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Small Intestinal Absorption of Bropirimine in Rats and Effect of Bile Salt on the Absorption

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Abstract

The intestinal absorption characteristics of a poorly water-soluble drug, bropirimine, were investigated by the in-situ small intestinal loop method using male Sprague-Dawley rats.

Bropirimine in solution was well absorbed in the overall small intestine, following first-order kinetics. The rate determining step for the disappearance of bropirimine from the small intestinal loop after dosing in the suspension was the dissolution process from suspension. Bropirimine was solubilized by sodium glycocholate.

The disappearance of bropirimine from the small intestinal loop was suppressed by sodium glycocholate contained in the solution, because of the loss of thermodynamic activity of bropirimine after its involvement in the micellar complex, not by the direct effect of bile salt on the permeability of intestinal mucosa. The disappearance of bropirimine was also suppressed by sodium glycocholate contained in the suspension.

The suppression by sodium glycocholate seemed to be caused by the greater influence of sodium glycocholate on the thermodynamic activity of bropirimine than on the dissolution from suspension.

Bropirimine, 2-amino-5-bromo-6-phenyl-4(3*H*)-pyrimidinone, is a biological-response modifier (immune modulator), which has established induction of interferon, and modulation of other lymphokines, antiviral and antitumour activity in various animal models (Stringfellow et al 1980; Hamilton et al 1982; Wierenga 1985; Eggermont et al 1986; Chang et al 1986). It is an orally active agent when given at high doses.

Because bropirimine is poorly water-soluble, the investigation of its intestinal absorption characteristics after administration of solution and suspension was needed before development of oral dosage forms with high doses. Bile salt increases the solubility and dissolution rate of poorly water-soluble drugs (Bates et al 1966a, b; Miyazaki et al 1979). It has been reported also that bile plays an important role in dissolution and absorption of poorly water-soluble drugs in the gastrointestinal tract (Miyazaki et al 1980; Aoyagi et al 1982; Shinkuma et al 1985). On the other hand, there are a few reports in which bile salt reduced the intestinal absorption of lipophilic drugs (Poelma et al 1989, 1990).

In the present study, intestinal absorption of bropirimine was investigated by a rat in-situ small intestinal closed-loop method. The study included investigations of the influence of site within the small intestine and the influence of bile salt on the absorption of bropirimine.

Materials and Methods

Materials

Bropirimine was supplied by The Upjohn Co. (MI, USA). Sodium glycocholate (purity 95·3%) was obtained from Tokyo Kasei Organic Chemicals (Tokyo, Japan). Carmellose sodium of practical grade was from Wako Pure Chemical Industries Ltd (Osaka, Japan). Other reagents used were of analytical grade.

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Solubility of bropirimine

The solubility of bropirimine was determined as follows: bropirimine (60 mg) was weighed and placed in a glass-stoppered centrifuge tube containing a solvent solution (30 mL). Phosphate buffer, acetate buffer, 0·1 M HCl, the first and second media of the disintegration test in the JP XII (pH 1·2 aqueous solution containing NaCl and HCl, and pH 6·8 aqueous solution containing KH_2PO_4 and NaOH, respectively) and saline containing sodium glycocholate at various concentrations were used as solvents. The tube was shaken at 100 strokes min^{-1} in a water bath at 37°C for 48 h; an aliquot was then collected after filtering through a Durapore filter (pore size 0·22 μm, hydrophilic polyvinylidene fluoride membrane; Nihon Millipore, Tokyo, Japan) to determine the solubility. The drug concentration of the filtrate was measured by HPLC. To investigate the adsorption of bropirimine to the filter, bropirimine solutions in the JP XII disintegration first medium (455 $\mu g\,mL^{-1}$), the JP XII disintegration second medium (23 $\mu g\,mL^{-1}$) and saline with 1 and 40 mM sodium glycocholate (25 and 88 $\mu g\,mL^{-1}$, respectively) were prepared, and the drug concentrations of the solutions before and after filtering were determined. Adsorption of drug to the filter was not observed.

Formulation used in the in-situ small intestinal loop study

Four bropirimine solutions (30 $\mu g\,mL^{-1}$) were prepared by dissolving the bulk drug in saline containing sodium glycocholate at various concentrations (0, 1, 10 and 40 mM). Two

bropirimine solutions (10 and 100 $\mu g\,mL^{-1}$) in saline containing sodium glycocholate at 40 mM were also prepared. Two bropirimine suspensions (200 and 500 $\mu g\,mL^{-1}$) were prepared as follows. The drug was suspended in saline containing carmellose sodium (0·25%) with a pestle and a mortar. A suspension of 500 $\mu g\,mL^{-1}$ with sodium glycocholate was prepared by suspending the drug in saline containing carmellose sodium (0·25%) and sodium glycocholate (40 mM) with a pestle and a mortar. To avoid significant changes in the particle sizes in suspensions from the point of thermodynamic equilibrium, the suspensions were prepared just before administration. The pH values of all the solutions and suspensions were between 6 and 7.

In-situ small intestinal loop study

The in-situ small intestinal loop study was carried out to estimate intestinal absorption of bropirimine according to the method described by Nishihata et al (1986). Male Sprague-Dawley rats, 200–230 g, were fasted for 16 h before the experiment during which water was freely available. After the rats were anaesthetized with sodium pentobarbitone (30 $mg\,kg^{-1}$, i.p.) the middle abdominal incision was performed. An intestinal loop (about 10 cm) was prepared, the drug formula (1 mL) was administered, and the abdomen was closed. The intestinal loop was excised at a designated time after administration to determine the remaining amount of bropirimine. After incision, the mucosa of the loop was rinsed with 15 mL saline three times and the rinse solutions were combined. The regional differences in absorption rate were investigated by preparing intestinal loops in the upper, middle or lower region of the small intestine. The upper jejunum, the lower ileum, and the middle part between these two were defined as the upper, lower and middle region of the small intestine, respectively. The absorption characteristics and the effect of sodium glycocholate were investigated using the upper region of the small intestine. It has been reported that the aqueous boundary layer in the closed-loop influences the drug absorption kinetics (Schurgers et al 1986). Moving and touching the loop during the experiment was minimized to minimize the effect on the aqueous boundary layer thickness, and a strictly identical procedure was employed to avoid a difference in the thickness variation between the experiments.

Assay of bropirimine by HPLC

The solubility of bropirimine was determined by HPLC as follows. Filtrate (500 μL) and 5 mL of an internal standard solution (3 $\mu g\,mL^{-1}$ *p*-hydroxethylbenzoate in methanol) were placed into a test tube and mixed vigorously for 30 s, followed by sonication in an ultrasonic cleaner (47 kHz; Yamato Scientific Co., Ltd, Tokyo, Japan) for 5 min. Ten microlitres of the mixed solution was injected onto the HPLC column. The HPLC system consisted of a liquid chromatograph (LC-6A: Shimadzu, Kyoto, Japan), an auto injector (SIL-6A; Shimadzu) with a system controller (SCL-6A; Shimadzu), a UV spectrophotometric detector (SPD-6A; Shimadzu) and a Chromatopac (C-R6A; Shimadzu). For the stationary phase, a reverse-phase column (STR ODS-H, 15 cm–4 mm; Shimadzu Techno Research, Kyoto, Japan) was used. The mobile phase was a mixture of water, methanol, and acetic acid (50 : 50 : 1, v/v/v) with a flow rate of 0·6 mL min^{-1}. Detection was performed at 254 nm. Regarding the reproducibility of the assay, the mean results ± s.d. were 21·8 ± 1·1, 450·8 ± 21·0, 26·4 ± 0·3 and 90·6 ± 1·3 $\mu g\,mL^{-1}$ bropirimine for 23 $\mu g\,mL^{-1}$ in the JP XII disintegration second medium, 455 $\mu g\,mL^{-1}$ in the JP XII disintegration first medium, 25 $\mu g\,mL^{-1}$ in saline with 1 mM of sodium glycocholate and 88 $\mu g\,mL^{-1}$ in saline with 40 mM of sodium glycocholate, respectively. Bropirimine samples obtained in the in-situ intestinal loop study were assayed as follows. The combined rinsed solution was diluted in a volumetric flask with saline to make a total volume of 100 mL, and then sonicated in the ultrasonic cleaner for 10 min. After centrifugation (1000 *g*, 10 min), 1 mL of the supernatant was collected, and 2 mL of an internal standard solution (0·5 or 2 $\mu g\,mL^{-1}$ *p*-hydroxyethylbenzoate in acetonitrile) was added gradually. After centrifugation (1000 *g*, 10 min), 2 mL of the supernatant was collected and evaporated under nitrogen at 50°C. The residue was dissolved in the mobile phase to prepare an assay sample. Bropirimine was assayed by HPLC under the conditions described except for the following changes; the mobile phase comprised a mixture of water, acetonitrile and acetic acid (70 : 20 : 1, v/v/v), a flow rate of 0·8 mL min^{-1} was employed, and an injection volume of 50 mL was used. Regarding the reproducibility of the assay, the mean results ± s.d. were 9·7 ± 0·5, 18·3 ± 0·9 and 51·0 ± 0·5 $\mu g\,mL^{-1}$ for 10, 20 and 50 $\mu g\,mL^{-1}$ bropirimine, respectively.

Results

Solubility of bropirimine

Table 1 shows the aqueous solubility of bropirimine at various pH values at 37°C. The solubility was poor in the pH range 2–8 but increased below pH 2. The solubility of bropirimine in saline increased in the presence of sodium glycocholate (Fig. 1), indicating the higher solubility (three times) in saline with 40 mM of the bile salt. However, the bile salt did not show a clear critical micellar concentration (CMC) in the solubility profile. To determine the CMC of sodium glycocholate, 30 $\mu g\,mL^{-1}$ bropirimine in saline

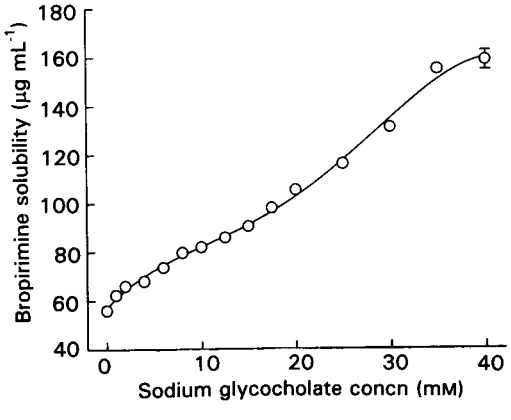

FIG. 1. Solubilization of bropirimine by sodium glycocholate in saline at 37°C. Each point represents the mean ± s.d. of four experiments.

Table 1. Solubility of bropirimine at 37°C.

Medium	pH of medium	pH of saturated solution	Solubility (mg mL^{-1})
0·1 M HCl	0·95	1·00	1·494
JP XII disintegration first medium	1·20	1·18	0·964
pH 2·0, 0·1M Phosphate buffer	2·01	2·04	0·186
pH 3·0, 0·1 M Phosphate buffer	3·09	3·10	0·069
pH 4·0, 0·1 M Acetate buffer	4·05	4·06	0·061
pH 5·0, 0·1 M Acetate buffer	5·03	5·03	0·057
pH 6·0, 0·1 M Phosphate buffer	5·96	5·96	0·059
JP XII disintegration second medium	6·85	6·88	0·065
pH 7·0, 0·1 M Phosphate buffer	7·02	7·02	0·065
pH 8·0, 0·1 M Phosphate buffer	8·03	8·01	0·135

containing sodium glycocholate at various concentrations were prepared and the osmotic pressure and surface tension of the solutions were determined at 25°C using a freezing point osmometer (OM 801; VOGEL, Germany) and a Wilhelmy tensiometer (ESB-V; Kyowa Science Co. Ltd, Tokyo, Japan), respectively. As shown in Fig. 2, the CMC on the basis of both these methods was approximately 7 mM.

Effect of bile salt on small intestinal absorption of bropirimine in rats

As shown in Fig. 3, bropirimine in solution disappeared from the rat intestinal loop with apparent first-order kinetics. The disappearance rate constant (k_{dis}) was calculated from the logarithm of the percent remaining in the loop plotted against time by linear regression and given in Table 2 with half-life ($t_{\frac{1}{2}}$). The lower site of the small intestine tended to have the smaller disappearance rate constant; however, there were no significant statistical differences in the percent of bropirimine remaining in the loop at each time point between the parts of the small intestine. The adsorption of bropirimine to the rat intestinal loop seemed to be negligible because the remaining percents of bropirimine extrapolated to time 0 were nearly 100%. Thus, the disappearance of bropirimine from the loop was considered to correspond to the absorption. These results indicated that bropirimine was well-absorbed in the overall small intestine. As bropirimine has a low aqueous solubility, bropirimine in suspension disappeared from the rat intestinal loop at a slower rate than that in solution. When the apparent disappearance rate after dosing in suspension was analysed according to first-order kinetics (Table 3), the results indicated that the absorption rate decreased as the bropirimine dose increased, i.e. the delay in apparent absorption of bropirimine in suspension indicates that the overall limiting step is the dissolution step of bropirimine rather than the absorption step.

The disappearance of bropirimine from the intestinal loop after dosing in solution was suppressed as the concentration of sodium glycocholate increased (Fig. 4, Table 4). However, when the concentration of sodium glycocholate was constant (40 mM), no differences were found in the rate of bropirimine disappearance between the drug concentrations

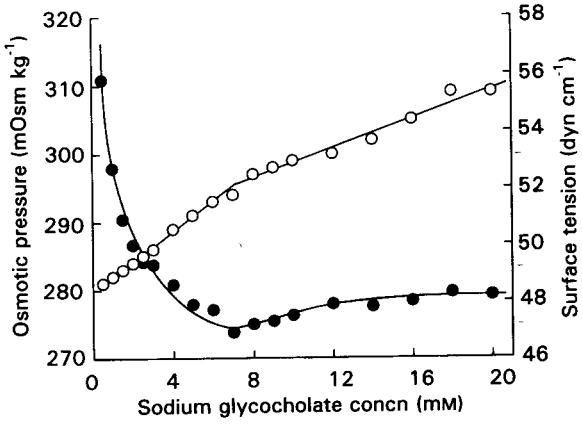

FIG. 2. Osmotic pressure (○) and surface tension (●) of 30 μg mL^{-1} bropirimine solution as a function of sodium glycocholate concentration. Each point represents the mean of two experiments.

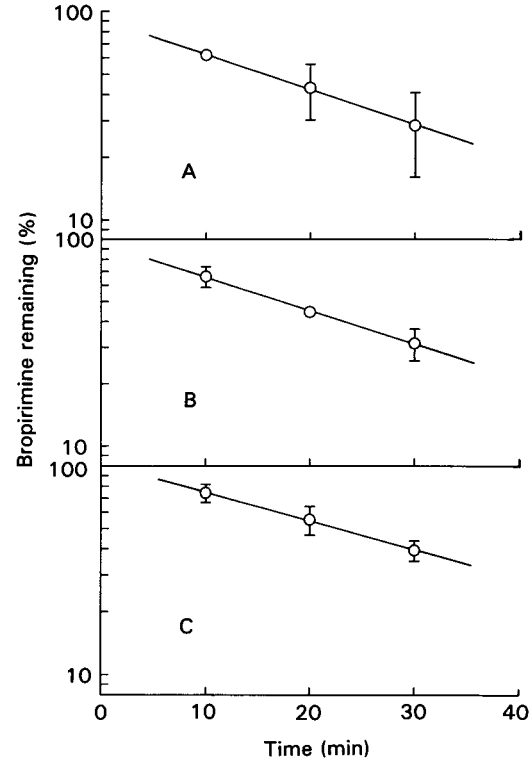

FIG. 3. Bropirimine (%) remaining in rat small intestinal loop after administration of 30 μg mL^{-1} bropirimine solution in the upper (A), middle (B), and lower (C) parts of the small intestine. Each point represents the mean ± s.d. of at least three rats.

Table 2. Parameters for disappearance of bropirimine from various sites of rat small intestine after administration in solution (30 $\mu g\,mL^{-1}$ in saline).

Loop site	k_{dis} (min^{-1})	$t_{\frac{1}{2}}$ (min)
Upper	0·0419	16·5
Middle	0·0378	18·4
Lower	0·0318	21·8

of 10, 30 and 100 $\mu g\,mL^{-1}$. The disappearance of bropirimine in the suspension of 500 $\mu g\,mL^{-1}$ was also suppressed by the presence of sodium glycocholate at 40 mM in the administered formula (Table 3).

Discussion

The absorption of bropirimine is considered to occur by simple diffusion kinetics through the intestinal wall, because the disappearance of bropirimine in the small intestine was consistent with first-order kinetics when administered in solution. The diffusion of a drug in a micellar solution can be described by the phase-separation model (Stilbs 1982). The apparent diffusion coefficient (D_{app}) is defined by equation 1:

$$D_{app} = sD_m + fD_f \quad (1)$$

where D_m and D_f are the diffusion coefficients of the drug in micelles and the free drug, respectively. The fraction of the drug solubilized in micelles (s) and the fraction of the drug free in solution (f) are calculated from the solubility data by equations 2 and 3:

$$s = (C_s - C_0)/C_s \quad (2)$$

$$f = 1 - s \quad (3)$$

where C_s is the solubility of the drug in the medium with micelles and C_0 is the solubility in the medium without micelles. Therefore, when the permeability of the intestinal mucosa is not affected directly by sodium glycocholate, the ratio (R) of the disappearance rate constants of bropirimine from rat intestional loop in the presence and absence of sodium glycocholate is expected to be similar to the ratio of the diffusion coefficients (D_{app}/D_f). R is calculated as the ratio of k_{dis} of the solution with sodium glycocholate and k_{dis} of the solution without sodium glycocholate; when the diffusion barrier allows only the passage of free drug, R is expected to be consistent with f. The values of R after administration of solution are listed with the values of f in Table 4. R was almost in agreement with f for a particular

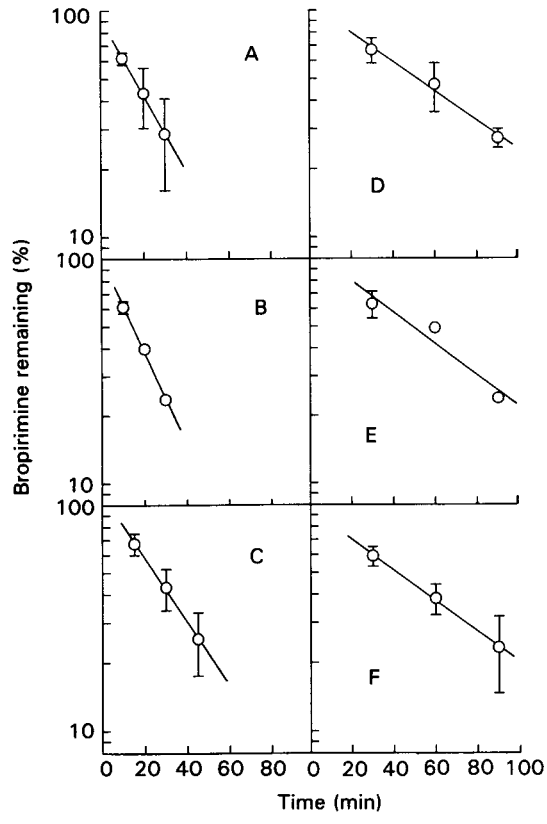

FIG. 4. Bropirimine (%) remaining in the upper part of the rat small intestinal loop after administration of bropirimine solution (A, B, C, D 30 $\mu g\,mL^{-1}$; E 10 $\mu g\,mL^{-1}$; F 100 $\mu g\,mL^{-1}$) with sodium glycocholate (A none; B 1 mM; C 10 mM; D, E, F 40 mM). Each point represents the mean ± s.d. of at least three rats.

micellar concentration. These results indicated again that the absorption of bropirimine was controlled by diffusion kinetics through the intestinal wall, and that the suppression of the absorption by sodium glycocholate was caused by the loss of thermodynamic activity of bropirimine due to the entrapping of bropirimine in the micelle, not by the direct effect on the permeability of the intestinal mucosa by bile salt. Sodium cholate and its conjugates have been shown not to affect the mucosal membrane permeability (Muranushi et al 1980; Yamaguchi et al 1986).

When the dissolution rate of bropirimine from suspension is significantly faster than the disappearance rate of bropirimine in solution from the rat intestinal loop, the dispersion medium of the suspension is the saturated solution of bropirimine until the solid bropirimine in suspension is dissolved completely. The disappearance from the rat

Table 3. Parameters for disappearance of bropirimine from the upper part of rat small intestine after administration in suspension with or without sodium glycocholate.

Bropirimine ($\mu g\,mL^{-1}$)	Sodium glycocholate (mM)	k_{dis} (min^{-1})	$t_{\frac{1}{2}}$ (min)	k_0 ($\mu g\,min^{-1}$)	$k_{1/3}$ (min^{-1})
200	0	0·0256	27·1	3·0	0·0070
500	0	0·0175	39·6	6·1	0·0051
500	40	0·0108	64·0	3·4	0·0031

Table 4. Parameters for disappearance of bropirimine from the upper part of rat small intestine after administration in solution with sodium glycocholate.

Concn		k_{dis}	$t_{\frac{1}{2}}$	R	f
Bropirimine ($\mu g\,mL^{-1}$)	Sodium glycocholate (mM)	(min^{-1})	(min)		
30	0	0·0419	16·5	1·00	1·00
30	1	0·0474	14·6	1·13	0·90
30	10	0·0336	20·6	0·80	0·68
30	40	0·0150	46·3	0·36	0·35
10	40	0·0161	43·0	0·38	0·35
100	40	0·0161	43·0	0·38	0·35

intestinal loop after administration of suspension would be expected to follow zero-order kinetics with a constant rate independent of the amount of bropirimine in suspension for a particular temperature:

$$Q = k_0 t \quad (4)$$

where Q is the amount of bropirimine which has disappeared from rat intestinal loop at time t and k_0 is the zero-order rate constant. In the case of suspension without sodium glycocholate, as shown in Fig. 5A, the plots of equation 4 with respect to the suspensions of 200 and 500 $\mu g\,mL^{-1}$ yielded apparently straight lines with good correlation (r = 0·999). However, the slopes of the two straight lines were not constant, i.e. the slope of the suspension of 500 $\mu g\,mL^{-1}$ was approximately twice that of the suspension containing 200 $\mu g\,mL^{-1}$ (Table 3). Thus, the assumption that the dissolution rate of bropirimine from suspension was significantly faster than the disappearance rate after administration of solution was not accepted. When the disappearance rate of bropirimine in the solution form from rat intestinal loop is significantly faster than the dissolution rate from suspension, the disappearance after administration of suspension is characterized by the dissolution from suspension. The release of drugs from suspension is described by the cube root law (equation 5) (Hixson & Crowell 1931):

$$W_0^{1/3} - W^{1/3} = kt \quad (5)$$

where W_0 is the amount of solid drug at time 0, W is the amount of solid drug at time t and k is the cube root dissolution rate constant. When it is assumed that the drug powder consists of spherical particles, k is expressed by equation 6:

$$k = [N\rho(\pi/6)]^{1/3}(2DC/\delta\rho) \quad (6)$$

where N is the number of solid particles of the drug, ρ is the density of the drug, D is the diffusion coefficient of the drug, C is the solubility of the drug and δ is the thickness of the diffusion layer. Equation 5 is divided through by $W_0^{1/3}$ to give equation 7:

$$1 - (W/W_0)^{1/3} = k_{1/3}t \quad (7)$$

where $k_{1/3}$ is $k/W_0^{1/3}$. The prerequisite in the cube root law is that a drug powder consists of uniformly-sized particles. However, it has been reported that the release of benzoic acid from crystals with a narrow particle size distribution also fitted the cube root law equation (Niebergall & Goyan 1963). Therefore, the disappearance rate after dosing in suspension was analysed according to the cube root law although the powder of bropirimine did not consist of uniformly-sized particles. Regarding the suspensions of 200 and 500 $\mu g\,mL^{-1}$ in saline, the plots of $(W/W_0)^{1/3}$ vs t fitted equation 7 (Fig. 5B). The $k_{1/3}$ of the suspension of 500 $\mu g\,mL^{-1}$ was smaller than that of the suspension of 200 $\mu g\,mL^{-1}$ (Table 3). The reason seemed to be that the degree of agglomeration of the drug particles in the suspension was higher with the increase in loading amount of bropirimine due to poor solubility of bropirimine. Both suspensions yielded y-axis intercepts smaller than 1. This can be explained by a part of bropirimine dissolved in suspension before administration of the suspension to the loop. With these analyses, it appears that the rate determining step for the overall disappearance of bropirimine from the rat intestinal loop after administration of suspension was the dissolution from suspension.

The disappearance of bropirimine after administration of the suspension of 500 $\mu g\,mL^{-1}$ with sodium glycocholate also fitted the cube root law equation (Fig. 5B). The $k_{1/3}$, however, was smaller than that of the suspension without sodium glycocholate (Table 3). If sodium glycocholate does not affect the values of D and δ of equation 6, the $k_{1/3}$ of the suspension with sodium glycocholate will be greater than that of the suspension without sodium glycocholate, because the C value of the former was approximately 2·8 times that of the latter. The R value related to D_{app}/D_f decreased depending upon the concentration of sodium glycocholate in the solution (Table 4) by entrapping bropirimine in the

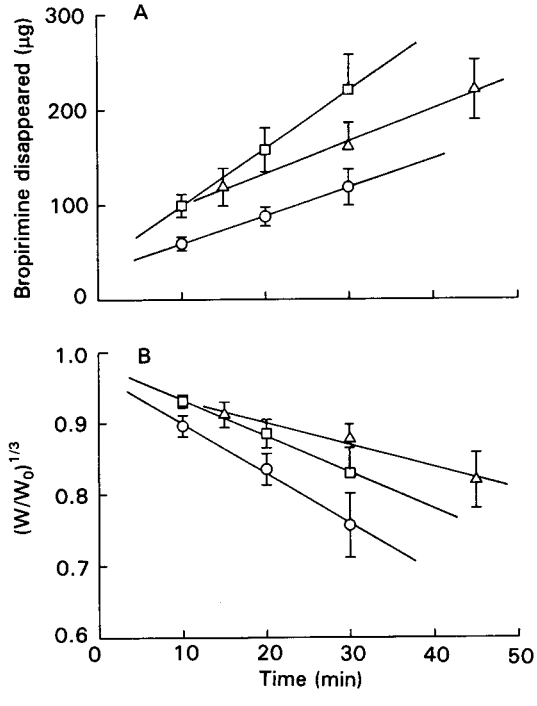

FIG. 5. Bropirimine disappearance from rat small intestinal loop (A) or cube root of the ratio of bropirimine remaining in rat small intestinal loop (B) after administration of bropirimine suspension. ○ 200 $\mu g\,mL^{-1}$ bropirimine suspension with 0 mM sodium glycocholate, □ 500 $\mu g\,mL^{-1}$ with 0 mM sodium glycocholate, △ 500 $\mu g\,mL^{-1}$ with 40 mM sodium glycocholate. Each point represents the mean ± s.d. of at least three rats.

micelle, which decreased the value of D_{app}. Thus, in the case of the suspension, the decrease in the $k_{1/3}$ value was due to the decrease in D_{app}, even when the solubility increased, i.e. the suppression of disappearance seems to be caused by the greater effect of sodium glycocholate in decreasing thermodynamic activity of bropirimine.

As bropirimine in solution is well-absorbed in the small intestine and dissolution was the rate determining step for the absorption of bropirimine in suspension, the development of oral dosage forms with fast dissolution rates of bropirimine will result in the achievement of improved bioavailability of bropirimine. The bioavailability of bropirimine after oral administration of the dosage forms may be reduced by food intake, since the absorption of bropirimine was suppressed by sodium glycocholate contained in the solution and suspension. However, bile secretion and other factors, such as gastric emptying rate, gastrointestinal motility, splanchnic blood flow, and acid secretion will also play a part in the effect of food on bioavailability (Welling 1977; Melander 1978; Toothaker & Welling 1980) and further investigations will be necessary for bropirimine to determine the dosing regimens.

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