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Effect of Simultaneous Administration of Drugs on Absorption and Excretion. X.[1] Plasma Protein Binding of Carbutamide in Rabbits

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The binding of carbutamide to rabbit plasma protein *in vivo* and *in vitro* was studied by using a new ultrafiltration technique. The binding of carbutamide to rabbit plasma protein *in vivo* markedly decreased with increasing plasma concentration of carbutamide. On the other hand, the binding of carbutamide to rabbit plasma protein *in vitro* hardly changed. Over one half of carbutamide in rabbit blood was observed to be as N^4-acetylcarbutamide. N^4-Acetylcarbutamide was highly bound to rabbit plasma protein, and caused a marked decrease in the binding of carbutamide to rabbit plasma protein *in vitro*. These results indicate that the difference of binding of carbutamide to rabbit plasma protein *in vivo* and *in vitro* is due to the displacement of carbutamide from plasma protein binding sites by N^4-acetylcarbutamide.

Keywords——carbutamide; plasma protein binding; ultrafiltration method; N^4-acetylcarbutamide; displacement; carbutamide—its metabolite interaction

Since only unbound fraction of drugs is permeable across biological membrane, the binding of drugs to plasma protein is an important factor affecting their pharmacokinetic behavior such as distribution and elimination.

Previous many works have demonstrated that sulfonylureas are highly bound to plasma protein.[3] However, despite the relative abundance of *in vitro* evidence for the binding of sulfonylureas to purified plasma albumin, there is no *in vivo* evidence for plasma protein binding of sulfonylureas in living body. In the present paper, plasma protein binding of carbutamide in rabbits was studied by using a new ultrafiltration technique.

Experimental

Materials——Carbutamide was kindly supplied by Ono Pharmaceutical Industry Co., Ltd., Osaka, Japan. N^4-Acetylcarbutamide was synthesized from sulfanilamide according to the following method. Other drugs were obtained from commercial sources.

Animals——Male rabbits weighing 2.5—3.5 kg were fasted about 24 hr prior to all experiments, but water was freely available.

Synthesis of N^4-Acetylcarbutamide——N^4-Acetylsulfanilamide:[4] N^4-Acetylsulfanilamide was synthesized by acetylation of sulfanilamide. mp 214—216°. N^4-Acetylcarbutamide:[5,6] To a cooled mixture solution of 10 ml of 1 N NaOH and 10 ml of acetone was added 0.01 mol of N^4-acetylsulfanilamide. To this solution was added dropwise with stirring 0.01 mol of *n*-butylisocyanate dissolved in 4 ml of acetone. The solution was controlled so that the temperatures of the reaction mixture did not exceed 10°. Stirring

1) Part IX: Y. Imamura, H. Nakashima, and H. Ichibagase, *Yakugaku Zasshi*, **98**, 1663 (1978).
2) Location: *5-1, Oe-honmachi, Kumamoto 862, Japan.*
3) *a*) H. Wishinsky, E.J. Glasser, and S. Perkal, *Diabetes*, **11**, supp., 18 (1962); *b*) J. Judis, *J. Pharm. Sci.*, **61**, 89 (1972); *c*) Y.W. Chien, H.J. Lambert, and T.K. Lin, *ibid.*, **64**, 961 (1975); *d*) M.J. Crooks and K.F. Brown, *J. Pharm. Pharmacol.*, **26**, 304 (1974); *e*) S. Goto, H. Yoshitomi, and M. Kishi, *Yakugaku Zasshi*, **97**, 1219 (1977).
4) T. Uno and M. Ueda, *Yakugaku Zasshi*, **80**, 1785 (1960).
5) B. Blank, F.A. Farina, J.F. Kerrin, and H. Saunders, *J. Org. Chem.*, **26**, 1551 (1961).
6) S. Goto, H. Yoshitomi, and M. Nakase, *Chem. Pharm. Bull.* (Tokyo), **26**, 472 (1978).

was continued for 2 hr with cooling and 30 min at room temperatures. The acetone was removed *in vacuo* at 20° and the aqueous residue was acidified with 2 N HCl. The cooled acid solution was filtered and the filter cake was washed with distilled water, dried and recrystallized from ethanol. mp 176—180°. *Anal.* Calcd. for $C_{13}H_{19}N_3O_4S$: C, 49.83; H, 6.11; N, 13.41. Found: C, 50.08; H, 6.18; N, 13.75.

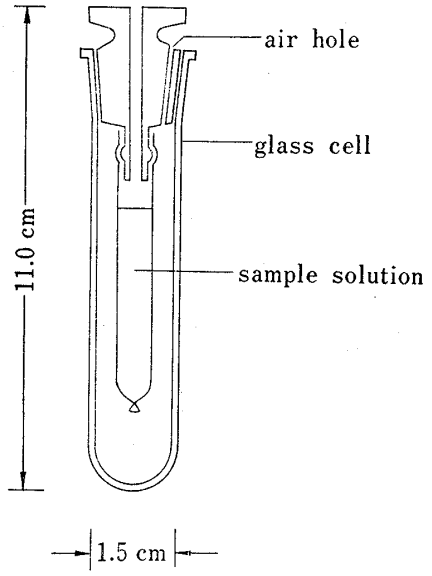

Fig. 1. Apparatus for Ultrafiltration

Ultrafiltration Method——Apparatus: Apparatus for ultrafiltration was shown in Fig. 1. Procedures: A Visking tubing (size 8/32) was knotted at one end with silk thread to form a bag and attached with a rubber band. The bag was dried at room temperatures for 90 min. Rabbit plasma (1.2 ml) was placed in the bag and centrifuged for 40 min at 2800 rpm. The centrifugation was carried out at low room temperatures (4—5°). A 0.1 ml of the ultrafiltrate was used for the determination of carbutamide concentration. The percentage of binding of carbutamide to rabbit plasma protein was calculated from the difference between carbutamide concentrations in rabbit plasma and its ultrafiltrate.

Electrophoresis Method——Sheet of cellulose acetate paper, 1.0×6.0 cm in size, and 0.06 M barbital buffer at pH 8.6 was used. Electrophoresis was carried out at 0.6 mA/cm for 50 min. Total protein concentrations in rabbit plasma and its ultrafiltrate were determined by the method of Lowry *et al.*[7] with bovine serum albumin as the standard.

Analytical Method——Bratton and Marshall method[8] was employed for measuring carbutamide, N^4-acetylcarbutamide and total carbutamide concentration. All samples were deproteinized with 10% trichloroacetic acid. Total and unchanged carbutamide concentration in rabbit blood after a single oral dose of carbutamide were determined after and before hydrolysis of the sample for 1 hr in a boiling water bath, respectively. N^4-Acetylcarbutamide concentration in ultrafiltrate of rabbit plasma after addition of N^4-acetylcarbutamide was determined after hydrolysis of the sample for 1 hr in a boiling water bath.

Results and Discussion

Some Factors Affecting Ultrafiltration

Adsorption of drugs to a cellulose membrane is an important determinant affecting ultrafiltration. If a drug is adsorbed to the cellulose membrane, the drug concentration in ultrafiltrate will be decreased as compared with the initial concentration. As shown in Table I, carbutamide concentration in ultrafiltrate was found to be lower than that in protein-free carbutamide solution. However, the ratio of carbutamide concentration in ultrafiltrate to carbutamide concentration in protein-free carbutamide solution did not depend on the change in the initial carbutamide concentration. These findings suggest that ultrafiltrate may be diluted with water remaining in the cellulose membrane,[9] and indicate that carbut-

Table I. Adsorption of Carbutamide to a Cellulose Membrane

Carbutamide concn. (μg/ml)		C_u/C_s
Carbutamide solution (C_s)	Ultrafiltrate (C_u)	
20	17.5	0.88
50	44.8	0.89
100	88.7	0.89
150	130.7	0.87
200	176.9	0.89

7) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
8) A.C. Bratton and E.K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

amide is apparently adsorbed to the cellulose membrane. Therefore, carbutamide concentration in ultrafiltrate was corrected on the basis of the ratio calculated from each experiment.

Leakage of protein into ultrafiltrate was studied by a electrophoresis method. Figure 2 shows a typical pattern of cellulose acetate paper electrophoresis in rabbit plasma and its ultrafiltrate. A little albumin was observed to be leaked into the ultrafiltrate. However, as can be seen from Table II, the total protein concentration in ultrafiltrate was about 1.0% of the total protein concentration in rabbit plasma. Thus the binding of carbutamide to rabbit plasma protein was determined by neglecting leakage of protein into ultrafiltrate.

Kurz *et al.*[10] have revealed that drug concentration in ultrafiltrate increases with increasing volume of ultrafiltrate, and pointed out that the relative volume of ultrafiltrate should not exceed 20% of total volume of plasma in order to keep low the errors. In our experi-

Table II. Leakage of Protein into Ultrafiltrate

Rabbit No.	Total protein concn. (g/dl)		Leaked (%)
	Rabbit plasma	Ultrafiltrate	
1	6.78	0.078	1.15
2	7.30	0.075	1.03
3	7.72	0.067	0.87
4	7.70	0.068	0.88
Mean	7.38	0.072	0.98
S.E.	0.24	0.003	0.07

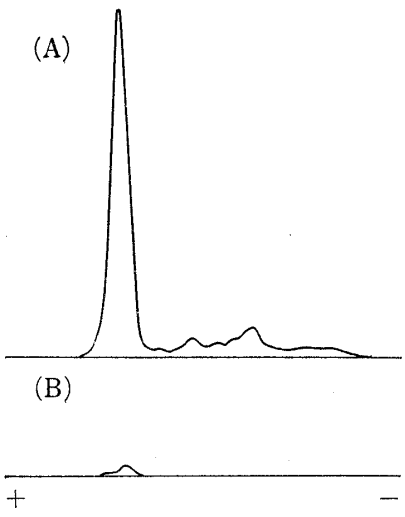

Fig. 2. Cellulose Acetate Paper Electrophoresis of Plasma and Its Ultrafiltrate

(A) rabbit plasma: 0.6 μl,
(B) ultrafiltrate: 1.2 μl.

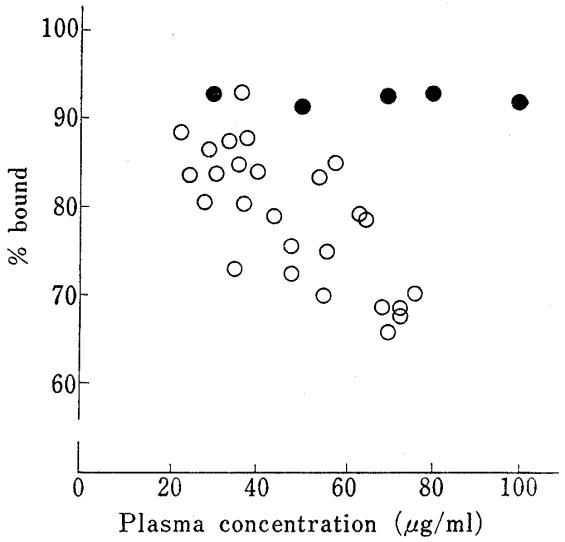

Fig. 3. Relationship between the Binding of Carbutamide to Rabbit Plasma Protein *in Vivo* or *in Vitro* and the Plasma Concentration of Carbutamide

The binding of carbutamide to rabbit plasma protein *in vivo* was determined in rabbit plasma after a single oral dose of carbutamide (200 mg/kg). Sampling of the plasma was made at 1 hr after dosing. The binding of carbutamide to rabbit plasma protein *in vitro* was determined in rabbit plasma after addition of carbutamide.
Key ○: *in vivo*, ●; *in vitro*.

9) E. Owada, R. Hori, and T. Arita, *Yakuzaigaku*, **33**, 125 (1973).
10) H. Kurz, H. Trunk, and B. Weitz, *Arzneim.-Forsch.*, **27**, 1373 (1977).

ments, the relative volume of ultrafiltrate was found to be about 15% of total volume of rabbit plasma. This finding seems to approximately satisfy the above experimental condition.

The Binding of Carbutamide to Rabbit Plasma Protein *in Vivo* and *in Vitro*

Figure 3 shows the relationship between the binding of carbutamide to rabbit plasma protein *in vivo* or *in vitro* and the plasma concentration of carbutamide. The binding of carbutamide to rabbit plasma protein *in vivo* markedly decreased with an increase in the plasma concentration of carbutamide. In addition, a significant negative correlation was observed between the binding of carbutamide to rabbit plasma protein *in vivo* and the the plasma concentration of carbutamide ($r=-0.733$, $p<0.001$). On the other hand, the binding of carbutamide to rabbit plasma protein *in vitro* hardly changed in the range of its drug concentration from 30 to 100 μg/ml.

It has been known that N^4-acetylcarbutamide is a major metabolite of carbutamide in rabbits.[11,12] Consequently, the effect of N^4-acetylcarbutamide on the binding of carbutamide to rabbit plasma protein *in vitro* was studied in order to elucidate the difference of binding of carbutamide to rabbit plasma protein *in vivo* and *in vitro*. As shown in Fig. 4, the binding of carbutamide to rabbit plasma protein *in vitro* markedly decreased by the addition of N^4-acetylcarbutamide. Furthermore, the binding of N^4-acetylcarbutamide to rabbit plasma protein *in vitro* was larger than that of carbutamide to rabbit plasma protein *in vitro* as shown in Fig. 5. These findings indicate that N^4-acetylcarbutamide strongly displaces carbutamide from plasma protein binding sites.

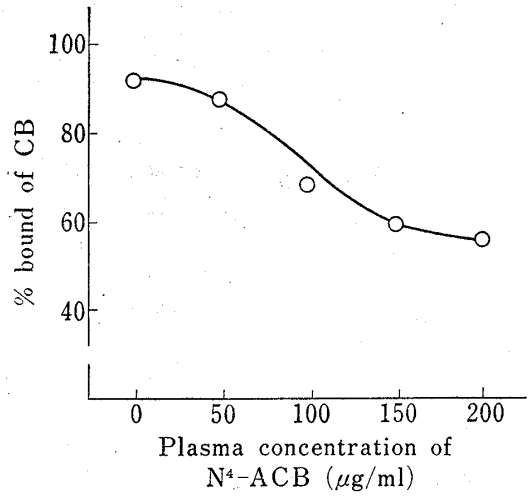

Fig. 4. Effect of N^4-Acetylcarbutamide (N^4-ACB) on Binding of Carbutamide (CB) to Rabbit Plasma Protein *in Vitro*

Key: plasma concentration of carbutamide; 50 μg/ml.

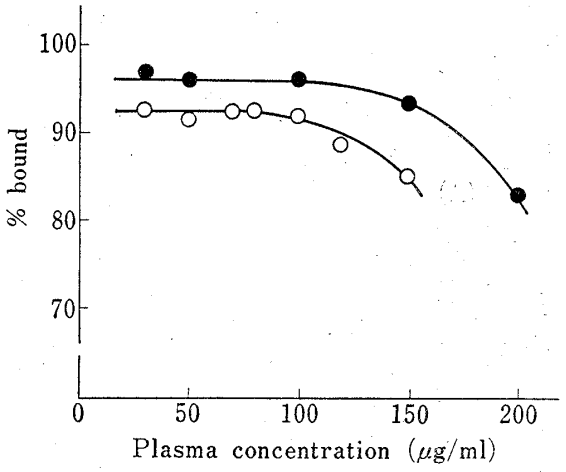

Fig. 5. Binding of Carbutamide and N^4-Acetylcarbutamide to Rabbit Plasma Protein *in Vitro*

Key: ○; carbutamide, ●; N^4-acetylcarbutamide.

Figure 6 shows the time course of carbutamide and N^4-acetylcarbutamide concentration in rabbit blood after a single oral dose of carbutamide. The blood concentration of N^4-acetylcarbutamide was calculated from the difference between total and unchanged carbutamide concentration in rabbit blood. It is evident from the result shown in Fig. 6 that from 50 to 70% of carbutamide in rabbit blood is in the acetylated form. Root[11] has also reported that from 20 to 60% of carbutamide in rabbit blood after a single oral dose of carbutamide is in the acetylated form. These data imply that the displacement of carbutamide from plasma protein binding sites by N^4-acetylcarbutamide can occur in rabbits.

11) M.A. Root, *J. Pharm. Exp. Ther.*, **119**, 468 (1957).
12) E.W. Maynert, *Ann. Rev. Pharmacol.*, **1**, 45 (1961).

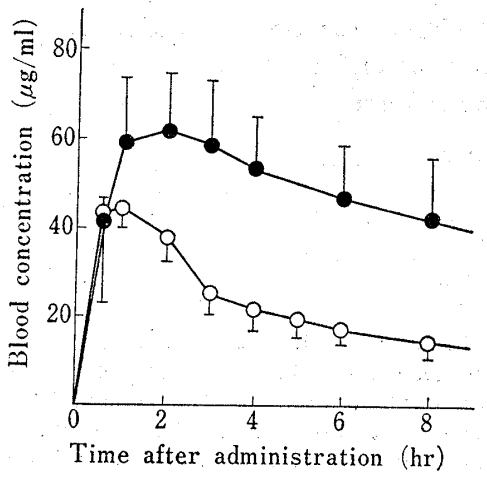

Fig. 6. Time Course of Carbutamide and N^4-Acetylcarbutamide Concentration in Rabbit Blood after a Single Oral Dose of Carbutamide

Values represent the mean ± S.E. of 4 rabbits.
Key: dose of carbutamide; 200 mg/kg,
○; carbutamide, ●; N^4-acetylcarbutamide.

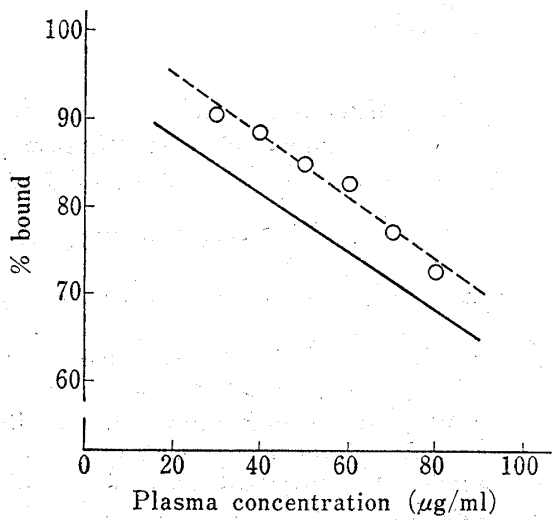

Fig. 7. Relationship between the Binding of Carbutamide to Rabbit Plasma Protein *in Vitro* and the Plasma Concentration of Carbutamide in the Presence of N^4-Acetylcarbutamide

Carbutamide and N^4-acetylcarbutamide in the 1:1 molar ratio were added to rabbit plasma at various concentrations.
Key: ——: regression line obtained in *in vivo* experiment, $Y=94.57-0.33X$,
----: regression line obtained in this experiment, $Y=102.42-0.36X$.

In order to clarify further the effect of N^4-acetylcarbutamide on the binding of carbutamide to rabbit plasma protein *in vitro*, carbutamide and N^4-acetylcarbutamide in the 1:1 molar ratio were added to rabbit plasma at various concentrations, and then the binding of carbutamide to rabbit plasma protein *in virto* was determined. As shown in Fig. 7, the plots for the percentage of binding of carbutamide to rabbit plasma protein *in vitro versus* the plasma concentration of carbutamide was found to be close to the regression line obtained in *in vivo* experiment.

From all results described above, it is concluded that the difference of binding of carbutamide to rabbit plasma protein *in vivo* and *in vitro* is due to the displacement of carbutamide from plasma protein binding sites by N^4-acetylcarbutamide.

The binding of drugs to plasma protein is well known, but most information has been derived from *in vitro* studies. Accordingly, an accumulation of knowledge concerning the binding of drugs to plasma protein *in vivo* is necessary in order to understand more detailed pharmacokinetic behavior of drugs. In this paper, we have provided evidence that the binding of carbutamide to rabbit plasma protein *in vivo* is markedly decreased by its major metabolite, N^4-acetylcarbutamide. Recently, Conard *et al.*[13] have reported that 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, which is a major metabolite of diphenylhydantoin,[14] is highly bound to human and rat plasma protein *in vitro*. This fact suggests that 5-(*p*-hydroxyphenyl)-5-phenylhydantoin may play a significant role in the binding of diphenylhydantoin to plasma protein *in vivo*.

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13) G.J. Conard, C.O. Haavik, and K.F. Finger, *J. Pharm. Sci.*, **60**, 1643 (1971).
14) T.C. Butler, *J. Pharm. Exp. Ther.*, **119**, 1 (1957).