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Preliminary Evaluation of Furosemide–Probenecid Interaction in Humans

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Abstract □ The pharmacokinetics and pharmacodynamics of intravenous furosemide, 40 mg, were studied in four healthy male subjects in a crossover fashion with and without probenecid pretreatment. In each study, 16 plasma and 10 urine samples were collected over 24 hr. Fluid and electrolyte urinary losses were replaced orally throughout the study. Unchanged furosemide and probenecid were measured using high-pressure liquid chromatography; urinary sodium was measured by flame photometry. Although probenecid caused marked changes in the pharmacokinetic parameters of furosemide (increased area under the curve, decreased plasma and renal clearance, increased half-life, and decreased fraction excreted unchanged in the urine), there was no significant difference in its gross 8-hr natriuretic and diuretic effect. However, analysis of the time course of natriuresis showed a pattern similar to that of the urinary furosemide excretion rate, whereas the plasma concentration was poorly correlated over the entire dose–response curve.

Keyphrases □ Furosemide—pharmacokinetics and pharmacodynamics with and without probenecid pretreatment, humans □ Probenecid—pharmacokinetics and pharmacodynamics of furosemide with and without probenecid pretreatment, humans □ Drug–drug interactions—pharmacokinetics and pharmacodynamics of furosemide with and without probenecid pretreatment, humans □ Pharmacokinetics—furosemide with and without probenecid pretreatment, humans

Furosemide is an anthranilic acid derivative used to treat edematous states of hepatic, cardiac, and renal origin (1–3). It is believed to act at the luminal surface of the nephron where it inhibits the active reabsorption of chloride in the ascending limb of the loop of Henle (4–7). Since furosemide is highly protein bound (8, 9), access to the lumen occurs primarily through active secretion *via* the nonspecific organic acid secretory pathway (5, 6, 10). Thus, any drug or chemical substance that competes for this pathway could prevent furosemide from reaching its site of action and thereby attenuate its diuretic response.

Probenecid is a weak organic acid that competes with furosemide for active secretion into the kidney lumen. This competition can prevent furosemide from achieving an adequate cellular or luminal concentration and thereby diminish its natriuretic and diuretic response. Previous studies in experimental animals supported this hypothesis and showed that probenecid can decrease the natriuretic action of furosemide (10, 11). Studies in humans evaluating the effect of probenecid on the pharmacokinetics and pharmacodynamics of furosemide are limited and less clear (12–14).

The present investigation was undertaken to clarify the mechanism by which probenecid alters the diuretic response of furosemide. An additional objective was to define, in humans, a relationship between the dose of furosemide, its concentration or amount in a measurable sampling compartment, and its diuretic effect.

EXPERIMENTAL

Materials and Methods—Four males, 21–33 years and 65–77 kg, participated as outpatients in this study. Each subject had a normal medical history, physical examination, and standard laboratory tests. Each subject received 40 mg of furosemide alone and after pretreatment with probenecid. An interval of at least 1 week elapsed between studies. Subjects fasted the night before and until at least 2 hr after administration of the diuretic. Identical lot numbers for each drug were used throughout the study.

Furosemide was administered intravenously over ~3 min, with the midpoint of the infusion considered as time zero. Probenecid (1 g) was ingested at bedtime the night before and on arising the morning of the study (30–60 min prior to furosemide administration). Blood samples (3 ml) to determine the drug concentration were obtained with an indwelling heparinized scalp vein needle at 0, 5, 10, 20, 30, 45, 60, 80, 100, 120, 180, 240, 300, 360, and 480 min and ~24 hr.

Voided urine was collected at 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 24 hr and at two times of spontaneous voiding at home between the 8- and 24-hr collections. After each voiding, subjects drank a volume of balanced electrolyte solution flavored with fruit syrup equal to their urinary volume to avoid dehydration and electrolyte depletion.

All 24-hr blood samples showed normal electrolytes, urea nitrogen, and creatinine. Sodium concentrations were measured with a flame photometer[1]. Statistical differences were determined using a paired t test.

Plasma samples of furosemide, with and without probenecid pretreatment, were analyzed using a rapid, sensitive, and specific high-pressure liquid chromatographic[2] (HPLC) assay developed in this laboratory (15). Samples were run on a μBondapak C_{18} reversed-phase column[3] (30 cm × 3.9 mm i.d.) using dual-channel UV detection[4] (0.01 aufs). A 20-μl aliquot containing the internal standard, phenobarbital sodium (0.25 mg/ml), was added to 0.20-ml plasma furosemide samples. The mixture was vortexed, and 0.40 ml of acetonitrile was added. The mixture was vortexed again and then centrifuged for 10 min. The supernate was transferred to a clean test tube and evaporated under nitrogen gas until ~0.15 ml of the solution remained. A solvent system of 25% acetonitrile in 0.01 *M* acetic acid, adjusted to pH 5.0 with sodium hydroxide, was employed to measure furosemide at 280 nm and pheno-

[1] Model 450, Corning Scientific Instruments, Medfield, Mass.
[2] Model 6000A, Waters Associates, Milford, Mass.
[3] Waters Associates, Milford, Mass.
[4] Model 440 absorbance detector, Waters Associates, Milford, Mass.

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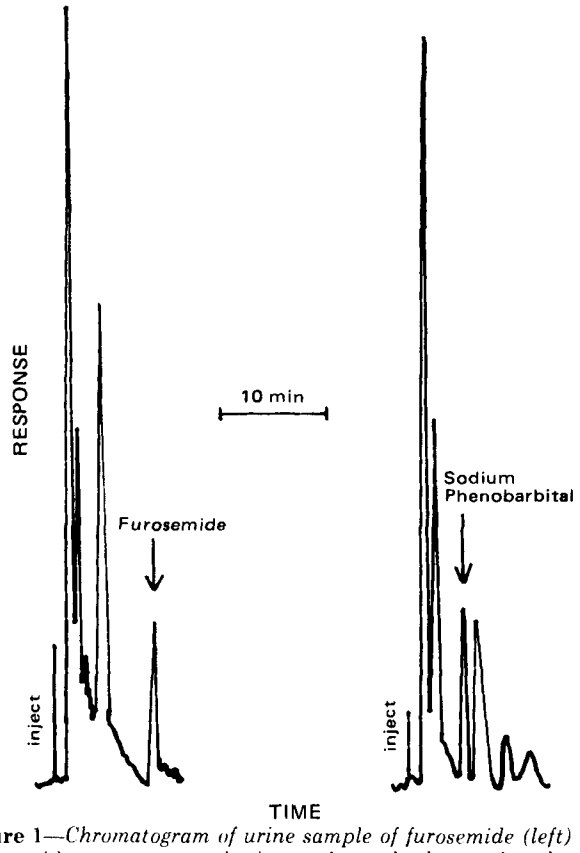

Figure 1—*Chromatogram of urine sample of furosemide (left) with probenecid pretreatment; the internal standard was phenobarbital sodium (right).*

barbital sodium at 254 nm. Urine samples of furosemide administered alone were prepared in a similar fashion, except that the addition of acetonitrile and the evaporation step were omitted.

During the assay of urine furosemide samples obtained following probenecid pretreatment, interfering peaks (possibly from probenecid metabolites) occurred in both the 280- and 254-nm detection channels. Therefore, it was necessary to develop a new assay system to separate the interfering peaks from those of furosemide and the internal standard, phenobarbital sodium. These urine samples were assayed for furosemide by HPLC[5] on a C_{18} reversed-phase column[3] using fluorescence detection[6]. The excitation and emission wavelengths of furosemide were set at 345 and 405 nm, respectively. The photomultiplier gain was set at 4, and the sensitivity range was set at 10. A 20-μl aliquot of the internal standard, phenobarbital sodium (2.5 mg/ml), still was measured by UV detection[7] at 254 nm (0.05 aufs).

With a solvent system of 30% acetonitrile in 0.015 *M* aqueous phosphoric acid solution at a flow rate of 2.0 ml/min, furosemide and phenobarbital sodium had retention times of 9.0 and 5.0 min, respectively (Fig. 1). A typical standard curve of the peak height ratio of furosemide to phenobarbital sodium over the concentration range of 0.82–20.50 μg/ml resulted in a linear least-squares regression equation of $y = 0.205x + 0.008$ ($r^2 = 0.999$). With fluorescent detection, concentrations as low as 8.20 ng/ml were measured.

Plasma samples of probenecid were prepared in a similar fashion to furosemide. A 40-μl aliquot of phenobarbital sodium (4 mg/ml) was used as the internal standard, and the solvent system (30% acetonitrile in 0.01 *M* acetic acid, adjusted to pH 5.0 with sodium hydroxide) had a flow rate of 2.0 ml/min. Both probenecid and phenobarbital sodium were measured using UV detection[7] at 254 nm. Plasma probenecid concentrations were fairly constant during the entire study and ranged from 100 to 165 μg/ml (Fig. 2).

Calculations—The half-life of furosemide, $t_{1/2}$, was determined by linear regression from the terminal portion of the urinary excretion rate *versus* midpoint time plots. The area under the plasma concentration–time curve, *AUC*, was calculated using the trapezoidal rule, extrapolated to infinity from the last measured concentration.

The volume of distribution at steady state, Vd_{ss}, was determined by the compartment-independent method of Benet and Galeazzi (16):

$$Vd_{ss} = \text{dose } (AUMC)/(AUC)^2 \qquad \text{(Eq. 1)}$$

where *AUMC* is the area under the curve of the first moment of the concentration–time curve, *i.e.*, $\int_0^\infty tC_p\,dt$. The total plasma clearance of furosemide, Cl_p, was calculated from:

$$Cl_p = \text{dose}/AUC \qquad \text{(Eq. 2)}$$

The total renal clearance, Cl_r, was estimated from:

$$Cl_r = Ae_\infty/AUC \qquad \text{(Eq. 3)}$$

where the amount of unchanged drug recovered in the urine at time infinity is represented by Ae_∞. The fraction of furosemide excreted unchanged in the urine, f_e, was calculated by dividing Ae_∞ by the intravenous dose. Nonrenal plasma clearance, Cl_{nr}, was calculated as the difference between the plasma and renal clearances. The fraction excreted by nonrenal routes, f_{nr}, was calculated by dividing the nonrenal clearance by the total plasma clearance.

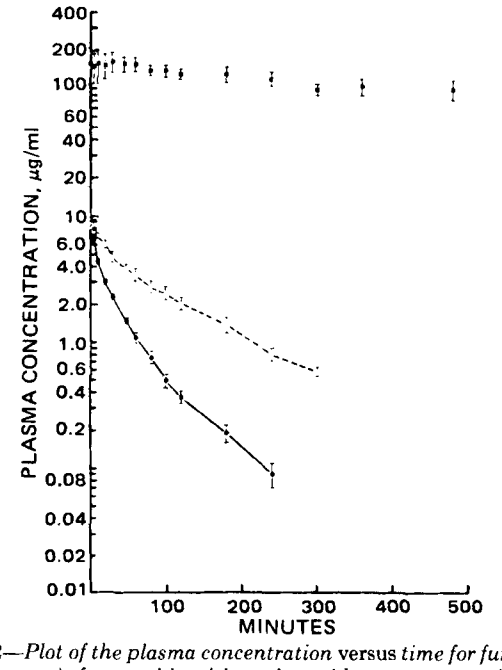

Figure 2—*Plot of the plasma concentration* versus *time for furosemide alone (●——●), furosemide with probenecid pretreatment (○- - -○), and probenecid (■). Data are expressed as the mean ± SEM (n = 4).*

RESULTS

The effects of probenecid on the pharmacokinetics of furosemide were analyzed in both plasma and urine (Table I). Mean plasma concentrations of furosemide with probenecid pretreatment were significantly increased at all time points except at 5 min (Fig. 2). The significant increase in *AUC* supports this observation [252 ± 24 (μg min)/ml for furosemide alone (Treatment I) and 785 ± 87 (μg min)/ml for furosemide with probenecid pretreatment (Treatment II); $p < 0.001$]. In addition, the total plasma clearance of furosemide was decreased significantly in the presence of probenecid (160 ± 15 ml/min for Treatment I and 51.4 ± 5.3 ml/min for Treatment II; $p < 0.001$). This difference in total plasma clearance was reflected by the significant increase in the half-life of furosemide (82 ± 5 min for Treatment I and 175 ± 17 min for Treatment II; $p < 0.001$) since the Vd_{ss} value was not altered significantly (8.44 ± 0.93 liters for Treatment I and 6.66 ± 1.78 liters for Treatment II; $p > 0.20$).

The total renal clearance of furosemide was reduced markedly with probenecid pretreatment (118 ± 17 ml/min for Treatment I and 23.1 ± 1.0 ml/min for Treatment II; $p < 0.002$), while the nonrenal plasma clearance did not change significantly (42 ± 12 ml/min for Treatment I and 28.3 ± 4.9 ml/min for Treatment II; $p > 0.10$). In addition, the fraction of furosemide excreted unchanged in the urine in the presence of probenecid was reduced significantly (0.74 ± 0.07 for Treatment I and 0.46 ± 0.05 for Treatment II; $p < 0.01$) with a corresponding increase in

[5] Perkin-Elmer Series 2, Norwalk, Conn.
[6] Fluorescence spectrophotometer 204-A, Perkin-Elmer, Norwalk, Conn.
[7] LC 250, Perkin-Elmer, Norwalk, Conn.

Table I—Pharmacokinetic Effects of Probenecid on Furosemide

Subject	Treatment	AUC, (μg min)/ml	Cl_p, ml/min	Vd_{ss}, liters	$t_{1/2}$, min	Cl_r, ml/min	Cl_{nr}, ml/min	f_e	f_{nr}
TP	Furosemide	225	178	8.85	85	122	56	0.68	0.31
	Furosemide with probenecid	723	55.3	6.00	185	22.8	32.5	0.41	0.59
RP	Furosemide	280	143	9.55	85	101	42	0.71	0.29
	Furosemide with probenecid	907	44.1	5.50	193	22.7	21.4	0.52	0.49
TT	Furosemide	242	165	7.62	75	139	26	0.84	0.16
	Furosemide with probenecid	787	50.9	5.81	160	22.3	28.6	0.44	0.56
DH	Furosemide	261	153	7.73	82	109	44	0.71	0.29
	Furosemide with probenecid	724	55.3	9.31	161	24.6	30.7	0.45	0.56
Mean ± *SD*	Furosemide	252 ± 24	160 ± 15	8.44 ± 0.93	82 ± 5	118 ± 17	42 ± 12	0.74 ± 0.07	0.26 ± 0.07
	Furosemide with probenecid	785 ± 87	51.4 ± 5.3	6.66 ± 1.78	175 ± 17	23.1 ± 1.0	28.3 ± 4.9	0.46 ± 0.05	0.55 ± 0.04
Level of significance		S ($p < 0.001$)	S ($p < 0.001$)	NS ($p > 0.20$)	S ($p < 0.001$)	S ($p < 0.002$)	NS ($p > 0.10$)	S ($p < 0.01$)	S ($p < 0.01$)

the fraction excreted by nonrenal routes (0.26 ± 0.07 for Treatment I and 0.55 ± 0.04 for Treatment II; $p < 0.01$).

Analysis of the urinary excretion rate of furosemide, with and without probenecid pretreatment, is shown in Fig. 3. Initially, the urinary excretion rate of furosemide with probenecid was significantly lower than that of furosemide when administered alone. However, after ~125 min, the two curves (Treatments I and II) intersect; at subsequent times, probenecid caused a significant increase in the furosemide excretion rate. This result was primarily due to the large difference in plasma furosemide concentrations at subsequent times between Treatments I and II (Fig. 2) since the renal clearance was reduced but was constant throughout each study.

The effect of probenecid on furosemide-induced natriuresis is shown in Fig. 4. The initial natriuretic response to furosemide when it was given concomitantly with probenecid was reduced compared to that of furosemide administered alone. The two curves (Treatments I and II) intersect at 100 min; at subsequent times, probenecid caused an increase in furosemide-induced natriuresis, similar to that seen for the urinary excretion rate of furosemide. Although differences in the sodium excretion rate were seen with and without probenecid pretreatment, they did not appear to be statistically different. Table II shows that the 8-hr sodium excretion (milliequivalents) was 291 ± 53 for Treatment I and 323 ± 106 for Treatment II ($p > 0.50$). The diuretic response (milliliters per 8 hr) was 2257 ± 422 for Treatment I and 2637 ± 632 for Treatment II ($p > 0.20$).

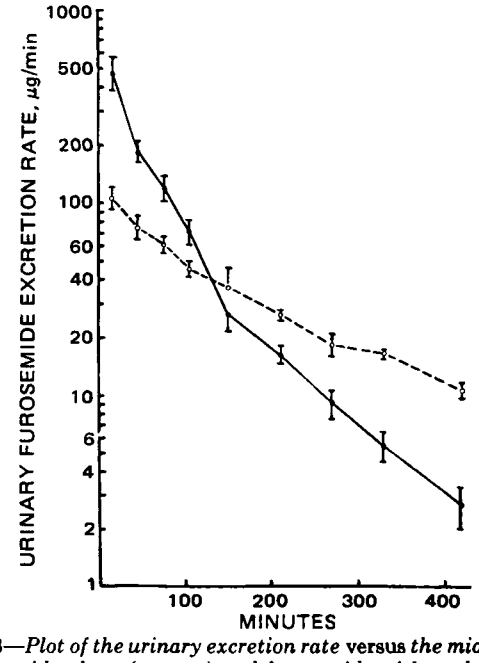

Figure 3—*Plot of the urinary excretion rate* versus *the midpoint time for furosemide alone (●—●) and furosemide with probenecid pretreatment (○---○). Data are expressed as the mean* ± SEM (n = *4).*

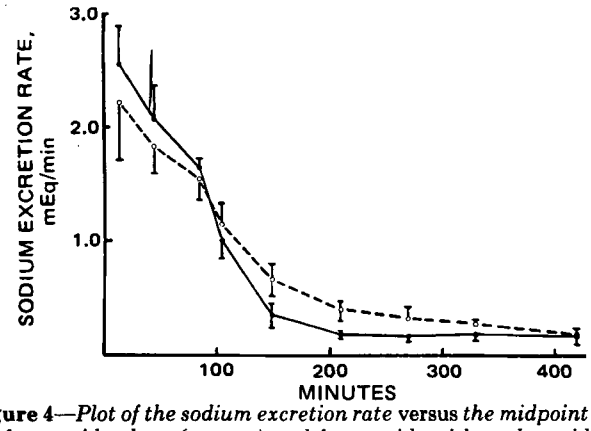

Figure 4—*Plot of the sodium excretion rate* versus *the midpoint time for furosemide alone (●—●) and furosemide with probenecid pretreatment (○---○). Data are expressed as the mean* ± SEM (n = *4).*

DISCUSSION

Experiments in animals suggested that the luminal concentration or amount of furosemide rather than its plasma concentration may be the critical determinant with respect to its natriuretic and diuretic effect (10, 11, 17, 18). Hook and Williamson (10) and Friedman and Roch-Ramel (11) demonstrated in the dog and cat, respectively, that probenecid (50 mg/kg iv) significantly inhibited furosemide-induced natriuresis. Since probenecid is highly secreted (19), it can compete for active transport and prevent furosemide from reaching the tubular fluid, thereby attenuating its natriuretic effect. However, human studies (12–14) do not

Table II—Effects of Probenecid on Furosemide Diuresis and Natriuresis

Subject	Treatment	Urine Volume, ml/8 hr	Sodium Excretion, mEq/8 hr
TP	Furosemide	2451	328
	Furosemide with probenecid	2117	232
RP	Furosemide	2251	262
	Furosemide with probenecid	2555	288
TT	Furosemide	1674	232
	Furosemide with probenecid	2329	296
DH	Furosemide	2653	343
	Furosemide with probenecid	3546	477
Mean ± *SD*	Furosemide	2257 ± 422	291 ± 53
	Furosemide with probenecid	2637 ± 632	323 ± 106
Level of significance		NS ($p > 0.20$)	NS ($p > 0.50$)

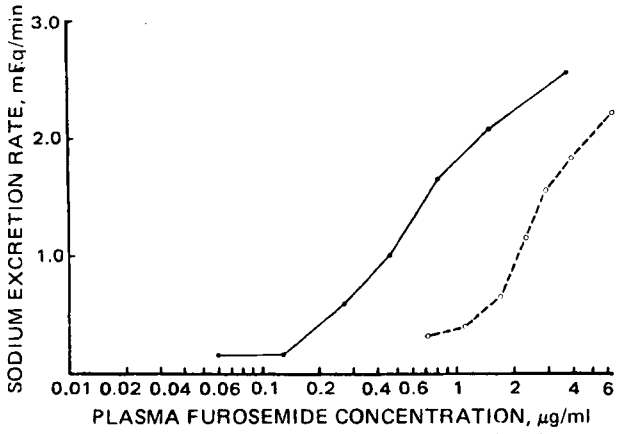

Figure 5—*Plot of the sodium excretion rate* versus *the plasma concentration for furosemide alone (●—●) and furosemide with probenecid pretreatment (O---O) (n = 4).*

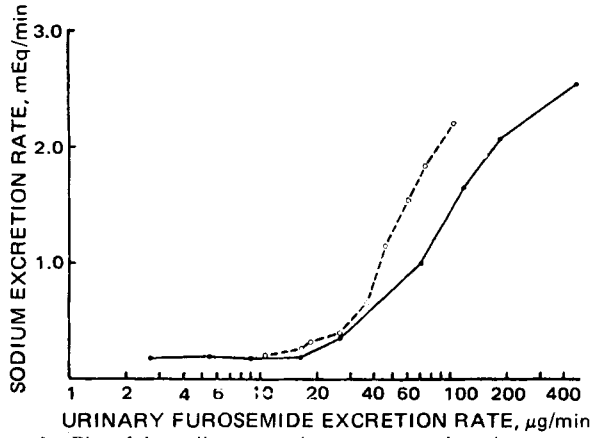

Figure 6—*Plot of the sodium excretion rate* versus *the urinary excretion rate for furosemide alone (●—●) and furosemide with probenecid pretreatment (O---O) (n = 4).*

corroborate these findings in animals. In contrast, probenecid caused either no change or a significant increase in the natriuretic response to furosemide.

The first study of the effect of probenecid on furosemide kinetics and natriuretic response in humans showed that probenecid significantly decreased the total plasma clearance (155 ml/min for Treatment I and 85 ml/min for Treatment II) and the total renal clearance (134 ml/min for Treatment I and 63 ml/min for Treatment II) of furosemide, and significantly increased the furosemide half-life (35.8 min for Treatment I and 60.8 min for Treatment II) (12). However, the 6-hr urine volume (5098 ml for Treatment I and 6164 ml for Treatment II) and the sodium excretion (578 mEq for Treatment I and 694 mEq for Treatment II) were not significantly different between treatments. In addition, the fraction of the dose excreted unchanged in the urine was not statistically altered with probenecid pretreatment, although three of the four subjects studied did excrete a smaller percentage. Therefore, the investigators (12) concluded that their results were consistent with the findings of a previous study (10), which suggested that the amount of furosemide in the tubular fluid is the main determinant of furosemide diuresis. Although we do not disagree with their conclusions, the previous investigators did not fully characterize the mechanism of this interaction between furosemide and probenecid. Since the time course of the natriuretic and diuretic response was not described, the previous investigators were considering only gross effects.

Homeida *et al.* (13) also demonstrated marked changes in furosemide pharmacokinetics with probenecid pretreatment. Similarly, these investigators noted that since the total proportion of unchanged drug reaching the renal tubule was not changed markedly, the total diuretic effect remained unaltered. However, this conclusion is suspect since our calculations of their data show an approximate 41% decrease in the fraction of the furosemide dose excreted unchanged in the urine (from 0.34 to 0.20) when the subjects were pretreated with probenecid. In addition, furosemide was assayed spectrofluorometrically, which is rather nonspecific, especially in urine. This method may account for the unusual values for the total, renal, and nonrenal plasma clearances reported in their control subjects, as suggested by Benet (20).

In a more recent study, the pharmacodynamic effect of probenecid on the response to furosemide in humans was quantified (14). Analysis of the time course of natriuresis and diuresis showed that probenecid actually decreased the response of furosemide for the first 60–90 min but increased the subsequent response sufficiently to result in a statistically greater overall effect. However, it was noted (14) that since the concentrations or amounts of furosemide in the urine were not compared with the response, a unifying hypothesis to explain the mechanism of a furosemide–probenecid interaction was not possible[8].

In the present investigation, the time course of furosemide in plasma and urine was compared with that of the natriuretic effect in an attempt to explain the mechanism for a furosemide–probenecid interaction. Although probenecid caused marked changes in the pharmacokinetic parameters of furosemide (Table I), there was no significant difference in its gross natriuretic and diuretic effect (Table II). Analysis of the time course for natriuresis (Fig. 4) shows that probenecid actually decreased the response for the first 100 min after furosemide administration. However, the subsequent response was increased sufficiently to result in no statistical difference in the mean 8-hr value for sodium excretion. Although a similar pattern was seen with respect to the urinary excretion of furosemide, the magnitude of this difference between treatments was statistically significant (Fig. 3).

Figure 5 shows that probenecid caused a significant shift to the right in the relationship between the plasma concentration of furosemide and its natriuretic effect. This observation suggests that higher plasma furosemide concentrations are needed in the presence of probenecid to produce a natriuretic response equivalent to that produced by lower concentrations when probenecid is absent. Figure 6 shows the relationship between the urinary furosemide excretion rate and the natriuretic effect. Although Treatments I and II were not parallel over the entire dose–response curve, the amount of furosemide excreted into the urine was better correlated with response than was the plasma furosemide concentration.

The shift to the left between the urinary excretion rate of furosemide and the effect (upper portion of Fig. 6) may be real or may be an artifact due to the limited number of subjects. However, a possible explanation for this finding may involve an interaction between probenecid and prostaglandins. Previous investigators hypothesized that prostaglandins mediate the natriuretic–diuretic effect of furosemide (21–25). Renal prostaglandins are synthesized primarily in the medulla (24, 26–28) and are released into the extracellular fluids (29). *In vitro* studies showed that prostaglandins accumulate in several tissues, including the renal cortex, as a result of an active transport mechanism (30, 31). In addition, it was shown that probenecid can inhibit the renal tubular transport of prostaglandins, presumably by competing for active transport into the urine (32, 33). Although this conclusion is speculation, this inhibition by probenecid of prostaglandin transport may result in a tubule that is more responsive to smaller amounts of furosemide in the urine and thus account for the shift to the left as described.

CONCLUSIONS

The mechanism by which probenecid alters furosemide-induced natriuresis is consistent with *in vitro* studies (6) indicating that furosemide acts at the luminal surface of the nephron. This study in humans, as well as previous animal studies (18), demonstrates that the urinary excretion rate of furosemide is a better indicator of natriuresis and diuresis than is the plasma concentration.

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Syntheses and Evaluation of Ketals, Hemithioketals, and Dithioketals of Conjugated Styryl Ketones Principally for Antineoplastic Activity

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Abstract □ Ketals, hemithioketals, and dithioketals of nuclear-substituted styryl ketones were prepared as latentiated forms of the ketones. This undertaking was based on the premise that there is increased acidity in tumors compared to normal tissue, and thus preferential regeneration of the ketone in neoplastic tissue may occur. Attempts to form 1,3-dioxolans of Mannich bases were unsuccessful. The prepared compounds did not possess significant anticancer properties, but analgesic, anti-inflammatory, antihistaminic, and antimicrobial activities were found in the prepared Mannich bases.

Keyphrases □ Antineoplastic activity—ketals, hemithioketals, and dithioketals of conjugated styryl ketones, synthesis and evaluation of activity □ Styryl ketones, conjugated—synthesis of ketals, hemithioketals, and dithioketals, evaluation for antineoplastic activity □ Mannich bases—synthesized from conjugated styryl ketones and acetophenones, evaluation for antineoplastic activity

A recurrent problem in the design of compounds for cancer chemotherapy is the synthesis of derivatives possessing selective toxicity for tumors. Biochemical differences between cancerous and normal cells have been claimed (1), including the increased acidity of certain malignant cells compared to normal tissue (2–4). The increased acidity of tumors has been ascribed to the greater rate of aerobic glycolysis in neoplastic tissue (5), which leads to increased lactic acid production. The pH of a number of tumors has been reported to be ~7.0 (2–4), although such measurements probably recorded the extracellular pH. Therefore, the pH of the intracellular fluid probably is even lower (6), and an average pH value for a number of tumorous tissues has been considered to be ~6.5 (7). Hence, a prodrug permitting the release of a cytotoxic agent under acidic conditions may afford selective lethality of tumors with ameliorated toxicity for normal tissue. Only a few attempts have been made to design compounds based on this pH differential (8–11).

Several investigations in this laboratory involved the preparation of some nuclear-substituted styryl ketones (I) and related Mannich bases (II) for evaluation as anticancer agents (12–14) and in other screens (15–17). While the ketones have not been evaluated against P-388 lymphocytic leukemia, some of the Mannich bases showed promising levels of activity in this screen (12); one compound (IIe) increased the mean survival time in mice by >40% (12). However, murine toxicity was found in II due at least partially to interference with mitochondrial function (18, 19). Therefore, prodrugs of both I and II were prepared, and both I and the prodrugs were evaluated in the P-388 screen so that anticancer activities and murine toxicities of the ketones (I and II) and their latentiated precursors could be compared. Since ketals, hemithioketals, and dithioketals are known to hydrolyze under acidic conditions but are stable in neutral or alkaline media (20), the preparation of these derivatives from I and II may

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