EFFECT OF FUROSEMIDE ON PLASMA CLEARANCE, ANTICOAGULANT EFFECT AND PROTEIN BINDING OF WARFARIN IN RATS

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(Received May 21, 1982)

The effect of furosemide on the elimination, anticoagulant effect and the in vitro and in vivo protein bindings of warfarin was examined in rats. The pharmacokinetic parameters of warfarin and prothrombin complex activity (PCA) after a single i.v. coadministration with warfarin (1.2 mg/kg) and furosemide (1.67 mg/kg) were not significantly different as compared with those in the group injected warfarin alone; however when coadministered with 5 mg/kg of furosemide, the elimination rate constant was significantly increased and PCA was markedly enhanced beyond 60 h after administration. The concurrent treatment with warfarin and a higher dose (10 mg/kg) of furosemide caused an increase in the anticoagulant effect of warfarin even at earlier periods after administration. Both the unbound warfarin concentration in serum at 30 min and the amount of warfarin extracted into liver at 2 h after a single i.v. dosing in the coadministered group were significantly increased as compared with those in the group received warfarin alone. Results from in vitro binding studies using bovine serum albumin and rat plasma showed a typical competitive nature of protein binding of warfarin and furosemide at the same binding sites. These results suggest that the interactions, such as the displacement of warfarin binding at albumin binding sites, between warfarin and furosemide are produced, when a high dose of furosemide was coadministered.

Keywords—warfarin; furosemide; elimination; anticoagulant effect; hepatic uptake; protein binding; displacement of warfarin from protein binding sites

INTRODUCTION

It is a well-known fact that coumarin anticoagulant drugs such as warfarin are strongly bound to plasma protein. The plasma protein binding of warfarin had been studied by many investigators. They found that above 98% of the drug was bound in undiluted serum obtained from human and animals. It has been established that highly protein-bound drugs, such as phenylbutazone, indomethacin, aspirin and ethacrynic acid, when administered concomitantly with warfarin, cause the displacement of warfarin from plasma protein binding sites, and consequently potentiate its anticoagulant effect. On the other hand, some drugs, antihistaminics and diuretics, are able to reduce the anticoagulant effect of warfarin. The drug displacement increases the amount of active anticoagulant and the availability of more free or unbound drug, which, in turn, leads to a change in the distribution and elimination kinetics of the affected drug.

Anticoagulants are commonly used in combination with other drugs such as diuretics and tranquilizers in order to improve a variety of symptoms accompanied with thromboembolic disorders. Furosemide, a potent diuretic, also corresponds to this case and has been often coadministered with warfarin. However, Sebille et al. and Sudlow et al. reported the competition of warfarin and furosemide for the same binding sites in human serum albumin in vitro. Clarifying a possible interaction between the two drugs in vivo is of great interest from a clinical point of view and in leading to safer coadministration of these two drugs. In this study, in order to estimate whether a competition of protein binding between warfarin and furosemide occurs in vivo, the effect of furosemide on the pharmacokinetics,
anticoagulant effect, hepatic uptake and \textit{in vivo}
and \textit{in vitro} plasma protein bindings of warfarin
were investigated in detail.

EXPERIMENTAL

Materials — 1) Experimental Animals: Male
Wistar rats weighing 270—350 g were used
throughout the study. The animals had free access
to MF diet (Oriental Yeast Co., Ltd.) and water
before and during the experiment. 2) Drug:
Potassium warfarin (warfarin, J. P. grade) and
furosemide (J. P. grade) were obtained from Eizai
Co., Ltd. and Yodogawa Pharm. Co., Ltd., respecti-
cively. Coumarterril, an internal standard for gas
liquid chromatography (GLC), was a gift from
Nihon Tokushu Noyaku Sei Co., Ltd. Pent-
fluorobenzyl bromide, thromboplastin (Bacto
Thromboplastin), and bovine serum albumin
(BSA, fraction V) were purchased from Tokyo
Chemical Ind. Co., Ltd., Difco Laboratories
(Detroit, Michigan, USA), and Sigma Chemical
Co., Ltd. (St. Louis, Missouri, USA), respecti-
vely. All other chemicals used were of special grade.

Treatment of Animals — Warfarin and
furosemide were administered intravenously as a
solution (0.1 ml/100 g) in saline, but the latter was
dissolved in a small amount of dimethylfor-
mamide and diluted with saline. In vivo
administration, warfarin and furosemide were
administered as a solution in saline and an
aqueous suspension in 2% acacia in volumes of
0.4 ml/100 g respectively to animals. The animals
were divided at random into 2 groups, each con-
sisting of 3—6 rats.

1) Rapid Single Intravenous Administration:
a) Animals were treated with a single intravenous
(i.v.) administration of warfarin (1.2 mg/kg, in a
some cases 0.6, 2.4 mg/kg) alone or b) warfarin
and furosemide (1.67, 5 or 10 mg/kg)

2) Repeated Oral Administration: a) Animals
were treated with every other day oral
administration of warfarin from day 1 to day 5
(0.6 mg/kg on day 1, thereafter 0.3 mg/kg) and
for 5 d with daily oral administration of
furosemide (2 mg/kg), (warfarin-furosemide
group), b) animals were treated orally with war-
farin alone as described in a) without furosemide
(warfarin group). After the final treatment, 0.5 ml
blood was collected at arbitrary intervals from tail
vein in a sodium citrate-treated syringe. The
plasma was separated immediately by centrifuga-
tion at 1400 \( \times g \) for 10 min.

Determination of Warfarin Concentration in
Serum — 1) Determination by GLC: Warfarin
concentrations in plasma samples were deter-
mined according to the assay procedure described
by Kaiser et al.\(^{10}\) with slight modifications (0.5 or
0.2 \( \mu g \) coumarterril as an internal standard and
0.1 ml plasma were used), by GLC method using
Hitachi 163 gas chromatography with an electron
capture detector. The U-shaped pyrex column (2
m \( \times \) 3 mm I.D.) was packed with 1% silicon OV-
17 on 100—200 mesh Chromosorb W-AW.
Operating conditions were: column temperature,
240°C; detector temperature, 275°C; nitrogen
carrier gas flow-rate, 75 ml/min.

2) Determination by Fluorescence Method:
Samples containing warfarin were determined by
the method of Laliberte et al.\(^{11}\) with slight
modifications, using ethylenechloride as an
extracting solution and 10% HCl in stead of
trichloroacetic acid. Warfarin was estimated from
its fluorescence with excitation at 320 nm and
emission at 385 nm by using a Hitachi 650—10
spectrofluorometer.

Determination of Free Warfarin Concentration in
Serum — Serum samples obtained at 30 min
after a single \( i.v. \) dose were subjected to
ultrafiltration by an immersible molecular separa-
tion unit (Immersible CX-10, nmwl 10000
Daltons, Millipore Co., Bedford Mass., USA).
The filter unit was immersed in 2 ml of serum at room
temperatures and aspirated. Warfarin concentra-
tion in the filtrate was determined by the GLC
method.

Measurement of Prothrombin Complex Activity
— Prothrombin complex activity (PCA) of
plasma samples were measured by the one-stage
prothrombin time method of Quick.\(^{12}\)

Determination of Free Fatty Acids in Plasma
— Free fatty acids (FFA) in plasma samples were
determined by the method of Novák.\(^{13}\)
Determination of Warfarin Concentration in Liver — Animals were fasted for 12 h prior to the experiment. Liver homogenate was prepared in 0.25 M sucrose-50 mM phosphate buffer, pH 7.4, at 2 h after a single i.v. dose. Warfarin concentration in the homogenate was determined according to the modified method of Kaiser et al.\textsuperscript{10} described in the GLC method.

Protein Determination — Protein concentration was determined by the method described by Lowry et al.\textsuperscript{14} with bovine albumin, fraction V, as a standard.

Urine Volume and Urinary Sodium and Potassium Excretions — Animals of both groups, each consisting of 4 rats were kept in metabolic cages separately and urine was collected every 6 or 12 h up to 72 h after a single i.v. dose of the drugs. Urinary sodium and potassium were measured by flame photometry, using a Hitachi 300 UHF plasma spectra scan.

In Vitro Binding of Warfarin to BSA and Rat Plasma — Equilibrium dialysis was used to assess protein binding of warfarin to BSA and rat plasma. The apparatus for equilibrium dialysis described by Goro et al.\textsuperscript{15} was used; the two compartments containing 3.0 ml each of drug solution (warfarin: 2—100×10^{-5} M, furosemide: 1.5—5×10^{-4} M) or diluted BSA or undiluted plasma were separated by washed dialysis cellulose membrane (cellulose tubing, C-65, Sanko Pure Chemical Co.). The drugs and BSA were dissolved in 0.067 M phosphate buffer, pH 7.4. The dialysis apparatus was shaken (100 oscillation/min in 5 cm amplitude) on a Toyo incubater (TC-1) for 24 h at 30°C, and each compartment was sampled for warfarin determination. Previous studies have shown that the equilibrium is reached in approximately 20 h under these conditions. Warfarin concentration was determined by the fluorescence method.

The Scatchard equation\textsuperscript{16} was applied to determine the association constant of the drug. The binding parameters were calculated by least-squares regression analysis by the following equations:

\[
\frac{r}{C_i} = \left( K_1 \cdot n_1 - K_2 \cdot r \right) + \left( K_2 \cdot n_2 - K_2 \cdot r \right)
\]

\[r = r_1 + r_2\]

where \(K_1\) and \(K_2\) are the association constants corresponding to \(n_1\) and \(n_2\), the number of primary and secondary binding sites, respectively; \(C_i\) is the free drug concentration, and \(r\) is the molar ratio of the bound drug to the binding protein assuming a molecular weight of 69000.

In Vitro Binding of Warfarin to Soluble Fraction of Liver — Liver homogenate was prepared in 0.067 M phosphate buffer, pH 7.4, and centrifuged at 78000 × g for 60 min. The supernatant obtained was dialyzed for 4 d at 4°C against warfarin (1 and 3×10^{-4} M) and furosemide (1.5 and 3×10^{-4} M) or with no added furosemide in 0.067 M phosphate buffer, pH 7.4, using a visking tube (18/32, Sanko Pure Chemical Co.). The concentration of warfarin inside and outside the bag were determined by the fluorescence method described by Laliberte et al.\textsuperscript{11}

Pharmacokinetic Analysis — The calculation and statistical analysis were carried out with the aid of a pocket computer, Sharp PC-1211. The area (AUC) under the plasma drug concentration-time curve after administration was determined by the trapezoidal rule up to the last sampling point. The AUC beyond the last observed plasma concentration (\(C_n\)) was extrapolated according to \(C_n/K_{el}\), where \(K_{el}\) is the elimination rate constant. The data following a single i.v. dose of warfarin were analyzed by using a one-compartment open model. The half-life is calculated as \(t_{1/2} = 0.693/K_{el}\). The pharmacokinetic parameters were calculated by least-squares regression analysis. The apparent volume of distribution (\(V_d\)) was estimated according to the following equation:

\[V_d = \text{Dose}/C_0\]

Total clearance (\(Cl\)) was calculated by the following equation:

\[Cl = V_d \times K_{el}\]
Statistical Methods — The data were compared by an analysis of variance. When the analysis indicated that a significant difference existed, statistical significance between two means was determined by the Student’s t-test with $p < 0.05$ as the criterion of significance.

RESULT

Plasma Concentrations of Warfarin after a Single i.v. Administration of Warfarin and Furosemide

As shown in Fig. 1, the plasma decay curve of warfarin could be described as a single first-order function following a rapid i.v. injection of the drug (1.2 mg/kg). Thus, it seems that the disposition of warfarin fits to the one-compartment open model. In the case of coadministration with furosemide (1.67 or 5 mg/kg, i.v.), the plasma concentration of warfarin also declined according to a single first-order function. Some pharmacokinetic parameters calculated are summarized in Table I. With furosemide (1.67 mg/kg), the elimination rate constant of warfarin was slightly increased (0.021±0.003 to 0.027±0.007 h⁻¹), but not significant, as compared with that in the group treated with warfarin alone, and no statistically significant differences between both groups were observed in other pharmacokinetic parameters. When the dose of furosemide was increased to 5 mg/kg, the elimination rate constant of warfarin was significantly increased ($p < 0.001$) and its half-life was significantly decreased ($p < 0.01$) as compared

![Graph](image)

**FIG. 1.** Semilogarithmic Plots of Plasma Warfarin Concentration after a Single i.v. Administration of Warfarin alone or in Combination with Furosemide

Dose: warfarin 1.2 mg/kg, furosemide 1.67 or 5 mg/kg body weight, i.v. ○; warfarin alone, □; warfarin-furosemide (1.67 mg/kg), ▲; warfarin-furosemide (5 mg/kg). Each point represents the mean ± S.D. of 3–5 rats. a) $p < 0.001$ and b) $p < 0.01$ respectively compared with warfarin alone.

TABLE I. Pharmacokinetic Parameters of Warfarin after a Rapid i.v. Injection of Warfarin alone or in Combination with Furosemide

<table>
<thead>
<tr>
<th></th>
<th>$K_e$ (h⁻¹)</th>
<th>$t_{1/2}$ (h)</th>
<th>$V_d$ (l/kg)</th>
<th>$Cl$ (ml/kg/h)</th>
<th>AUC (μg·h·ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin alone</td>
<td>0.021±0.003</td>
<td>34.8±6.5</td>
<td>0.17±0.03</td>
<td>3.29±0.55</td>
<td>381.9±72.4</td>
</tr>
<tr>
<td>Warfarin -furosemide 1.67 mg/kg</td>
<td>0.027±0.007</td>
<td>27.1±6.6</td>
<td>0.14±0.02</td>
<td>3.86±0.84</td>
<td>343.2±58.6</td>
</tr>
<tr>
<td>Warfarin -furosemide 5 mg/kg</td>
<td>0.036±0.010a)</td>
<td>20.5±6.3b)</td>
<td>0.21±0.02</td>
<td>7.44±2.12a)</td>
<td>175.3±59.8a)</td>
</tr>
</tbody>
</table>

*Each value represents the mean ± S.D. of 3–5 rats. a) $p < 0.001$, b) $p < 0.01$; compared with warfarin alone.
with those in the group dosed warfarin alone, as shown in Table I, indicating that the remarkably enhanced elimination of warfarin was induced by the coadministration of furosemide at a relatively higher dose. Additionally, a 5 mg/kg dose of furosemide coadministered with warfarin increased the total clearance of warfarin by 2.3 times (3.29±0.55 to 7.44±2.12 ml/kg/h), although the $V_d$ was not significantly changed after coadministration of a higher dose of furosemide.

**Plasma Concentrations of Warfarin after Repeated Oral Administration of Warfarin alone or in Combination with Furosemide**

The plasma concentrations of warfarin after repeated oral treatment of the drug with or without furosemide (warfarin was given at the dose of 0.6 mg/kg on day 1 and 0.3 mg/kg on day 3 and 5, furosemide was given at daily dose of 2 mg/kg) are shown in Fig. 2. In both groups receiving warfarin alone and warfarin plus furosemide, the mean maximum plasma concentrations ($C_{\text{max}}$) of warfarin were attained at 9 h after oral administration. However, the $C_{\text{max}}$ level and AUC after coadministration of furosemide were slightly lower than that in rats receiving warfarin alone, but not significant, as shown in Table II.

**PCA following Administration of Warfarin alone or in Combination with Furosemide**

The time course of anticoagulant effect of warfarin following a rapid i.v. injection of warfarin alone (1.2 mg/kg) or in combination with furosemide (5 mg/kg) is shown in Fig. 3A. There was no difference in PCA between both groups up to 36 h after administration; however PCA in the group coadministered with furosemide was markedly recovered beyond 60 h after administration; the PCA at 72 h after administration in the groups treated with the combined drugs and with warfarin alone were 61.3±15.4 and 13.3±12% of the control, respectively ($p<0.01$). This indicates the remarkable decrease

![Graph](image)

**FIG. 2. Semilogarithmic Plots of Plasma Warfarin Concentration after Repeated Oral Administration of Warfarin alone or in Combination with Furosemide**

The animals were treated for 5 d with daily dosing of furosemide (2 mg/kg), and with every other day dosing of warfarin (on day 1; 0.6 mg/kg, and days 3 and 5; 0.3 mg/kg, respectively). Each point represents the mean ± S.D. of 4 rats. ●: warfarin alone, ○: warfarin-furosemide.

<table>
<thead>
<tr>
<th></th>
<th>$K_{el}$ (h$^{-1}$)</th>
<th>$t_{1/2}$ (h)</th>
<th>$K_a$ (h$^{-1}$)</th>
<th>AUC (µg·h/ml)</th>
<th>$C_{max}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin alone</td>
<td>0.019±0.006</td>
<td>38.5±10.6</td>
<td>0.330±0.120</td>
<td>233±84</td>
<td>3.65±0.75</td>
</tr>
<tr>
<td>Warfarin + furosemide</td>
<td>0.022±0.007</td>
<td>34.9±11.5</td>
<td>0.385±0.068</td>
<td>181±64</td>
<td>3.15±0.65</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 4 rats.

**TABLE II. Pharmacokinetic Parameters of Warfarin after Repeated Oral Administration of Warfarin alone or in Combination with Furosemide**
in anticoagulant effect of warfarin beyond 60 h. In a separate experiment, the concurrent administration of 1.2 mg/kg of warfarin and 1.67 mg/kg of furosemide did not cause any significant difference in PCA as compared with the group treated with the same dose of warfarin alone.

In order to estimate the change in PCA at the earlier period after administration, animals were

![Graph](image)

**FIG. 3. Effect of Warfarin Administered either alone or in Combination with Furosemide on Prothrombin Complex Activity of Rats**

Prothrombin complex activity was measured by the method of Quick after administration of the drugs. (A) warfarin 1.2 mg/kg and furosemide 5 mg/kg, i.v., (B) warfarin 0.6 mg/kg and furosemide 10 mg/kg, i.v. ● warfarin alone, ○ warfarin-furosemide. Each point represents the mean ± S.D. of 3–4 rats. a) \( p < 0.001 \), b) \( p < 0.01 \) and c) \( p < 0.02 \) respectively compared with warfarin alone.

**TABLE III. Free Warfarin Concentration in Serum and Hepatic Uptake of Warfarin**

<table>
<thead>
<tr>
<th></th>
<th>Concentration of unbound drug (ng/ml)</th>
<th>Free fraction (%)</th>
<th>Warfarin (ng)</th>
<th>Warfarin (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>liver weight</td>
<td>liver protein</td>
</tr>
<tr>
<td>Warfarin alone</td>
<td>39.14 ± 9.55</td>
<td>0.29 ± 0.09</td>
<td>229 ± 15</td>
<td>1.54 ± 0.10</td>
</tr>
<tr>
<td>Warfarin-furosemide</td>
<td>68.92 ± 11.78(^a)</td>
<td>0.44 ± 0.12</td>
<td>365 ± 98(^a)</td>
<td>2.42 ± 0.57(^b)</td>
</tr>
</tbody>
</table>

Blood specimens and liver samples were collected at 30 min and at 2 h, respectively, after a single i.v. dose of warfarin alone (2.4 mg/kg) or in combination with furosemide (10 mg/kg). Each value represents the mean ± S.D. of 4–7 rats.

\( a\) \( p < 0.02 \), \( b\) \( p < 0.01 \); compared with warfarin alone.
coadministered with a high dose of furosemide (10 mg/kg) and warfarin (0.6 mg/kg), and the PCA was compared with that in the group receiving warfarin alone. This result is shown in Fig. 3B. At 12–24 h after administration, the PCA in the group coadministered with furosemide decreased more than that in the group receiving warfarin alone; the PCA at 24 h after administration in the warfarin-furosemide-treated group was 13.9±4.3% of the control, and the group receiving warfarin alone was 26.1±5.1% (p < 0.02). These results indicate that the concurrent treatment with warfarin and a higher dose of furosemide caused an increase in the anticoagu-

FIG. 4. The Cumulative Urinary Volume and Sodium Excretion after a Single i.v. Dose of Warfarin alone or in Combination with Furosemide
(A) warfarin alone (1.2 mg/kg), (B) warfarin (1.2 mg/kg) and furosemide (1.67 mg/kg). Each value represents the mean ± S.D. of 4 rats. a) p < 0.001, b) p < 0.02 and c) p < 0.05 in (A) vs. (B).
lant effect of warfarin even at earlier periods after administration.

Concentrations of Plasma Free Fatty Acids

The concentrations of FFA in plasma before and 3 and 6 h after a single i.v. administration of warfarin alone (1.2 mg/kg) or in combination with furosemide (5 mg/kg) were determined to clarify the contribution of the endogenous FFA to the elimination of warfarin. The FFA concentration (0.27±0.08 Eq/l) at 3 h after a single i.v. coadministration with warfarin and furosemide was slightly lower than that (0.40±0.16 Eq/l) in the group injected with warfarin alone, but it not significant. There was no difference in the FFA levels of both groups at 6 h after administration (with furosemide, 0.31±0.06 Eq/l, and without furosemide; 0.34±0.20 Eq/l).

Free Warfarin in Serum and Hepatic Uptake of Warfarin

The unbound warfarin concentrations in serum at 30 min and the amount of warfarin extracted by liver at 2 h after a single i.v. dose of warfarin alone (2.4 mg/kg) or in combination with furosemide (10 mg/kg) are shown in Table III. The concentrations of unbound warfarin were significantly increased by the coadministration with furosemide as compared with those in warfarin-treated group (p < 0.02). This result suggests that the combination of a higher dose of furosemide and warfarin causes the displacement of warfarin from protein binding sites. In addition, in the group given both warfarin and furosemide the amount of warfarin extracted by liver was also increased (229±15 to 365±98 ng/g liver weight), suggesting that the coadministration with a higher dose of furosemide may inhibit the formation of prothrombin complex in liver at initial periods after administration.

Effect of Furosemide on Urinary Volume and Sodium and Potassium Excretions

The cumulative urinary volume and sodium and potassium excretions after a single i.v. dose of warfarin (1.2 mg/kg) alone or in combination with furosemide (1.67 mg/kg) are shown in Fig. 4. The diuretic effect of furosemide was markedly exerted up to 6 h after administration, and the

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FIG. 5. Binding of Warfarin as a Function of Furosemide Concentration in 0.067 M Phosphate Buffer Containing Bovine Serum Albumin

The experiment was done using equilibrium dialysis technique at 30°C and pH 7.4. Warfarin concentration; 2×10⁻⁴ M, BSA concentration; 5×10⁻⁵ M.

FIG. 6. Scatchard Plots for the Binding of Warfarin to Bovine Serum Albumin in the Absence (●) and Presence (○, △, ▲) of Furosemide

Furosemide concentration; ○ 1.5×10⁻⁴ M, ▲ 3×10⁻⁴ M, △ 5×10⁻⁴ M, warfarin concentration; 2-100×10⁻⁵ M, BSA concentration; 5×10⁻⁵ M. Each point represents the mean ± S.D. of 3-6 experiments.
Furosemide-Warfarin Interaction

TABLE IV. Binding Parameters for Interaction of Warfarin with Bovine Serum Albumin

<table>
<thead>
<tr>
<th>Furosemide concentration</th>
<th>$K_1^{a) b)}$</th>
<th>$K_2^{a) d)}$</th>
<th>$n_1^{b) d)}$</th>
<th>$n_2^{b) d)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.722±0.308</td>
<td>0.769±0.132</td>
<td>2.37±0.18</td>
<td>2.95±0.78</td>
</tr>
<tr>
<td>$1.5\times10^{-4}$M</td>
<td>3.392±0.520</td>
<td>0.253±0.106</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$3\times10^{-4}$M</td>
<td>1.855±0.381</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$5\times10^{-4}$M</td>
<td>0.598±0.080</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$^{a)}$ Association constant ($\times 10^4$M$^{-1}$), $^{b)}$ the number of binding sites. Each value represents the mean ± S.D. of 3–6 experiments. $^{c)}$ p < 0.01, $^{d)}$ p < 0.001; compared with in the absence of furosemide.

potassium excretion up to 6 h was similar in the rats administered with warfarin alone and in combination with furosemide.

Binding of Warfarin to BSA and Rat Plasma in Vitro

The percent binding of warfarin to BSA (50 $\mu$M) in the presence of furosemide at various concentrations is shown in Fig. 5. When the furosemide concentration was increased from 0 to 2 mM with fixed concentrations of BSA (50 $\mu$M) and of warfarin (200 $\mu$M), the bound fraction of warfarin was reduced from 66 to 18%.

Scatchard plots were constructed to characterize this reduced binding and to estimate the binding parameters for warfarin in the presence of furosemide, as shown in Fig. 6. The binding of warfarin to BSA showed a two-phase characteristic. Some binding parameters calculated from the linear portions of the respective plots are shown in Table IV. The association constants, $K_1$ and $K_2$, for warfarin were 4.72±0.31 and 0.77±0.13 ($\times 10^{4}$) M$^{-1}$ respectively, in the absence of furosemide. Since the $K_1$ value corresponding to primary binding site was higher than the $K_2$ value, warfarin seems to have the affinities predominantly to the binding site I over a range of the drug concentrations used. The association constants ($K_1$ and $K_2$) for warfarin binding and the number of binding sites decreased with increasing furosemide concentrations, although the $K_2$ and $n_2$ were not obtained at higher furosemide concentrations. This suggests a typical displacement of warfarin binding by furosemide.

Scatchard plots of warfarin binding to undiluted rat plasma are shown in Fig. 7. The number of the primary binding site, $n_1$ (in this

FIG. 7. Scatchard Plots for the Binding of Warfarin to Rat Plasma in the Absence (●) and Presence (○) of Furosemide

Warfarin concentration: $2-100\times 10^{-5}$ M, furosemide concentration: $5\times 10^{-4}$ M.

urinary volume and sodium excretion (2.21±0.38 mg/ml) in the group coadministered with furosemide were significantly increased as compared with those (1.33±0.22 mg/ml) in the group received warfarin alone (p < 0.05). However, there was no significant difference in those between both groups beyond 6 h after administration. This suggests that the diuretic effect of furosemide is transient. The total urinary

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case the \( n \) was expressed as mM concentration, was slightly decreased from 0.36 to 0.20 mM by 0.5 mM furosemide added; however the \( K \) value was unchanged.

**Binding of Warfarin to Soluble Fraction of Liver in Vitro**

The displacement of warfarin from liver soluble protein binding sites by furosemide was examined in vitro. The values of \( C_f/C_{tot} \) (\( C_f \) is the concentration of free drug and \( C_{tot} \) is the total concentration of drug) of warfarin (10^{-4} M) in the presence of 1.5 and 3 \( \times 10^{-4} \) M furosemide tended to only slightly increase (0.87 \( \pm \) 0.02 and 0.86 \( \pm \) 0.02, respectively) as compared with that in the absence of furosemide (0.83 \( \pm \) 0.04), although differences were not significant.

**DISCUSSION**

Several investigators have reported the interaction between warfarin and other drugs in laboratories and clinics, and most of the investigations reported were on the displacement of warfarin from protein binding sites by anti-inflammatory drugs, analgesics and sulfonamides. It was also shown that furosemide, which is frequently used with warfarin clinically, could displace warfarin from protein binding sites in vitro. A possible interaction between the coumarins and furosemide is of great importance from a clinical point of view. In order to understand in detail the displacement of warfarin by furosemide and clarify whether the drug interactions between warfarin and furosemide occur in vivo, the plasma concentration, prothrombin complex activity, plasma protein binding and liver uptake of warfarin were measured after coadministration with furosemide. Additionally, in vitro plasma binding to the serum albumin and liver soluble fraction were also studied in the presence of furosemide.

We have found that the \( t_{1/2} \) is 34.8 h and the apparent distribution volume is 0.17 l/kg after a single i.v. injection of warfarin (1.2 mg/kg) to rats (Fig. 1 and Table 1). This result partly agreed with those of Yacobi et al. (1972) (\( t_{1/2} \) 5–28 h, \( V_d \) 102–320 ml/kg) and Pyorala (1973) (\( t_{1/2} \) 5–70 h) in rats. When the animals were treated with a single injection of both warfarin (1.2 mg/kg) and furosemide (1.67 mg/kg), the pharmacokinetic parameters were not significantly changed as compared with those in animals receiving warfarin alone. On the other hand, when the dose of furosemide was increased by 3-fold (5 mg/kg), the pharmacokinetic parameters of warfarin were significantly altered (\( p < 0.01 \)), that is, \( K \) and \( t_{1/2} \) significantly increased, and AUC decreased as compared with those in group treated with warfarin alone (Fig. 1 and Table 1). These data suggest that a higher dose of furosemide affects the disposition of warfarin, consequently the pharmacologic effect. Nilsson et al. (1974) reported that the mean prothrombin time in subjects who were coadministered with furosemide (80 mg/d, a general dosage) and warfarin (50 mg/d) was similar to that in those receiving warfarin alone. Their data are in accord with our results that PCA in the group injected with warfarin alone was similar to that in the rats coadministered with a low dose of furosemide (warfarin; 1.2 mg/kg, furosemide; 1.67 mg/kg), whereas PCA in animals treated with warfarin and a high dose (5 mg/kg) of furosemide returned to normal level relatively earlier than that after warfarin alone (Fig. 3A). These results reflect well the difference in the plasma concentration of warfarin between both groups. Thus, it is considered that anticoagulant activity disappeared relatively rapidly after the coadministration of a high dose of furosemide and warfarin as a result of the interaction, such as displacing effect.

To clarify the alteration of anticoagulant activity at the earlier period post-dosing, a small amount (0.6 mg/kg) of warfarin and the increased amount (10 mg/kg) of furosemide were coadministered to rats. The anticoagulant activities at 12 and 24 h after administration were more potent in animals coadministered with furosemide than those in the group with warfarin alone (Fig. 3B). This observation suggests that the combination of warfarin and a high dose of furosemide induced the potent inhibition of prothrombin synthesis in liver even at earlier
periods after dosing, probably due to the increased liver uptake of free warfarin. This assumption was clearly demonstrated by the fact that the plasma free fraction and the liver uptake of warfarin were increased 1.5 and 1.6 times respectively in the coadministered group as compared with those in the group received warfarin alone (Table III). However, the increased free fraction of warfarin in plasma may induce a rapid elimination of the drug, partially due to the increased renal excretion based on the diuretic effect of furosemide by direct action on the renal tubules.28) This was evidenced by the increase in the $K_{el}$ and $Cl$ (Table I) and the decrease in the anticoagulant effect of the drug at the later period in the group coadministered with furosemide (Fig. 3A).

Warfarin is highly bound to plasma protein (almost exclusively to the albumin fraction),26,27) and plasma binding fraction of warfarin is 98.5–99.8% in rats,2,28) and furosemide is also a highly protein binding drug (96–98%).29,30) In vitro binding experiment by us demonstrated that warfarin had two binding sites, high and low affinity binding site, on bovine serum albumin or rat plasma (Fig. 6 and 7). This result accords with the data described by Sebille and Thuaud.8) Our study revealed a significantly reduced binding of warfarin to BSA in the presence of a high concentration of furosemide. The decreased abscissa intercepts of the Scatchard plots in Fig. 6 suggest a displacement of warfarin binding by furosemide at the high affinity binding sites predominantly (Table IV). The experiment using rat plasma also showed that the $n_1$ value of the high affinity binding site was decreased by furosemide addition. Sebille and Thuaud8) had shown the competition of furosemide and warfarin for the same binding sites of human serum albumin in vitro.

The reason why the displacement of warfarin from plasma binding sites did not occur in lower concentration of furosemide, but did in the high concentration of the drug is considered that in low concentration of both drugs the binding sites on whole albumin molecules are not saturated and both drugs can bind to the sites separately, but in high concentration of furosemide the binding sites are filled up and consequently warfarin is displaced from the sites by furosemide.

Since it was known in the in vitro study using human serum albumin that the high contents of fatty acids inhibit the plasma protein binding of warfarin,31,32) the plasma protein binding of warfarin may be affected by fatty acids in the plasma. We determined the contents of fatty acids in plasma in the group treated with warfarin alone or in combination with furosemide. There was no significant difference in the contents of endogenous fatty acids between both groups. Thus, the decreased anticoagulant effect and the increased $K_{el}$ and $Cl$ in rats coadministered with furosemide are not ascribed to the fatty acids in plasma.

In conclusion, the interactions, such as the drug displacement from albumin binding, between warfarin and furosemide were found, when a high dose of furosemide was coadministered. As a result, the free fraction of warfarin in plasma was increased and its anticoagulant activity was enhanced at earlier periods after coadministration of warfarin and a high dose of furosemide as a result of the increased warfarin uptake by liver. In contrast, the warfarin displacement from albumin binding enhanced the drug elimination from plasma and induced relatively rapid disappearance of the anticoagulant effect. Since the anticoagulant activity of warfarin in vivo is strongly affected if a high dose of furosemide (a 5 mg/kg dose of furosemide in rats corresponds to the dose level which is 2–3 times higher than a standard dose in human) is coadministered with the anticoagulant, it is necessary to take much care in the treatment with both warfarin and furosemide.

REFERENCES


