Quantitative Investigation of Renal Handling of Drugs in Dogs with Renal Insufficiency

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Abstract □ A quantitative analytical method for studying renal handling of drugs in dogs with mild renal impairment is described. Renal damage was induced experimentally by pretreatment with mercuric chloride or neomycin. Analytical results of renal handling in those animals indicated a reduction in maximum transport of secretion, while the affinity of drugs to secretion site and reabsorption showed slight or no change. These results were consistent qualitatively with other renal function test values which demonstrated the state of glomerular or proximal renal tubular function. Evidence for the applicability of the proposed analytical method for quantitative validation of functional changes in the nephron in renally impaired animals, as well as the precise determination of the site of damage, was demonstrated. This work holds considerable promise for the study of dosage adjustments in patients with renal disease.

Keyphrases □ Renal insufficiency—effect on the renal handling of drugs, dogs, sulfamethizole, cephalexin, ampicillin, quantitative analytical method □ Sulfamethizole—renal handling in dogs, quantitative analytical method, effect of renal insufficiency □ Cephalexin—renal handling in dogs, quantitative analytical method, effect of renal insufficiency □ Ampicillin—renal handling in dogs, quantitative analytical method, effect of renal insufficiency

In the pharmacokinetics of drugs, renal excretion plays an important role. Recent reviews (1–3) have shown that the pharmacokinetics of drugs change in patients with renal failure necessitating dosage adjustments. However, there have been few reports on the quantitative measurement of renal excretion alterations. In previous papers (4–6) we have introduced a new and simple analytical method for studying renal handling of drugs in rabbits, dogs, and humans under normal conditions. Quantitative analyses of glomerular filtration, renal tubular secretion, and renal tubular reabsorption were performed simultaneously.

Patients with renal failure show complicated patterns of renal functional impairment. In some cases, such as the early phase of pyelonephritis, kidney stone, and prostatic hypertrophy, the patients have selective renal tubular dysfunction. However, changes in renal handling of drugs in such patients have not been investigated.

In this paper, we focused on the application of our method to disease models in dogs. Thus, dogs with acute renal insufficiency (induced by nephrotoxic compounds) were chosen as the model for selective renal tubular damage in humans. The changes of renal functions and renal handling of drugs on each of the transport processes were followed in these dogs. On the simultaneous measurement of the processes mentioned, the analysis indicated the great potential of the method for diagnosing the site of the dysfunction and/or the degree of severity of nephron disorders affecting the rates of urinary excretion of drugs in patients with renal impairment.

Table I—Effects of Mercuric Chloride on Renal Tubular Secretion and Reabsorption of Sulfamethizole in a 7.5-kg Dog

	V_{max}[b], μmol/min	K_m[c], μM	R[d]	V'_m[e], μmol/min/kg
Control	17	100	0.10	2.3
24 h[a]	16	100	0.20	2.1
48 h[a]	11	98	0.21	1.5
72 h[a]	7	99	0.23	0.9

[a] Time after mercuric chloride injection. [b] Maximum velocity of secretion. [c] Michaelis constant. [d] Reabsorption fraction. [e] Corrected V_{max}.

EXPERIMENTAL SECTION

Materials—Sulfamethizole was of JP IX grade[1]. Cephalexin monohydrate[2], sodium ampicillin[2], and neomycin sulfate[3] were obtained commercially. All other chemicals were reagent grade.

Analytical Methods—*Sulfamethizole*—Plasma and urine samples were treated with deproteinizing reagents (7) and then analyzed by the procedure of Bratton and Marshall (8), using 2-dimethylaminoethyl-1-naphthylamine as the coupling reagent. Unbound (protein-free) sulfamethizole concentrations in plasma were determined by ultrafiltration using a membrane cone[4].

Cephalexin and Ampicillin—Drug concentrations in plasma and urine were determined fluorometrically (9, 10). Unbound drugs in plasma were assayed after ultrafiltration using cellulose tubing[5].

Inulin and Creatinine—Inulin concentrations in plasma and urine were measured by a modification of the method of Dische and Borenfreund (11). Creatinine levels in plasma and urine were analyzed by Jaffe's reaction using picric acid (12).

Other Urinalyses—Urine lysozyme activity was determined by the method of Litwack (13), and urine glutamic oxaloacetic transaminase (GOT) activity was assayed using 2,4-dinitrophenylhydrazine as the color reagent (14). Blood urea nitrogen (BUN) was measured by the modified diacetylmonoxime method (15). Urinary protein was estimated using urinalysis reagent strips[6].

Animal Model—Male mongrel dogs weighing 5–15 kg were used. The animals each received a single 15-mg/kg im injection of mercuric chloride or 45-mg/kg sc injections of neomycin sulfate daily for 7 d. These doses of nephrotoxic compounds were chosen to produce the selective renal tubular damage and the mild disease states, which changed slowly. Under these conditions, we could follow the changes in renal function and renal handling of drugs with time. These dosages did not cause diarrhea, and food intake and body weight did not change more than 10% throughout the experimental period. For the dogs with acute renal insufficiency induced by mercuric chloride, renal clearance experiments were performed before and after mercuric chloride injection. For the neomycin-treated dogs, the experiments were carried out before injection, 3 and 7 d after the first injection, and 1 and 2 weeks after the final injection.

Renal Clearance Experiment—Animals were anesthetized with pentobarbital (27 mg/kg ip). Each animal received a constant infusion of 4% (w/v) mannitol and 0.3% (w/v) inulin in saline at 3 mL/min throughout the experiment. Blood samples were taken by venipuncture. Urine samples were collected *via* a catheter. Urinary pH was measured immediately after collection.

Each drug was dissolved in saline and given intravenously. Urine was collected for 2 h beginning 5 min[1] after injection and at 10-min intervals thereafter. During this 2-h experiment, 6–10 blood samples were drawn, and total and unbound drug were determined in the plasma as described under *Analytical Methods*. Blood collection was performed at 1 min before the midpoint of the selected urine collection period in order to correct the delay time of drug appearance from blood to urine.

The glomerular filtration rate (GFR) was determined by inulin clearance. Clearance ratios were calculated for each sample using the unbound drug concentration in plasma (Pf) and expressed as the excretion ratio (ER) using the following:

$$ER = \frac{U \cdot V}{GFR \cdot Pf} \qquad \text{(Eq. 1)}$$

[1] Eizai Co. Ltd.
[2] Toyo Jozo Co. Ltd.
[3] Meiji Seika Co. Ltd.
[4] Centriflo CF-50A; Amicon Co. Ltd.
[5] 8/32; Visking Co.
[6] G. P. Pretest Wako; Wako Pure Chemicals.

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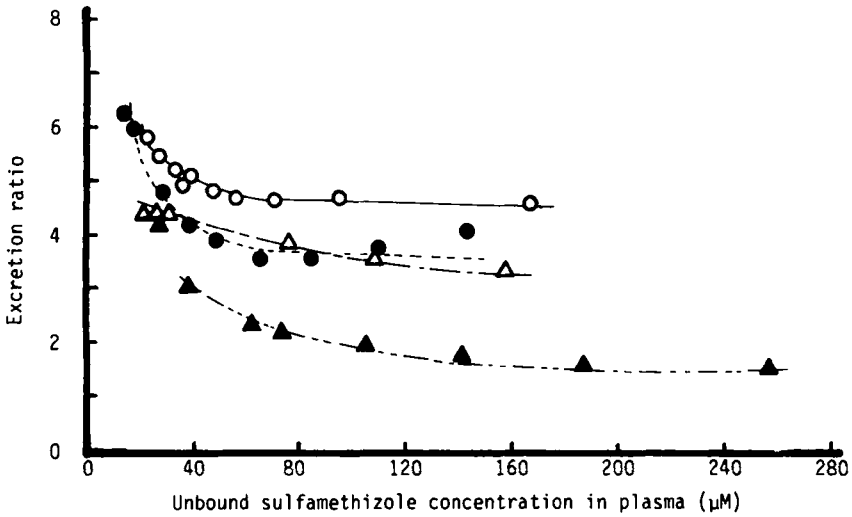

Figure 1—*Representative plot of renal clearance of sulfamethizole in a dog and computer-simulated curves before and after mercuric chloride injection. Key: (○) control (before mercuric chloride injection); (●) 24 h; (△) 48 h; (▲) 72 h.*

where U is drug concentration in urine and V is the urine flow rate. In some animals, the clearance experiments were repeated two to three times within 2–7 d under normal conditions to check the reproducibility of these experiments; the excretion ratios among the experiments were almost the same. The last clearance experiment was used as "control" in those cases.

Computer Analyses—Computer analyses were carried out using the following equation as previously described (4–6):

$$ER = \left(1 + \frac{V_{max}}{(K_m + Pf) \cdot GFR}\right) \cdot (1 - R) \qquad \text{(Eq. 2)}$$

where V_{max} is the maximum velocity of secretion, K_m is the Michaelis constant, and R is the reabsorption fraction.

RESULTS

The effects of disease on the renal clearance of various drugs injected intravenously were investigated in dogs with mild renal insufficiency induced by the administration of mercuric chloride. Figure 1 shows typical patterns of sulfamethizole secretion through the kidney and computer-simulated curves using the data listed in Table I. Excretion ratios of sulfamethizole declined as a function of time after administration of mercuric chloride. Using each curve, the maximum velocity of secretion (V_{max}), affinity constant of drug to the site of secretion (K_m), and tubular reabsorption fraction (R) were calculated. The analytical results indicated that the treatment with mercuric chloride markedly reduced the V_{max}, but K_m remained unchanged (Table I, Fig. 2). Also, administration of mercuric chloride produced an increase in renal tubular reabsorption as the urine pH decreased (Fig. 2), but glomerular filtration rate (GFR), creatinine clearance (Ccr), and BUN remained unaltered. Regarding the renal tubular functions, however, urine lysozyme activity, urine GOT activity, and urinary protein increased with time after mercuric chloride injection.

Concomitantly, the effects of disease on the renal clearance of ampicillin (Fig. 3a) and cephalexin (Fig. 3b) were also studied 48 h after the injection of mercuric chloride. Similar results as those for sulfamethizole were obtained using either drug; *i.e.*, V_{max} diminished substantially (50–60% of control), while K_m and R showed no significant change compared with the control values. Urine lysozyme activity increased markedly, while GFR decreased slightly.

Renal clearance experiments for sulfamethizole were then carried out on neomycin-treated dogs. Figure 4 shows representative results of this experi-

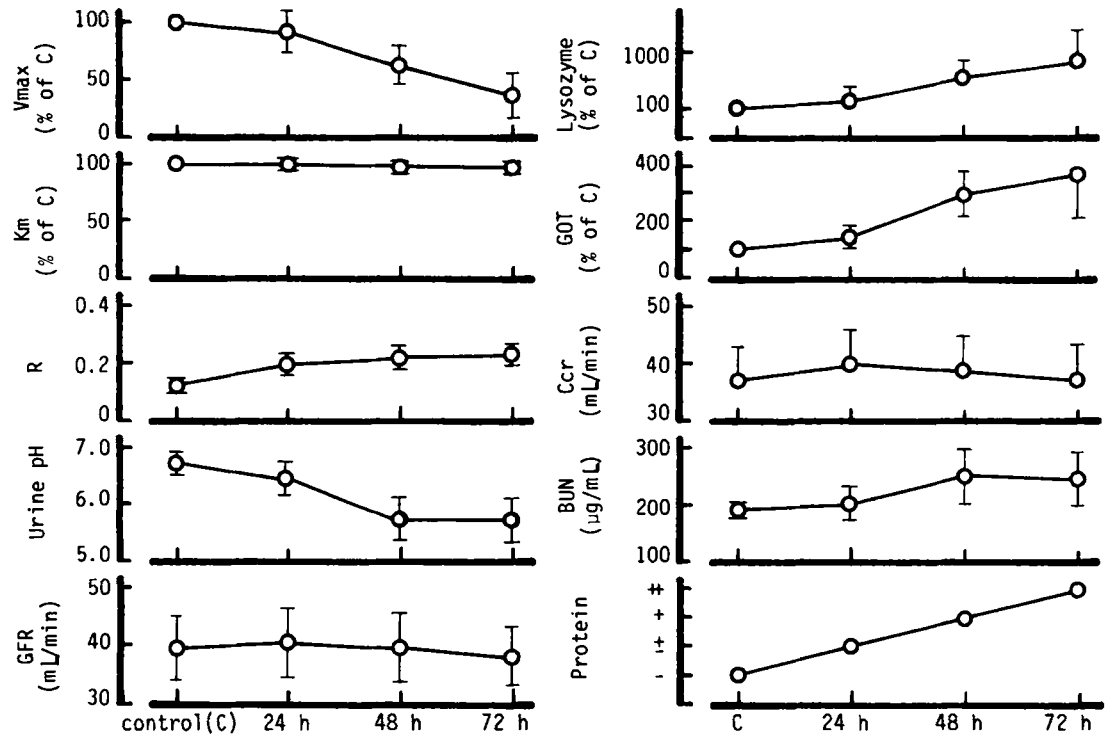

Figure 2—*Effects of mercuric chloride on various renal functions in dogs. Data represent mean ± SD of three animals. Key: (V_{max}) maximum velocity of secretion of sulfamethizole; (K_m) Michaelis constant for secretion of sulfamethizole; (R) reabsorption fraction of sulfamethizole; (GFR) glomerular filtration rate; (Lysozyme) lysozyme activity in urine; (GOT) glutamic oxaloacetic transaminase activity in urine; (Ccr) creatinine clearance; (BUN) blood urea nitrogen; (Protein) urinary protein; (C) control.*

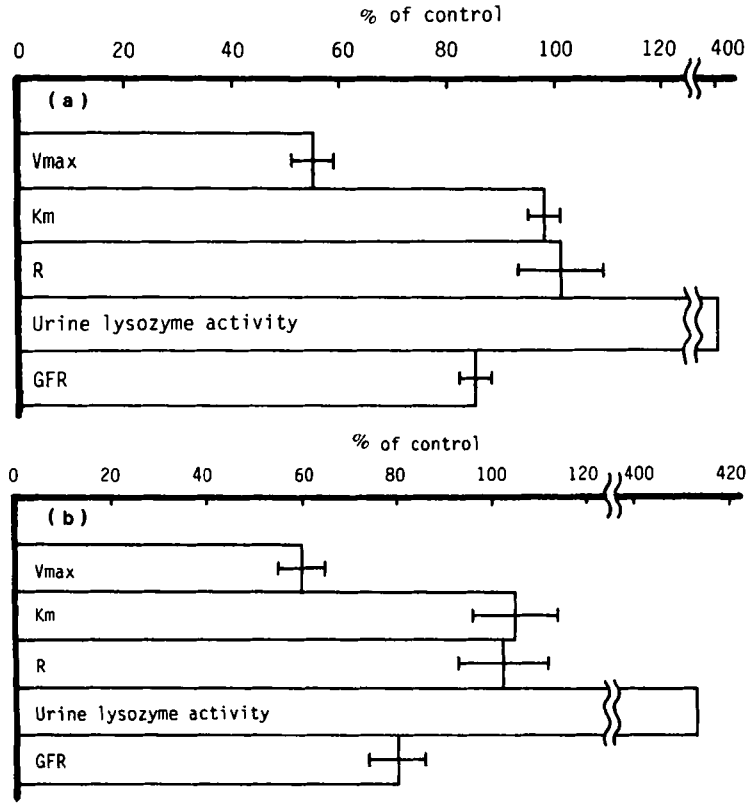

Figure 3—*Effects of mercuric chloride on the renal clearances of ampicillin (a) and cephalexin (b) and renal function of dogs 48 h after injection. Data represent mean ± SD of three animals. Abbreviations are as described in Fig. 2.*

ment with computer-simulated curves, and Table II presents the analytical data. The administration of neomycin decreased the excretion ratio for sulfamethizole, with a gradual recovery on the cessation of neomycin effect with time (Figs. 4 and 5). Our analysis clearly demonstrated the substantial reduction of V_{max} during the neomycin treatment (Table II, Fig. 5: 3 and 7 d). One and two weeks after the end of neomycin treatment, V_{max} increased and approached the values of the control, although K_m and R remained unmodified throughout the experiments (Table II, Fig. 5). Slight increases in lysozyme and GOT excretion in urine were detected (Fig. 5). GFR and Ccr were decreased ~20% as compared with the controls, but BUN showed no increase, except 7 d after the start of neomycin treatment (Fig. 5).

DISCUSSION

Some patients with renal failure show a nonparallel decrease of glomerular and tubular functions. However, the changes in renal handling of drugs in such patients are unknown. Thus, experiments were performed to clarify those quantitative changes using our proposed method. As a subclinical study, mercuric chloride- or neomycin-treated dogs were chosen as the model of selective renal tubular impairment.

Induction of renal impairment by mercuric chloride is well known. Histological studies have shown primary damage in the proximal tubular cells (16, 17). Thus, dogs treated with mercuric chloride were used as a model for the study of the effect of acute renal disease on drug clearance.

Dogs treated with mercuric chloride showed no significant change in the indices of glomerular filtration (GFR, Ccr, and BUN) as compared with the normal condition (Fig. 2). However, the urine pH decreased (Fig. 2), indicating alteration of the kidney function. The significant increases of lysozyme, GOT, and protein in the urine indicated renal tubular damage.

Lysozyme and GOT in urine have been reported to reflect the proximal tubular cell function (18, 19). As these two enzymes are actively reabsorbed by the proximal tubular cells, they are excreted into urine in minute amounts

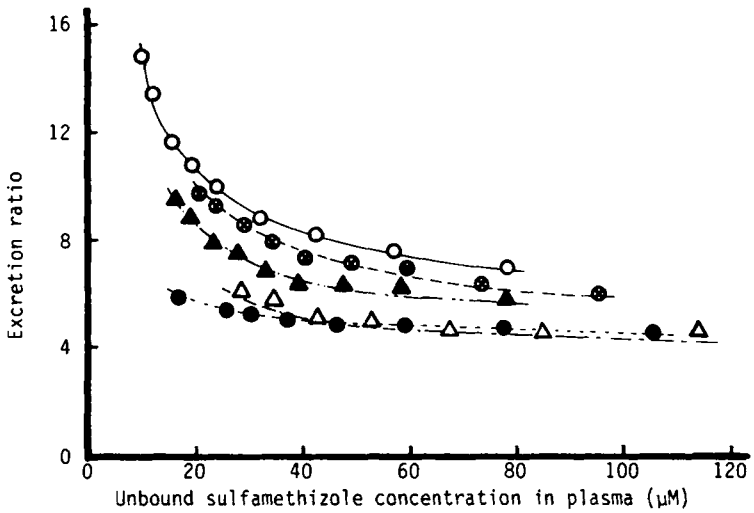

Figure 4—*Representative plot of renal clearance of sulfamethizole in a dog and computer-simulated curves before, during, and after neomycin injections. Key: (○) control (before injection); (●) 3 d and (△) 7 d after the first neomycin injection; (▲) 1 week and (⊗) 2 weeks after the final neomycin injection.*

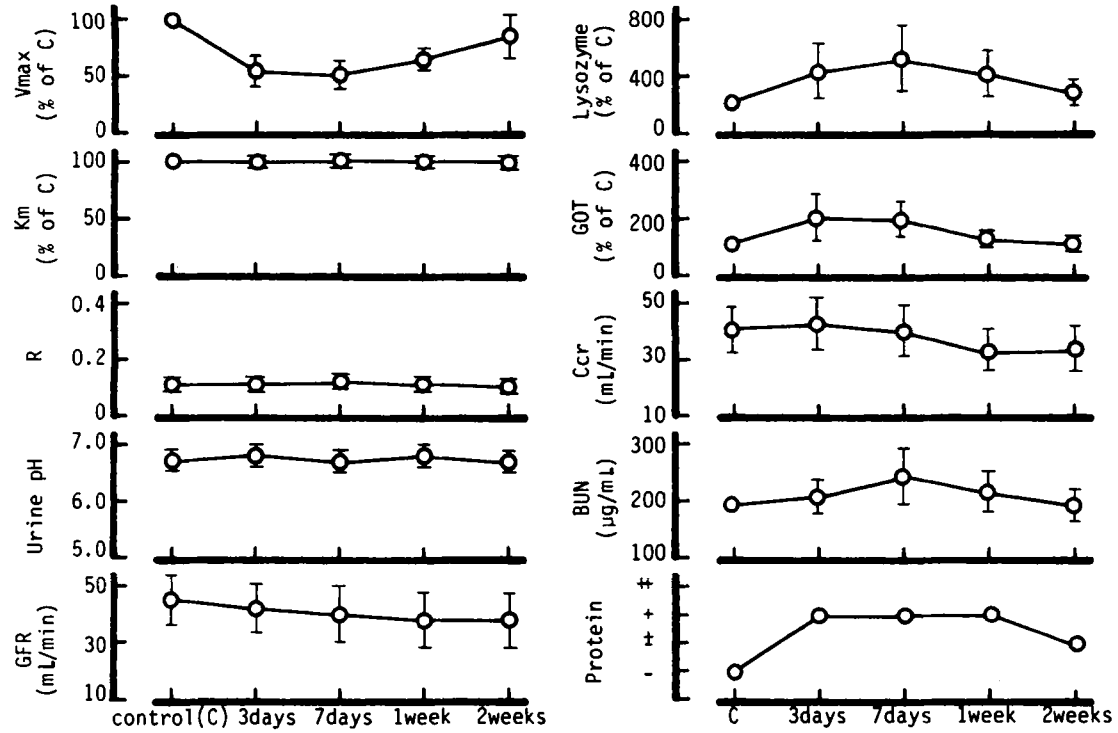

Figure 5—*Effects of neomycin on various renal functions in dogs. Data represent mean ± SD of three animals. Abbreviations are as described in Fig. 2.*

under normal conditions. The excreted amounts of these enzymes increase as the number of injured renal tubular cells increase. Increased lysozyme and GOT activities in urine, therefore, indicate damage of the proximal renal tubules (Fig. 2). Further, the increase in urinary protein has been considered due to cell debris from damaged tubular cells.

Results gathered in the study of sulfamethizole using the clearance method fitted the above findings with good qualitative compatibility, *i.e.*, a marked decrease in maximum velocity of secretion, V_{max}, and no change in the affinity constant of secretion, K_m, which suggested the reduction in the number of intact proximal tubules. Also, the absence of alteration in reabsorption for cephalexin and ampicillin, as shown in Fig. 3, and the pH-dependent changes in the reabsorption for sulfamethizole supported the finding that renal tubular reabsorptive function was intact. The analytical data clearly demonstrated that the reduction in excretion ratios were mainly due to decreases in V_{max} for secretion.

Specific proximal tubular cell damage has also been reported for neomycin (20, 21). Our results indicated only mild damage to the proximal tubules, as shown in Fig. 5. Subsequently, signs of recovery from damage caused by neomycin injections were detected. Under this slightly impaired condition (Ccr remained the same or decreased slightly), the excretion ratios were substantially diminished, and the analytical data revealed that this reduction was due to the decrease in V_{max} (Table II). These results were consistent with the early report of neomycin effects by Gol'dberg (20). He reported changes in the transport maximum of *p*-aminohippurate (Tm PAH) and Ccr under the conditions we used, with greater reduction of Tm PAH than of Ccr. Present results also showed a greater reduction of V_{max} than of Ccr.

Although body weights did not change more than 10% over the course of the experiment, since the dogs varied in weight from 5 to 15 kg, V_{max} per unit weight (V'_m) was calculated. V'_m showed small variation among dogs (Tables I and II).

Table II—Effects of Neomycin on Renal Tubular Secretion and Reabsorption of Sulfamethizole in a 15-kg Dog

	V_{max}[c], μmol/min	K_m[d], μM	R[e]	V'_m[f], μmol/min/kg
Control	33	90	0.90	2.2
3 d[a]	18	89	0.10	1.1
7 d[a]	18	98	0.12	1.2
1 week[b]	21	95	0.10	1.4
2 weeks[b]	28	93	0.09	1.9

[a] Days after the first neomycin injection. [b] Weeks after the final neomycin injection. [c] Maximum velocity of secretion. [d] Michaelis constant. [e] Reabsorption fraction. [f] Corrected V_{max}.

In conclusion, the quantitative data gathered on the renal handling of drugs in dogs with experimentally induced acute renal insufficiency provided conclusive evidence for the reliability of the clearance method for qualitatively testing renal function. Functional changes in the nephron could be also quantitatively estimated.

Recently, a kinetic model (using normal dogs) of the renal excretion of iodopyracet has been proposed (22). That method has not been useful clinically since data fitting for plasma concentrations must be done prior to the calculation of parameters for urinary excretion rates. Our equation was simple and suitable for clinical application in patients with renal failure and was found to be more appropriate than that method using the Akaike's information criterion (23, 24).

This study suggests the use of the described method in dosage adjustments for patients with renal disease. This possibility is now being tested clinically in patients with renal failure where the renal tubular functions are reduced more than their creatinine clearance.

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Aqueous Conversion Kinetics and Mechanisms of Ancitabine, a Prodrug of the Antileukemic Agent Cytarabine

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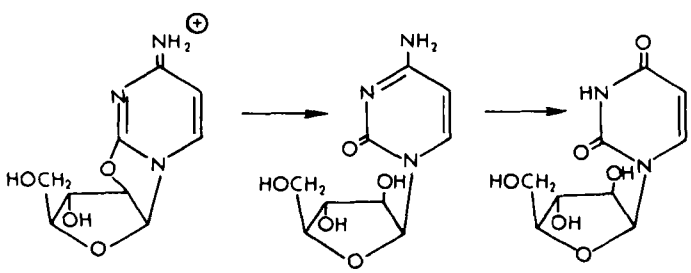
Abstract □ The kinetics of conversion of the prodrug ancitabine to the anticancer drug cytarabine have been studied in aqueous solutions in the pH range of 1.5–10.7, temperature range of 19.5–80.0°C, ionic strength range of 10^{-4} to 1.5, and in the presence of several general-base catalysts. Under all conditions ancitabine was quantitatively converted to cytarabine. The pH–rate profiles were linear with slope = 1 in alkaline pH, becoming pH independent in the region of maximum stability at pH ≤ 4, where buffer catalysis was found to be insignificant and $k_{obs} \cong (1.12 \times 10^{11}\ h^{-1})\exp\{-10121\ deg/T\}$. At 30°C, pH ≤ 4, it is calculated that an aqueous ancitabine solution will maintain 90% of its initial concentration for 12 d. A novel method for measuring general-base catalysis in competition with predominating specific-base catalysis and in the presence of secondary salt effects at constant ionic strength was developed. Three mechanisms of hydrolytic prodrug conversion are proposed: nucleophilic hydroxide addition, general base-assisted nucleophilic water attack, and spontaneous water attack.

Keyphrases □ Ancitabine—conversion to cytarabine, kinetics, aqueous solutions □ Cytarabine—conversion of ancitabine, aqueous solutions, kinetics □ Kinetics—ancitabine conversion to cytarabine, aqueous solutions

Ancitabine (I), a prodrug of the antileukemic agent cytarabine (II), has been shown to be more effective than II in several animal tumor systems (1, 2) (Scheme I). The pharmacological activity of I is attributed to its conversion to II rather than any direct effect on nucleic acid synthesis (3, 4). Although incubation of I with Ehrlich ascites carcinoma cells demonstrated very little uptake of I, significant intracellular concentrations of II were nonetheless observed (5).

Intravenous doses of I are primarily excreted unchanged in urine together with II and its inactive metabolite, 1-β-D-arabinosyluracil (III) (3, 6, 7). The efficacy of II is limited by its rapid deamination to III (6). It has been suggested that a depot form of II might be useful in maintaining effective levels (8).



I II III

Scheme I

Unlike II, I is not phosphorylated nor deaminated (6, 9, 10). A prodrug may extend the biological duration of the drug through slow prodrug absorption or rate-limiting conversion to the drug (11). Ancitabine may act as a reservoir through hydrolytic production of II (8).

In vivo conversion of I is thought to be chemical rather than enzymatic hydrolysis (8, 12, 13). The purpose of this study was to investigate the kinetics and mechanisms of prodrug hydrolysis in aqueous solutions. Since I, like II, is not orally active (6), it is important to define its stability for preparation and storage of parenteral formulations.

EXPERIMENTAL SECTION

Stability in Sodium Hydroxide and Hydrochloric Acid Solutions Using Thin-Layer Chromatography—Samples of 0.01 M ancitabine (I)[1] in 0.1 M HCl and 0.05 M NaOH at 50°C were taken over a 4-d period, and 10-μL aliquots were spotted together with reference samples of I, II[2], III[3], and cyclouridine[4] on 20 × 20-cm TLC plates (0.25 mm silica gel GF)[5]. The plates were developed to 10 cm in water-saturated 1-butanol–propanol (3:1), air dried, and examined at 254 nm. A time zero plate was also impregnated with I_2 vapor, and 0- and 1-h plates were sprayed with sulfuric acid and heated. This procedure was also used periodically in the kinetic studies to identify the components in the reaction mixtures.

Spectrophotometric Analysis of Ancitabine and Cytarabine in Mixtures—Absorbance spectra from 220 to 330 nm were obtained for synthetic mixtures to determine which wavelengths could best be monitored for reaction time-course changes[6]. These showed an isosbestic point at 267 nm.

Beer's law plots in 0.1 M HCl provided molar absorptivities (ε) at 240 and 290 nm of 7.25×10^3 and 4.70×10^2 for I and 1.38×10^3 and 1.05×10^4 for II. Known concentrations in mixtures were successfully calculated using:

$$10^5 \cdot [I] = 13.914A_{240} - 1.828A_{290} \quad \text{(Eq. 1)}$$

$$10^5 \cdot [II] = 9.604A_{290} - 0.623A_{240} \quad \text{(Eq. 2)}$$

which were derived from simultaneous equations for total absorbance, A, at 240 and 290 nm for mixtures of I and II.

Analysis of Three-Component Mixtures—Reverse-phase HPLC with

[1] 2,2′-Anhydro-(1-β-D-arabinofuranosyl)-cytosine; Sigma Chemical Co., St. Louis, Mo.
[2] Cytarabine; The Upjohn Co., Kalamazoo, Mich. The chemical name is 1-β-D-arabinofuranosylcytosine, the IUPAC name is arabinosylcytosine, and the common name is cytosine arabinoside (ara-C).
[3] Uracil-β-D-arabinofuranoside; Sigma Chemical Co., St. Louis, Mo.
[4] β-D-O^2,2′-cyclouridine; Terra-Marine Bioresearch, La Jolla, Calif.
[5] Type TLP 109; New England Nuclear, Boston, Mass.
[6] Model 250 spectrophotometer with model 6051 recorder, model 2451A automatic cell positioner, and model 6047 thermosensor; Gilford Instruments, Oberlin, Ohio.

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