that stable monolayers can be obtained which, through the activity of hydrolytic agents, become soluble and pass into the aqueous subphase. It is possible, therefore, to perform kinetic studies on a monolayer of pivampicillin.

The results of the acid and alkaline hydrolysis of monomolecular films of pivampicillin and the first results of hydrolysis by β-lactamases have been presented previously (7). In the present study the kinetic processes of hydrolysis by an esterase and β-lactamase on monomolecular films of pivampicillin are amplified and debated.

EXPERIMENTAL SECTION

For this study a surface manometer, designed and built in our laboratory based on the pseudo-zero-order system proposed by Verger *et al.* (6), was used; this manometer was described in detail in a previous work (10). It has a barostatic system, allowing the maintenance of constant pressure and the direct registration of the variation of the monolayer area through time. Sörensen buffer (pH 7) was used as the base liquid of the film, filling both sections of the trough (11). The buffer was prepared with twice-distilled water, potassium phosphate[1], and disodium phosphate[1].

Purified technical-quality pivampicillin[2] was used for preparation of the substrate monolayer; a 10^{-5} M solution of this substance in 20% absolute ethanol-80% petroleum ether (bp 40-60°C) was used. The solvent evaporated spontaneously within ~60 s after extension, leaving a monomolecular film of the antibiotic over the aqueous surface. In each case, a variable volume of solution was spread; the resulting film was compressed, reducing its surface until the desired surface pressure for the kinetic studies was obtained. The initial surface concentration (C) of the substrate molecules in the film was calculated from the amount of penicillin extended and the total area (in cm^2) occupied initially by the film in both compartments.

While under constant agitation, 10-100 μL of enzyme solution was injected under the monolayer in the reaction compartment. The injected enzymes were an esterase and a penicillinase. The esterase used (type I from dog liver) is a carboxyesterase (E.C. 3.1.1.1)[3]. One unit of this enzyme hydrolyses 1.0 μmol of ethyl butyrate/min at pH 8 and 25°C. The enzyme (100 U/mg of protein) was used in a buffered phosphate solution at pH 8, at a concentration of 1000 IU/mL. The penicillinase, from *Bacillus cereus* (40,000 IU/mg), is a penicillinamide β-lactamhydrolase (E.C. 3.5.2.6)[4]. One international unit inactivates 1 U of penicillin/min at pH 7 and 25°C. It was used in a buffered phosphate solution at pH 7, at a concentration of 400,000 IU/cm^3.

The recording of the kinetic process (variation of the film area against time) started immediately after enzyme addition and continued for 60-120 min. The number of hydrolyzed molecules was calculated from the displacement of the mobile barrier, taking into account that a 1-cm displacement corresponds to a surface decrease of 17.5 cm^2 and to a number of hydrolyzed molecules equal to $17.5C$ ($molecules/cm^2$). A linear representation was obtained in the zero-order trough by plotting the number of hydrolyzed molecules against time. The type of slope allowed us to calculate the velocity of the hydrolytic process ($molecules{\cdot}cm^{-2}{\cdot}min^{-1}$).

RESULTS

The injection of dog liver esterase under the monolayer of pivampicillin, at final concentrations from 100 to 10,000 IU/100 mL of subphase and at starting film pressures from 1×10^{-3} to 2×10^{-2} $N{\cdot}m^{-1}$, produced neither solubilization of the monolayer nor a real decrease of the starting pressure of the film over a 2-h period. After the injection of the enzyme, however, the surface pressure of the monolayer increased. This demonstrates penetration of the esterase in the penicillin film, but without a hydrolytic effect on pivampicillin.

The injection of penicillinase produced a rapid hydrolysis and solubilization of the pivampicillin, which manifested itself in a decrease in the monolayer area at constant surface pressure, after the short induction time (τ) necessary for the enzyme to penetrate the monolayer. The rate at which the substrate molecules leave the surface depends on the surface pressure of the film and the quantity of the enzyme injected.

In all cases, the activity of the process (hydrolyzed molecules by unit of surface), after the induction time (τ), was directly proportional to time, *i.e.*, it was a pseudo-zero-order process. As a result, the graphic representation of the activity against time was a straight line during the initial 60 min, after which the line curved slightly as the process decreased.

Figure 2 shows the hydrolytic activity (in hydrolyzed molecules per square centimeter of monolayer surface) in the reaction compartment *versus* time at different surface pressures after the injection of 4000 IU of penicillinase. After a short induction time (τ) the curve straightened until the final stage of the process. When the surface pressure of the film increases, the reaction rate also increases, tending to stabilize at high pressures (Fig. 3).

The activity of the hydrolytic process is strongly influenced by the amount of injected enzyme. This can be seen in Fig. 4, where the hydrolyzed substance is set against time, at a constant surface pressure of 4.2×10^{-3} $N{\cdot}m^{-1}$, for different amounts of injected penicillin in the reaction compartment (4000-12,000 IU). As can be seen in Fig. 5, the reaction rate (hydrolyzed molecules per milliliter and per minute) increases with the amount of enzyme, giving a straight line.

The induction time (τ) decreases when the surface pressure of the film increases, stabilizing at high pressures (Fig. 6). The induction time also decreases when the amount of injected enzyme increases.

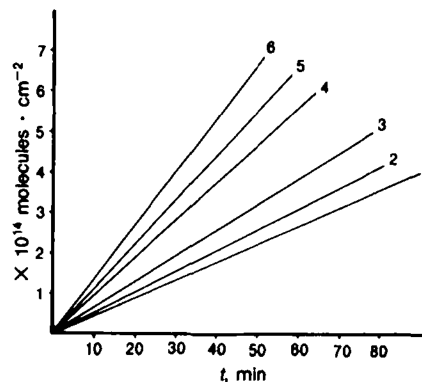


Figure 2—*Activity of the hydrolysis of pivampicillin films by penicillinase (4000 IU) at different film surface pressures. Key: (1) 4.2×10^{-3} $N{\cdot}m^{-1}$; (2) 6.7×10^{-3} $N{\cdot}m^{-1}$; (3) 8.4×10^{-3} $N{\cdot}m^{-1}$; (4) 12.4×10^{-3} $N{\cdot}m^{-1}$; (5) 13.9×10^{-3} $N{\cdot}m^{-1}$; (6) 16.7×10^{-3} $N{\cdot}m^{-1}$.*

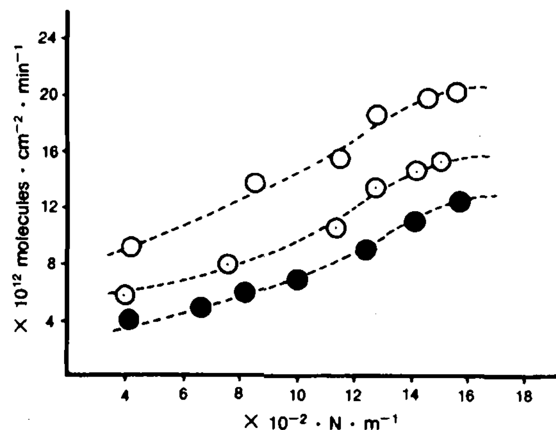


Figure 3—*Hydrolysis velocity of pivampicillin films as a function of the film surface pressure after the injection of different quantities of penicillinase. Key: (○) 12,000 IU; (⊙) 8000 IU; (●) 4000 IU.*

[1] The highest purity obtainable; E. Merck, Darmstadt, F.R.G.
[2] Obtained and purified by Antibióticos, S.A., León, Spain.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Serva Feinbiochemica, Heidelberg, F.R.G.

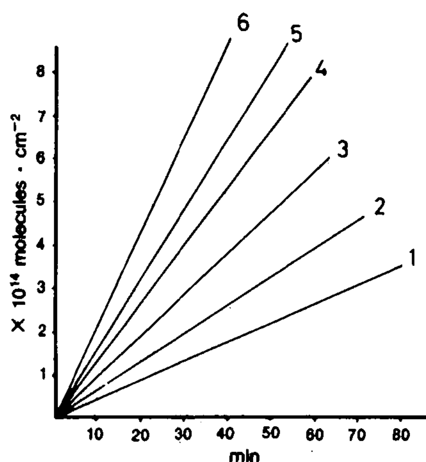


Figure 4—*Activity of the hydrolytic process of pivampicillin films by injecting different amounts of penicillinase. Key: (1) film at 4.2 × 10^{-3} N·m^{-1}, injection of 4000 IU; (2) film at 4.2 × 10^{-3} N·m^{-1}, injection of 8000 IU; (3) film at 4.2 × 10^{-3} N·m^{-1}, injection of 12,000 IU; (4) film at 16.7 × 10^{-3} N·m^{-1}, injection of 4000 IU; (5) film at 16.7 × 10^{-3} N·m^{-1}, injection of 8000 IU; (6) film at 16.7 × 10^{-3} N·m^{-1}, injection of 12,000 IU.*

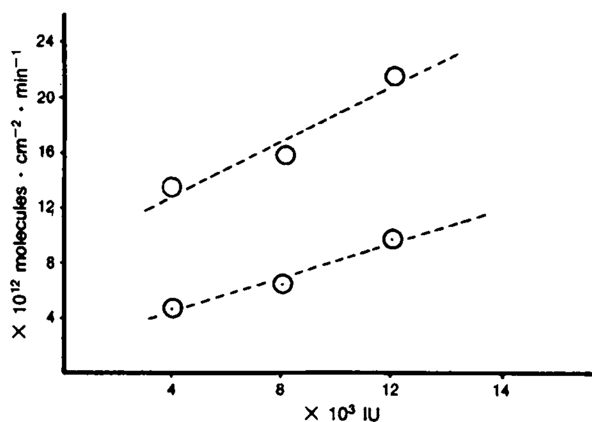


Figure 5—*Hydrolysis velocity of pivampicillin films at surface pressures of 4.2 × 10^{-3} (⊙) and 16.7 × 10^{-3} N·m^{-1} (○) as a function of the amount of injected enzyme.*

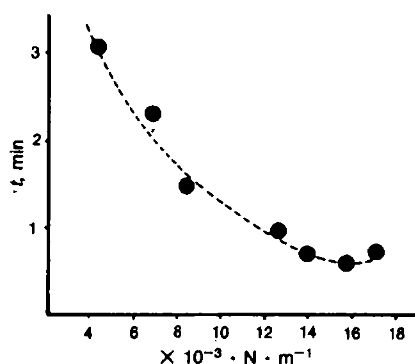
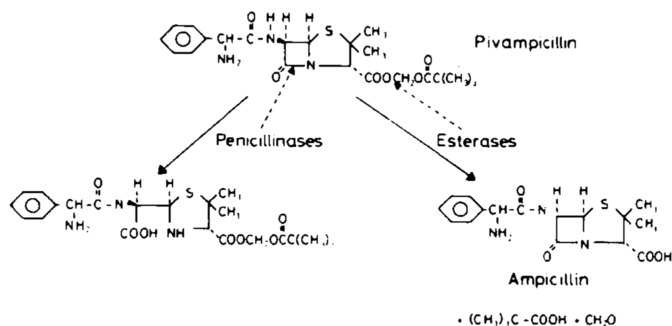


Figure 6—*Induction time of the hydrolysis of pivampicillin films after the injection of 4000 IU of penicillinase as a function of the film surface pressure.*

DISCUSSION

The pivampicillin molecule has two points especially sensitive to the hydrolytic process (Scheme I): the ester bond which forms the nucleus of 6-aminopenicillanic acid with the pivaloyl alcohol, and the β-lactamic bond of the 6-aminopenicillanic acid. Hydrolysis of the ester bond is produced through the effect of the blood esterases once the antibiotic has been absorbed, converting the antibiotic into active ampicillin, while the nonactive pivaloic ester (pivampicillin) is better absorbed through the digestive tract than the ampicillin itself (8).



Scheme I—Hydrolysis process of the pivampicillin by esterases and penicillinases.

The *in vitro* studies with bulk material performed by Yokota *et al.* (12) seem to indicate that the enzyme responsible for this hydrolytic process is a carboxyesterase (E.C. 3.1.1.1). But the results reported in the present study indicate that this enzyme is inactive, even when tested at very high concentrations. This fact can be interpreted in different ways: (*a*) lack of specificity of the enzyme with the substrate, which would contradict the results obtained by Yokota; (*b*) liberation of insoluble products, which is not very likely because the pivaloic alcohol and the ampicillin are extraordinarily soluble under the current experimental conditions; or (*c*) inactivation of the esterase enzyme in the interface (the more probable of the three interpretations). The latter would be in agreement with the results obtained in 1958 by Sarda and Desnuelle (13), who, using triacetin as a substrate in the monolayer, found very low hydrolytic activity for the esterase compared with that of the pancreatic lipase normally used in kinetic studies of lipolysis. Finally, the possibility exists that the ester group, due to the relatively large size of the molecule of pivampicillin, must be situated in the air-water interface in order to be reached by the enzyme molecules penetrating the monolayer from the aqueous subphase.

Compared with this poor hydrolytic esterase effect, the penicillinase produced a quick hydrolysis of the pivampicillin, with a rupture of the β-lactamic bond and solubilization of the penicillin, which let the monolayer pass into the aqueous subphase. Pivampicillin is normally considered more resistant than ampicillin to the action of β-lactamase enzymes, but more sensitive than other "resistant" penicillins. The resistance of pivampicillin to the penicillinase is due to the steric effect that results from the esterification of the ampicillin carboxyl group. This chemical modification makes access of the enzyme to the active site in the β-lactamic nucleus more difficult (9). In spite of this, β-lactamase has been shown in this report to be very active in the monolayer, producing hydrolysis of all the molecules in a few minutes.

The process for enzymatic reactions in monolayers, according to a general scheme proposed by Verger *et al.*, (14), takes place in three stages. In the first stage, the enzyme penetrates the monolayer in a reversible process, with a penetration constant K_p and a desorption constant K_d; with time an equilibrium is reached, so that the amount of the enzyme penetrated is equal to that desorbed. In the second stage, also reversible, an enzyme-substrate complex (of the Michaelis-Menten type) is made, with a formation constant K_m. Finally, the enzyme-substrate complex is broken, freeing the enzyme (which remains in the monolayer) and the soluble reaction product(s) (which dissolves in the aqueous subphase), with a velocity regulated by a catalytic constant K_{cat}.

This model has been discussed based on the results obtained by Dervichian (15). However, it adapts well to the results obtained in studying the enzyme kinetics of penicillin hydrolysis in monolayers by penicillinases.

At the beginning of this process, the penicillinase has to penetrate the monolayer, improving the velocity of reaction until it reaches an equilibrium between the adsorption and desorption of enzyme molecules, and the number of molecules which exist in the monolayer remains constant. This requires an induction time (τ), after which the reaction rate is constant (a pseudo-zero-order process).

The results obtained fit also with the equation proposed by Verger *et al.* (14):

$$V_m = \frac{K_{cat} E_0 S}{K_d / K_p \cdot K_m}$$

where V_m is the velocity of the process under steady-state conditions, *i.e.*, when the concentration of the intermediate products remains constant and, thus, the velocities of formation and decomposition of the enzyme-substrate complex are equal; E_0 is the enzyme concentration in the subphase; S is the surface concentration of substrate in the monolayer; K_m is the Michaelis-Menten interfacial constant ($K_{cat} + K_d/K_p$); and K_p and K_d are the penetration and desorption constants of the enzyme. As can be seen in this equation, the hydrolysis rate of the pivampicillin must increase proportionately with the increase of the enzyme concentration, which agrees with the results shown in Fig. 5.

The increase of the reaction rate with increasing surface pressure in the monolayer can be interpreted only when the increase in the pressure causes a higher surface concentration. One must remember, however, that the increase of surface pressure reduces the penetration capacity of the enzyme in the monolayer, as shown by Verger *et al.* (16) using radioactive enzymes. These two phenomena are the reason that a pressure of maximum activity can normally be reached, varying according to the enzyme and substrate used, after which the velocity of reaction decreases. In the process of the hydrolysis of pivampicillin by penicillinase, it has been impossible to reach this maximum value. However, using Fig. 4, one can assume that this maximum is found at $1.7\text{-}1.8 \times 10^{-2}\ N \cdot m^{-1}$.

Finally, one fact in this study that might be considered paradoxical is the decrease of the induction time (τ) when the surface pressure of the monolayer is increased (Fig. 6). That is, the enzyme penetrates more quickly in monolayers which are tightly compacted than in the monolayers in which the molecules of penicillin are free, leaving large intermolecular spaces. This effect can be interpreted as a result of the improvement of the "quality of the interface," especially as to the packing of the substrate molecules, which [as was demonstrated by Slotboom *et al.* (17)] influence the kinetic parameters of the enzyme, especially increasing the value of the penetration constant K_p.

REFERENCES

(1) L. Michaelis and M. L. Menten, *Biochem. J.*, **49,** 333 (1913).
(2) A. H. Hughes, *Biochem. J.*, **29,** 437 (1935).
(3) C. W. Garner and L. C. Smith, *Biochim. Biophys. Acta*, **39,** 672 (1970).
(4) J. W. Lagocki, N. D. Bayd, J. H. Law, and F. J. Kezdy, *J. Am. Chem. Soc.*, **92,** 2923 (1970).
(5) D. G. Dervichian, *Biochimie*, **53,** 25 (1971).
(6) R. Verger and G. H. de Haas, *Chem. Phys. Lipids*, **10,** 127 (1973).
(7) O. Valls, A. Alsina, and M. Alvarez, *Anal. Reale Acad. Farm.* **47,** 51 (1981).
(8) W. von Daefne, E. Drederiksen, E. Gundersen, F. Lund, P. Mork, H. J. Petersen, K. Roholt, L. Tybring, and W. O. Gottfredsen, *J. Med. Chem.* **13**(4), 607 (1978).
(9) J. P. Hou and J. W. Poole, *J. Pharm. Sci.*, **62,** 787 (1973).
(10) O. Valls, A. Alsina, and M. Alvarez, *Cien. Ind. Farm.* **1,** 23 (1982).
(11) J. Sörensen, *Biochem. Zeits*, **21,** 131 (1909).
(12) T. Yokota, *et al.*, *Jap. J. Pharmacol.*, **24,** suppl. 78 (1974).
(13) L. Sardá and P. Desnuelle, *Biochim. Biophys. Acta*, **30,** 513 (1958).
(14) R. Verger, M. C. E. Mieras, and G. H. de Haas, *J. Biol. Chem.*, **248,** 4023 (1973).
(15) D. G. Dervichian, *Biochimie*, **53,** 25 (1971).
(16) R. Verger, J. Rietsch, M. C. E. Dam-Mieras, and G. H. de Haas, *J. Biol. Chem.*, **251**(10), 3128 (1976).
(17) A. J. Slotboom, R. Verger, H. M. Verheij, P. H. M. Baartmans, L. L. M. van Deenen, and G. H. de Haas, *Chem. Phys. Lipids*, **17,** 128 (1976).

Time-Dependent Elimination of Cinoxacin in Rats

MASAHIRO KOIKE ˣ, RYO NORIKURA, KENJI MIZOJIRI, and KOICHI SUGENO

Received May 19, 1983, from *Shionogi Research Laboratories, Shionogi & Co., Ltd., Osaka, Japan.* Accepted for publication March 8, 1984.

Abstract ☐ The effect of the variation of urinary pH on the pharmacokinetics of the acidic antibacterial agent, cinoxacin (pK_a 4.60), was examined. Urinary pH of 24-h fasted rats remained at about pH 6 during the daytime, while that of nonfasted rats was high (about pH 7.5) in the morning and gradually decreased to a pH similar to that of the fasted rat in the afternoon. The free fraction of cinoxacin in fasted rat sera in the morning was similar to that in nonfasted rats despite the longer half-life of cinoxacin in fasted rats. In the afternoon the free fraction was slightly different despite similar cinoxacin elimination in fasted and nonfasted rats. These findings seemed to exclude the contribution of protein binding from the causes of increased cinoxacin elimination in nonfasted rats in the morning. Elimination rate constants of cinoxacin obtained with a one-compartment open model correlated well with urinary pH 30 min after injection, suggesting that the urinary pH plays a more important role in cinoxacin elimination. When cinoxacin was orally administered to fasted rats at 11:00, the area under the plasma concentration-time curve was threefold larger than in nonfasted rats. As found with the intravenous administration, this difference may be explained by the prolonged half-life caused by decreased urinary pH after fasting. This study revealed the time-dependent elimination of cinoxacin in nonfasted rats, which is related to physiological change of urinary pH caused by food intake.

Keyphrases ☐ Cinoxacin—time-dependent elimination, effect of fasting, urinary pH, rats ☐ Urinary pH—effect of fasting on cinoxacin elimination, diurnal alteration, rats

The chronopharmacological aspects of drugs have been discussed in many review articles (1-3). Some attribute these phenomena to the diurnal oscillation of pharmacological sensitivity to drugs (4) and others to the diurnal variance of plasma levels (or levels at the receptor site) without alteration of the sensitivity to the drugs. Alteration of plasma levels can be expressed by pharmacokinetic parameters such as absorption, distribution, metabolism, and excretion (5-9). Circadian rhythms of hepatic drug-metabolizing ability have been especially well investigated (10-13), but the causes of the rhythmicity in absorption, distribution, and excretion have not been thoroughly investigated in spite of being frequently described (9).

We noticed a diurnal rhythm of urinary pH in normally fed rats and found that the rhythm disappeared with fasting. We tried to clarify the effect of such diurnal alteration of urinary pH and fasting on the pharmacokinetics of drugs in the rat. The model compound was cinoxacin (1-ethyl-1,4-dihydro-4-oxo[1,3]dioxolo[4,5-*g*]cinnoline-3-carboxylic acid). Cinoxacin is an acidic (pK_a 4.60), antibacterial agent, which is excreted almost completely unchanged in rat urine (14, 15).

EXPERIMENTAL SECTION

Materials—Male Sprague-Dawley rats, weighing 240–270 g, were purchased commercially[1] and were housed in group cages with wooden chips on the floor in a room with constant temperature (24 ± 1°C) and humidity (55

[1] Clea Japan, Inc., Tokyo, Japan.

© 1984, American Pharmaceutical Association