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·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.

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Time-Dependent Elimination of Cinoxacin in Rats

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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.

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Table I—Biochemical Examination of Rat Sera [a]

Examination	Sera at 09:30		Sera at 15:30	
	Fasted, $n = 5$	Nonfasted, $n = 6$	Fasted, $n = 6$	Nonfasted, $n = 4$
Total bilirubin, mg/dL	0.34 ± 0.06[b]	0.17 ± 0.05	0.32 ± 0.04[b]	0.18 ± 0.05
Albumin, g/dL	3.12 ± 0.11[c]	3.22 ± 0.07[d]	3.35 ± 0.15	3.40 ± 0.08
Total protein, g/dL	5.38 ± 0.15[d]	5.60 ± 0.21[c]	5.92 ± 0.27	5.90 ± 0.12
Non-esterified fatty acid[e], mEq/L	0.52 ± 0.08[b,c]	0.15 ± 0.05[d]	0.58 ± 0.11[b]	0.26 ± 0.10

[a] Mean ± *SD*. [b] Significant difference at $p < 0.01$ compared with nonfasted rats. [c] Significant difference at $p < 0.05$ compared with sera at 15:30. [d] Significant difference at $p < 0.01$ compared with sera at 15:30. [e] Calculated as oleic acid.

± 5%). Under a 12-h light-dark cycle, rats were given free access to solid animal chow[2] and water. One-half of the rats were transferred to a cage with a stainless steel net floor (mesh size: 1.5 × 1.5 cm) 24 h prior to drug administration to prevent coprophagy (± 1) and deprived of chow but not water. Cinoxacin was obtained commercially[3]. ^{14}C-Labeled cinoxacin was synthesized by the method of Nagasaki *et al.* (16). Its specific activity was 37.2 μCi/mg and radiochemical purity was >98%. All other chemicals were of reagent grade.

Animal Experiments—Fasted and nonfasted rats, under light ether anesthesia, were catheterized in the right external jugular vein by Upton's method (17) and urine-collecting adapters were attached to their penises. The animals were then placed in individual cages[4] and left for >2 h to eliminate the effect of ether. Next, 20 mg/kg of ^{14}C-labeled or unlabeled cinoxacin (10 mg/mL of aqueous solution with an equivalent amount of sodium hydroxide) was intravenously administered *via* the caudal vein. At 12:00, [^{14}C]cinoxacin was used and at 15:00, unlabeled cinoxacin was used. The rats were periodically forced to void urine by using ether. Blood was taken *via* the catheter and immediately centrifuged to obtain the plasma. In the case of oral administration, [^{14}C]cinoxacin was used and blood sampling was done nonserially. Twenty mg/kg of [^{14}C]cinoxacin (10 mg/mL of aqueous solution prepared similarly to the intravenous injection) was orally administered and blood samples were taken *via* the aorta after abdominal incision under light ether anesthesia. Cinoxacin in plasma was determined by fluorometry (15) and the urinary pH was measured. When ^{14}C-labeled cinoxacin was administered, radioactivity was measured with a liquid scintillation counter[5]. Diurnal changes of urinary pH in noncatheterized rats were measured as described above without cinoxacin administration.

Biochemical Examination of Serum and Protein Binding—At 09:30 and 15:30, fasted and nonfasted rats were lightly anesthetized with ether and blood was withdrawn *via* the aorta after an abdominal incision. Serum was obtained by centrifugation using a serum separation aid[6]. Total bilirubin, albumin, and total protein in the serum were determined with an autoanalyzer[7] and nonesterified fatty acids were determined with a kit[8]. Free cinoxacin in the serum was measured with an ultrafiltration method *in vitro*. Five microliters of cinoxacin (6.0 mg/mL, pH 7.4 phosphate buffer solution) was added to 2 mL of the serum to make a final concentration of 15 μg/mL. After this mixture had been left for 1 h at 25°C, the serum was centrifuged in a membrane cone[9]. The free cinoxacin in the filtrate was determined by fluorometry.

Calculation of Pharmacokinetic Parameters and Statistical Analysis—The cinoxacin elimination rate constant in plasma was calculated using MULTI (18), a nonlinear least-squares program, based on a one-compartment open model. For statistical analyses, the paired *t* test and regression analysis were used.

RESULTS AND DISCUSSION

The rat which we usually use as an experimental animal takes food exclusively at night and rarely during the daytime. Biological rhythmicity probably exists based on the food intake rhythm. The effect of food and diet on pharmacokinetic parameters, *e.g.*, absorption, distribution, metabolism, and excretion, have been reviewed (19-21). Therefore, time-dependent pharmacokinetics may be considered in relation to the rhythmicity of food intake. We examined the alteration of cinoxacin pharmacokinetics as a function of time and feeding.

[2] Type CA-1; Clea Japan, Inc.
[3] Eli Lilly Co., Indianapolis, Ind.
[4] Ballman cage Model KN-326; Natsume Seisakusho, Tokyo, Japan.
[5] Model 1215 Rackbeta; LKB-Wallac, Turku, Finland.
[6] Separaid; HATCO International, Los Angeles, Calif.
[7] Model SMA plus MICRO; Technicon Instruments Corp., N.Y.
[8] NEFA Test-C *Wako;* Wako Pure Chemical Industries, Ltd., Osaka, Japan.
[9] Centriflo CF-25; Amicon Corp., Lexington, Mass.

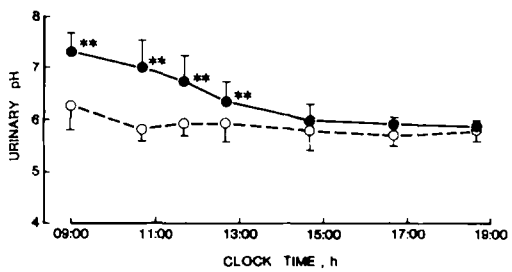

Figure 1—*Diurnal variance of urinary pH in fasted and nonfasted rats. The means and* SD *values from 15 rats are shown. Key: (●) nonfasted rats; (○) fasted rats; (**) significantly different from fasted rats at* $p < 0.01$.

In fasted and nonfasted rats, at either 09:30 or 15:30, serum was examined for factors affecting the protein binding of drugs (22-24). As shown in Table I, total bilirubin and nonesterified fatty acids increased markedly with fasting both in the morning and afternoon. Furthermore, a slight increase was observed in the afternoon in albumin, total protein, and nonesterified fatty acids in fasted and nonfasted rats. These results alerted us to the possibility that protein binding might be changed by time and feeding.

Next, we examined the diurnal rhythm of urinary pH and the effect of feeding. As shown in Fig. 1, urinary pH in fasted rats was constant during the daytime, whereas in nonfasted rats it was high in the morning and decreased slightly in the afternoon to the level of the fasted rats. This variance of urinary pH is likely to affect the renal excretion rate for drugs with pK_a values similar to urinary pH (25).

We selected cinoxacin as a model compound to investigate the effect of this variance of physiological parameters on pharmacokinetics. Renal excretion of cinoxacin is reported to be dependent on urinary pH because of renal reabsorption (26-29).

Table II shows serum protein binding of cinoxacin *in vitro*. Significant differences were detected between the serum of fasted and nonfasted rats at 15:30 but not at 09:30. Though the extent of the difference was small, it could affect the cinoxacin pharmacokinetics. Therefore, we investigated the cinoxacin elimination from plasma after intravenous administration in fasted and nonfasted rats in the morning and afternoon. Figure 2 shows the time course of plasma levels of cinoxacin after intravenous administration at 12:00 to fasted and nonfasted rats having different urinary pH values. As expected, plasma elimination was slower in fasted rats than nonfasted rats, and urinary excretion during the initial 1 h in fasted and nonfasted rats was 45.8 and 73.7% and at 2 h, 62.6 and 83.8% of the dose, respectively (data not shown). These results suggest that cinoxacin elimination was rapid especially in nonfasted rats, and the elimination was affected by minor difference of urinary pH at the time of and for at least 1 h after drug administration. This can be easily explained by greater renal reabsorption due to lower urinary pH in fasted rats (26-29).

On the other hand, when cinoxacin was injected at 15:00, it was similarly eliminated from plasma in both rat groups (Fig. 3), in spite of different serum protein binding ratios between fasted and nonfasted rats (Table II). This

Table II—*In Vitro* Serum Protein Binding of Cinoxacin in Rats

Rat	Unbound fraction, %[a]	
	Sera at 09:30	Sera at 15:30
Fasted	6.36 ± 0.38[b]	6.65 ± 0.25[c,d]
Nonfasted	6.54 ± 0.45[c]	6.00 ± 0.47[b]

[a] Percent of total concentration (15 μg/mL) ± *SD*. [b] $n = 5$. [c] $n = 6$. [d] Significant difference at $p < 0.05$ compared with sera in nonfasted rats at 15:30.

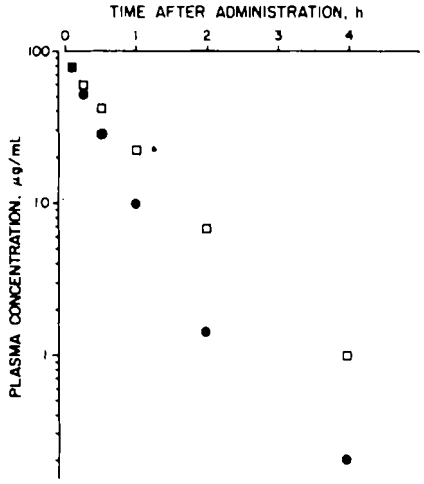

Figure 2—*Average plasma levels of cinoxacin after intravenous administration of 20 mg/kg of [^{14}C]cinoxacin to three fasted or nonfasted rats at 12:00. Key (□) fasted rats; (●) nonfasted rats.*

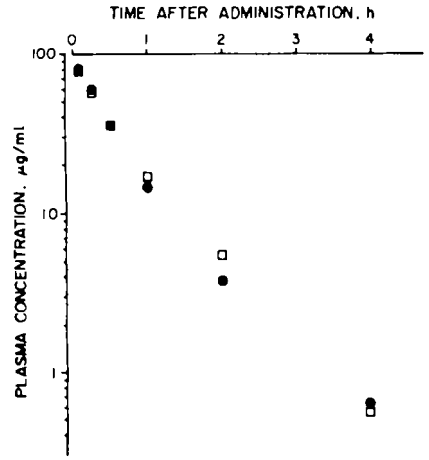

Figure 3—*Average plasma levels of cinoxacin after intravenous administration of 20 mg/kg of unlabeled cinoxacin to three fasted or nonfasted rats at 15:00. Key: (□) fasted rats; (●) nonfasted rats.*

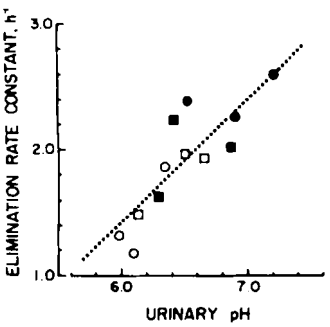

Figure 4—*Correlation between urinary pH 30 min after administration of 20 mg/kg of ^{14}C-labeled or unlabeled cinoxacin and elimination rate constant obtained from fasted or nonfasted rats at 12:00 or 15:30. Key: (●) nonfasted rats at 12:00; (○) fasted rats at 12:00; (■) nonfasted rats at 15:00; (□) fasted rats at 15:00.*

suggests that urinary pH plays a more important role than protein binding in cinoxacin elimination from the plasma. As previously described, cinoxacin elimination in nonfasted rats administered at 12:00 was rapid (Fig. 2) and >70% of the dose was excreted in urine until 13:00; furthermore, a higher pH

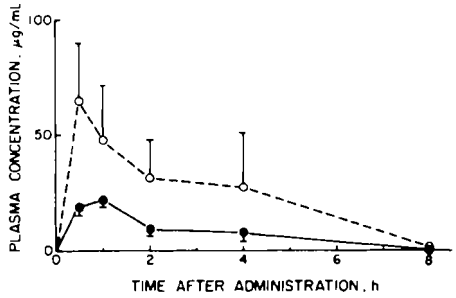

Figure 5—*Plasma levels of cinoxacin after oral administration of 20 mg/kg of [^{14}C]cinoxacin in fasted or nonfasted rats at 11:00. Means and* SD *values from three rats. Key: (○) fasted rats; (●) nonfasted rats.*

in urine was maintained at least until ~13:00 (Fig. 1). These results accounted for the faster elimination in nonfasted rats than fasted rats administered the drug at 12:00.

We investigated the correlation between urinary pH 30 min after intravenous administration and the elimination rate constant calculated from the values in Figs. 2 and 3. Figure 4 shows a significant correlation between the elimination rate constant obtained with the one-compartment open model and the urinary pH ($r = 0.8427$, $p < 0.01$). As the slope of the regression line is 1.033, if urinary pH decreases from 7 to 6, the elimination rate constant decreases from 2.43 to 1.40. Thus, diurnal variance of urinary pH in normally fed rats has an important effect on cinoxacin elimination, though in fasted rats changes in cinoxacin elimination do not arise because urinary pH remains constant during the daytime.

Figure 5 shows the plasma levels of cinoxacin orally administered to fasted and nonfasted rats at 11:00. As expected, higher plasma levels were observed in fasted rats. The area under the plasma concentration–time curve (AUC) calculated by the trapezoidal rule was 125.8 μg·h/mL in fasted rats and 40.9 μg·h/mL in nonfasted rats. The larger AUC in fasted rats is due to the lower urinary pH and in addition, a higher peak level may be partly attributable to a faster gastric emptying rate, as mentioned by Watanabe *et al.* (30).

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Determination of Benzalkonium Chloride by Gas Chromatography

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Abstract □ A new, simple, and useful approach for the analysis of benzalkonium chloride is presented. A gas chromatograph (GC) has been used to pyrolyze benzalkonium chloride in a specific and reproducible manner to yield two tertiary amines for each homologue of benzalkonium chloride present. These are separated by GC and are used to determine the homologue composition of the benzalkonium chloride. These determinations can be made with an analysis time of 25 min/sample.

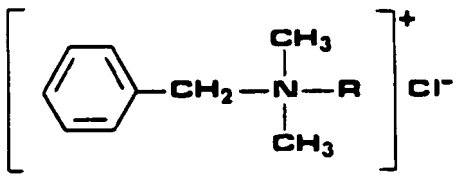
Keyphrases □ Benzalkonium chloride—determination by GC □ GC—determination of benzalkonium chloride

Benzalkonium chloride is used in pharmaceuticals as an antimicrobial preservative and is a mixture of homologous compounds (I), where the R group represents an *n*-alkyl chain which can vary from 10–18 carbon atoms. The relative amounts of the different *n*-alkyl species in benzalkonium chloride solutions greatly affect the antimicrobial spectrum and activity on the mixture (1). The current methods for analysis of benzalkonium chloride solutions according to the USP (2) rely upon titrimetric analysis of the total alkylbenzyldimethylammonium chloride based upon potassium iodate equivalents. Additionally, the ratio of *n*-alkyl components of benzalkonium chloride can be determined by microhydrogenation followed by solvent extraction and gas chromatography (GC). The ratio of alkyl components is then calculated and must meet specific USP requirements. These requirements state that the C_{12} homologue must comprise at least 40% of the total benzalkonium chloride content and that the C_{14} homologue must be at least 20%. Furthermore, these two homologues together must comprise not less than 70% of the total content.

Other methods have been reported for the determination of benzalkonium chloride, for example, ion-pairing techniques (3–5), direct titration involving tetraphenylboron sodium (6) and iodate (7). Later methods of analysis have included MS using laser ionization (8), chemical ionization (9), and HPLC (10).

GC methods include one based on a modified Hofmann degradation of benzalkonium chloride with subsequent analysis of the formed benzyldimethylamine and the corresponding alkene (11). Another method involves chemical derivatization of the benzalkonium chloride to introduce specific functional groups into the derivatives which are amenable to GC with enhanced detectability using electron capture and nitrogen-specific thermionic detecting systems (12).

The procedure presented here determines directly the alkyl chain-length ratios of benzalkonium chloride species in samples of benzalkonium chloride in an easy, sensitive, and reliable operation.



(I)

EXPERIMENTAL SECTION

Apparatus—Measurements were carried out using a GC[1] fitted with a flame ionization detector. The column used was a 0.9 m (2 mm i.d.) glass column packed with OV17 (3%) on Chromosorb W HP (80/100 mesh). A 10-μL syringe[2] was used to inject samples. The chromatograms were recorded on a potentiometric recorder[3] and peak area measurements were obtained using an integrator[4].

Mass spectrometric measurements for identification of the GC pyrolysis products were conducted using a spectrometer[5] operating in the electron impact ionization mode. The mass spectrometric data was handled by a data system[6].

Reagents, Solvents, and Standards—The carrier gas for the GC work was nitrogen; for MS work, the carrier gas was helium. The commercial samples of benzalkonium chloride were obtained from various manufacturers. Samples of benzalkonium chloride for analysis were prepared as solutions in methanol (0.5%). For confirmation, a benzalkonium chloride standard was obtained[7]. Individual pure benzalkonium chloride standards were examined with R = C_{14}, C_{16}, and C_{18}. The standards were also examined as solutions in methanol (0.5%).

[1] Model 204; Pye-Unicam.
[2] S.G.E.
[3] Model 2S; Servoscribe.
[4] Model 3390A; Hewlett Packard.
[5] Models MS30 and MS50; Kratos.
[6] Model DS55; Kratos.
[7] USP Convention Sample.

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