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Pharmacokinetics of Probenecid Following Oral Doses to Human Volunteers

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Abstract □ The pharmacokinetics of probenecid were examined following single 0.5-, 1.0-, and 2.0-g oral doses to healthy male volunteers. Doses were administered following overnight fast, according to a randomized design. Plasma levels of probenecid were determined by high-pressure liquid chromatography (HPLC), using sulfamethazine as the internal standard. Mean peak probenecid levels of 35.3, 69.6, and 148.6 μg/ml were obtained at 3–4 hr following the 0.5-, 1.0-, and 2.0-g doses, respectively. Probenecid levels from the 0.5- and 1.0-g doses declined in apparent monoexponential fashion, with mean elimination half-lives of 4.2 and 4.9 hr. Interpretation of the 2.0-g data by a kinetic model incorporating first-order elimination resulted in a plasma drug half-life of 8.5 hr. When first-order elimination was replaced by a Michaelis–Menten-type function, the mean value of the resulting V_m/K_m ratios was 0.20, equivalent to a plasma drug half-life [$0.693/(V_m/K_m)$] of 3.8 hr. Plasma probenecid curves from all three dosages were successfully fitted to the saturable elimination model using nonlinear regression and numerical integration routines. The results suggest that probenecid elimination may be saturable at therapeutic dose levels.

Keyphrases □ Probenecid—pharmacokinetics following oral doses to humans, high-pressure liquid chromatography, elimination □ Pharmacokinetics—probenecid following oral doses to humans, high-pressure liquid chromatography, elimination □ High-pressure liquid chromatography—probenecid following oral doses to humans, pharmacokinetics

Probenecid is used for the treatment of gout and gouty arthritis and also as an adjuvant in therapy to prolong the plasma levels of other compounds, particularly the β-lactam antibiotics (1).

Although it has been used clinically for several years, the pharmacokinetics of probenecid are not well documented. It is efficiently absorbed after oral doses (2–4) with peak plasma concentrations occurring at 1–5 hr. Probenecid is 83–95% bound to plasma proteins at concentrations of 20–176 μg/ml (3). It is cleared from the body predominantly by metabolism, which occurs mainly by side-chain oxidation and glucuronide conjugation (3, 5). Only 5–11% of orally dosed probenecid is excreted in unchanged form in urine (6, 7).

The rate at which probenecid is cleared from plasma was shown to be dose-dependent in dogs (8) and humans (3). In the latter study, the plasma half-life of probenecid increased from 3–8 hr following a 0.5-g iv dose, to 6–12 hr following a 2.0-g iv dose in three subjects.

Since probenecid is extensively metabolized, dose-dependent elimination may be due to saturation of one or more metabolic pathways. However, a subsequent study demonstrated an unchanged pattern of urinary metabolites from oral doses of 0.5, 1.0, and 2.0 g of probenecid (6).

In view of the uncertainty regarding the nature and possible dose-dependency of probenecid pharmacokinetics, this study was undertaken to examine plasma levels of probenecid following 0.5-, 1.0-, and 2.0-g oral doses to healthy male volunteers.

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Table I—Mean Plasma Probenecid Concentrations Following Single Oral Doses of 0.5, 1.0, or 2.0 g of Probenecid

Dose	Concentration of Probenecid, μg/ml												
	0.5 hr	1 hr	1.5 hr	2 hr	2.5 hr	3 hr	4 hr	6 hr	12 hr	24 hr	30 hr	36 hr	48 hr
0.5 g													
Mean	3.4	13.8	22.2	25.6	27.7	32.9	31.7	23.1	9.1	1.7	—[a]	—	—
SD[b]	2.1	8.2	14.9	19.3	17.2	8.5	4.5	4.0	1.7	0.6	—	—	—
1.0 g													
Mean	1.8	15.5	31.2	40.6	48.6	51.6	65.7	56.3	32.2	5.7	2.6	—	—
SD	1.8	19.7	30.3	41.9	40.3	34.6	28.0	12.3	7.5	3.0	2.0	—	—
2.0 g													
Mean	11.3	39.4	61.7	85.2	124.2	103.5	120.1	122.4	79.7	32.6	18.9	7.5	1.1
SD	11.6	28.2	33.2	45.1	77.8	60.1	31.7	21.6	12.6	9.9	6.7	3.6	1.1

[a] Below the limit of detection. [b] $n = 5$.

EXPERIMENTAL

Protocol—Five healthy male volunteers (20–39 years, 53–86 kg), whose weight–height relationships were within ±10% of normal (9), participated in the study after giving informed consent. Subjects were instructed to take no other medication for 1 week before the study and during the study. Subjects received single doses of 0.5, 1.0, and 2.0 g of probenecid[1] on three separate occasions, at least 1 week apart, according to a randomized design. Doses were administered with 250 ml of water at 8 am following an overnight fast. The tablets were swallowed whole. Food and water were withheld until 4 hr postdose, then normal eating and drinking were resumed.

Blood samples (~10 ml) were taken from a forearm vein into heparinized evacuated tubes[2] immediately before and then serially to 48 hr postdose. Plasma was separated by centrifugation and stored at −20° until assayed. No deterioration of samples was observed during storage.

Assay—Probenecid concentrations in plasma were determined by a modification of a previously described high-pressure liquid chromatographic (HPLC) procedure (10). Plasma (1 ml), pH 4 phosphate buffer (1 ml), sodium chloride (1 g), 0.1–0.3 ml of methanol containing 25–75 μg/ml sulfamethazine as internal standard, and ether (8 ml) were combined in a 15-ml centrifuge tube, vortex mixed for 15 sec, and shaken for 30 min on a horizontal shaker. After centrifuging at 3000×*g* for 10 min, 5 ml of the ether layer was transferred to a clean tube, and evaporated to dryness in a water bath at 40° under nitrogen. The residue was reconstituted in 1.0 ml of chromatographic mobile phase by vortex mixing for 30 sec, and 20 μl of this was injected onto the column.

The chromatographic system consisted of a solvent pump[3], a fixed-volume (20 μl) sample injection valve[4], a 10-μm particle size reversed-phase octadecyl column (4.6 mm × 250 mm)[5], and a fixed-wavelength (254 nm) UV detector[6]. All chromatograms were recorded at a chart speed of 10 cm/hr.

The mobile phase was 27.5% acetonitrile in 0.01 *M* phosphate buffer, at pH 6, and the flow rate was 1 ml/min. Under these conditions, the retention times of probenecid and sulfamethazine were 9 and 6 min, respectively. There was no interference from endogenous substances. Concentrations of probenecid were determined by peak height ratios. Standard curves using each individual's predose plasma were run together with each batch of postdose plasma samples.

The extraction efficiency of probenecid in this system was 94.3 ± 5.8% *SD* ($n = 20$). The chromatographic assay was linearly responsive to probenecid concentrations between 1 and 200 μg/ml, and assay reproducibility was within 10% of the mean in this concentration range. Probenecid metabolites, all of which are more polar than the parent compound, do not interfere with the assay for probenecid under these assay conditions (10).

Reagents—Human plasma for assay development was purchased[7]. Probenecid[8] and sulfamethazine[9] were of reference standard quality. All other chemicals and solvents were analytical reagent grade and were used as received.

Data Analysis—Individual probenecid profiles in plasma resulting from the three doses were examined using two different pharmacokinetic models. The first of these was a one-compartment kinetic model with first-order appearance of drug into and loss of drug from plasma and an absorption lag time. With this model, plasma concentrations of probenecid were fitted to Eq. 1 in which F is the fraction of the oral dose (D) which is absorbed into the systemic circulation, V_d is the apparent homogenous distribution volume for probenecid, k_a and k_{el} are first-order rate constants for appearance and loss of drug, respectively, and t_0 is the absorption lag time (11):

$$C = \frac{FD}{V_d}\left(\frac{k_a}{k_a - k_{el}}\right)\left(e^{-k_{el}(t-t_0)} - e^{-k_a(t-t_0)}\right) \qquad \text{(Eq. 1)}$$

Initial estimates of parameter values were obtained by standard graphical procedures. Final estimates were obtained by nonlinear regression analysis using the program NREG (12) on a digital computer[10].

The second model, which was investigated primarily as a result of the plasma data resulting from the 2.0-g dose of probenecid, is different from the first in that the elimination phase is described in terms of a saturable Michaelis–Menten-type term. The rate equation for time-dependent change in probenecid plasma levels with this model is:

$$\frac{dc}{dt} = \frac{FDk_a}{V} e^{-k_a(t-t_0)} - \frac{V_m C}{K_m + C} \qquad \text{(Eq. 2)}$$

where V_m and K_m are Michaelis–Menten-type functions describing the maximum rate of probenecid elimination and the drug concentration when the rate of elimination is one-half the maximum value, respectively, and all other parameters are as described for Eq. 1. This rate equation cannot be solved analytically, and nonlinear regression analysis was done by coupling the program NREG with the numerical integration program DGEAR (13). Initial estimates of the values of V_m and K_m were obtained graphically by a previous method (14). Final computer estimates of values for k_a and FD/V_d from Eq. 1 were used as initial estimates for these parameters when fitting data to Eq. 2.

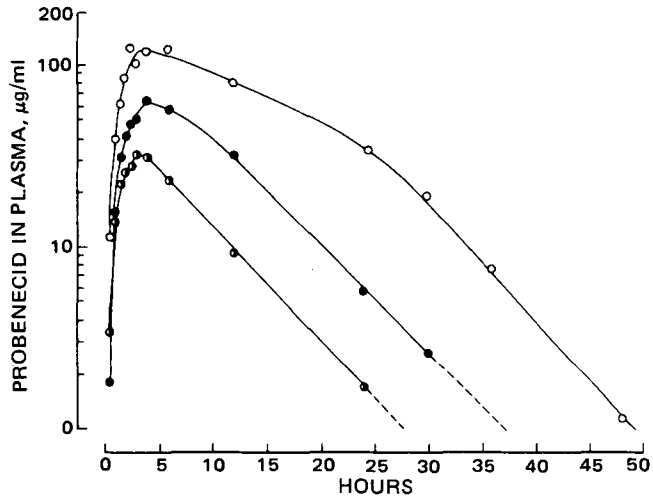

Figure 1—*Mean plasma levels of probenecid in five subjects following single oral doses. Key: 0.5-g (◑); 1.0-g (●); and 2.0-g (○) doses of probenecid.*

[1] Probenecid 500-mg tablets USP, lot No. E002Erl, Mylan Pharmaceuticals Inc., Morgantown, W.Va.
[2] Vacutainer.
[3] Model 110, Altex Scientific Inc., Berkeley, Calif.
[4] Model 210, Altex Scientific Inc., Berkeley, Calif.
[5] Lichrosorb C-18, Altex Scientific, Berkeley, Calif.
[6] Model 153, Altex Scientific, Berkeley, Calif.
[7] American Red Cross, Madison, Wis.
[8] Merck, Sharp and Dohme, West Point, Pa.
[9] Sigma Chemical Co., Saint Louis, Mo.
[10] Univac model 1100.

Table II—Pharmacokinetic Parameter Values (±1 *SD*) for Probenecid [a]

Parameter	Value 0.5-g Dose	1.0-g Dose	2.0-g Dose	Statistic[b]
C_{max}[c] (μg/ml)	35.3 ±8.5	69.6 ±26.9	148.6 ±47.4	C > B > A[d]
t_{max}[e] (hr)	3.1 ±0.9	3.9 ±1.3	3.5 ±1.5	NSD[f]
$AUC^{0\rightarrow 48hr}$[g] (μg hr/ml)	292 ±66	772 ±237	2109 ±429	C > B > A
$AUC^{0\rightarrow 48hr}/D$[h] (hr/ml × 10^{-6})	584 ±131	772 ±237	1054 ±214	C > A, B
k_a (hr^{-1})	0.98 ±0.49	0.56 ±0.41	0.66 ±0.29	NSD
t_0 (hr)	0.80 ±0.71	1.1 ±0.6	0.56 ±0.32	NSD
k_{el} (hr^{-1})	0.18 ±0.05	0.15 ±0.03	0.08 ±0.004	A, B > C
$t_{1/2(el)}$[i] (hr)	4.2 ±1.1	4.9 ±0.8	8.5 ±0.4	C > A, B
FD/V[j] (μg/ml)	50 ±10	109 ±22	176 ±30	C > B > A
F/V^k (10^{-6}/ml)	100 ±21	109 ±22	80 ±10	B > C
r^{2l}	0.98 ±0.02	0.98 ±0.02	0.95 ±0.07	NSD

[a] The model-dependent parameter values were obtained by fitting plasma data to Eq. 1. [b] Differences between doses are significant if $p < 0.05$. [c] Observed maximum concentration of probenecid in plasma. [d] A = 0.5-g dose, B = 1.0-g dose, and C = 2.0-g dose. [e] Time of C_{max}. [f] No significant differences. [g] Area under plasma–probenecid curve (0–48 hr), calculated by trapezoidal rule. [h] Area normalized for administered dose. [i] Calculated from $t_{1/2(el)} = \ln 2/k_{el}$. [j] Fraction of the dose absorbed, expressed as a concentration in its volume of distribution. [k] FD/V_d normalized for administered dose. [l] Coefficient of determination, $r^2 = (\Sigma obs^2 - \Sigma dev^2)/\Sigma obs^2$.

Statistical analysis of plasma levels, and also pharmacokinetic parameter values, resulting from the three doses were examined by ANOVA for crossover design. Significant effects between specific doses were examined by Tukey's test (15). Differences between pharmacokinetic parameter values, and also coefficients of determination, obtained by nonlinear regression analysis of plasma data using the first-order and saturable kinetic models were examined by paired *t*-test.

RESULTS

The mean plasma levels of probenecid obtained from the three doses are given in Table I, and the data are summarized graphically in Fig. 1. Some model-independent pharmacokinetic parameter values and also pharmacokinetic constants obtained after fitting individual data sets to Eq. 1 are given in Table II.

Following the 0.5-g dose, plasma probenecid levels reached a mean peak value of 35.3 μg/ml at 3 hr and then declined in apparent monoexponential fashion. Probenecid could be detected in all subjects up to 24 hr postdose. Following the 1.0-g dose, the mean peak probenecid level increased twofold to 69.6 μg/ml and occurred at 4 hr postdose. Drug levels again declined in apparent monoexponential fashion and could be detected in all subjects up to 30 hr postdose.

Individual plasma probenecid profiles following the 2.0-g dose were different from those resulting from the lower doses. The mean peak level was again doubled, compared with that from the 1.0-g dose, to 148.6 μg/ml and occurred at 3.5 hr. After peak levels had been reached from this dose, plasma levels of probenecid declined initially at a slower rate compared with the lower doses, and the rate of decline in the logarithm of plasma levels *versus* time during this period was curvilinear. It was not until 24–30 hr after dosing, when probenecid plasma levels had fallen to similar values to those obtained from the 0.5- and 1.0-g doses, that the rate of loss of probenecid from plasma became log-linear, with a similar rate to that obtained from the lower doses. Following the 2.0-g dose, probenecid could be detected in the plasma of all subjects at 36 hr, and in three of the five subjects at 48 hr, postdose.

Table III—Pharmacokinetic Parameter Values (±1 *SD*) Obtained by Analyzing Plasma Probenecid Levels from the 2.0-g Dose According to First-order or Saturable Kinetic Models

Parameter	Value First-Order Model[a]	Saturable Model[b]	Statistic[c]
k_a (hr^{-1})	0.66 ± 0.29	0.63 ± 0.26	NS[d]
t_0 (hr)	0.56 ± 0.32	0.40 ± 0.52	NS
k_{el} (hr^{-1})	0.082 ± 0.504	—[e]	
$t_{1/2(el)}$ (hr)	8.5 ± 0.4[f]	3.8 ± 1.1[g]	S[h]
V_m (μg/ml·hr)	—	8.6 ± 2.1	
K_m (μg/ml)	—	46.6 ± 26.5	
V_m/K_m (hr^{-1})	—	0.20 ± 0.06	
FD/V_d	176 ± 30	160 ± 19	NS
r^2	0.95 ± 0.07	0.96 ± 0.04	NS

[a] Data was fitted to Eq. 1. [b] Data was fitted to Eq. 2 with numerical integration. [c] Data compared using a paired *t* test. [d] Not significant ($p > 0.05$). [e] Does not apply. [f] Calculated from $t_{1/2(el)} = \ln 2/k_{el}$. [g] Calculated from $t_{1/2(el)} = \ln 2/(V_m/K_m)$. [h] Significant ($p < 0.05$).

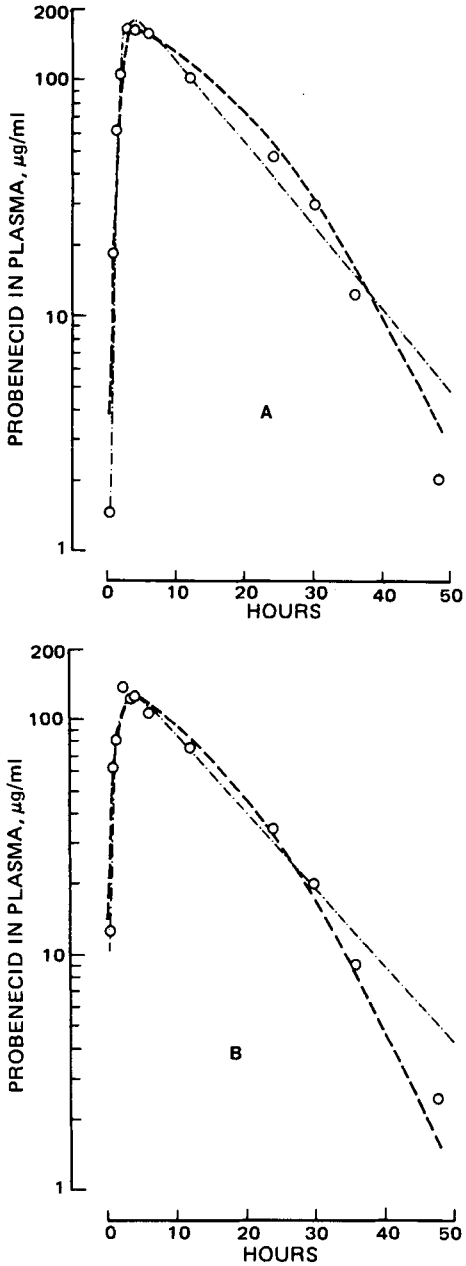

Figure 2—*Plasma levels (○) of probenecid in two subjects and nonlinear regression curves obtained from Eq. 1 (·--) and Eq. 2 (---). Key: (A) subject 1; (B) subject 2.*

Although the value of C_{max} was dose-proportional between the three dosages of probenecid, the 0–48-hr area under the plasma curve, $AUC^{0\rightarrow 48\ hr}$, increased 2.6-fold between the 0.5- and 1.0-g doses, and 2.9-fold between the 1.0- and 2.0-g doses. When the areas were normalized for the administered dose, there appeared to be a progressive increase in the values with increasing dose, and the value for the 2.0-g dose was significantly larger than those from the other two doses.

While the increases in area values were greater than expected with increasing dose size, the opposite effect was observed in the values of FD/V_d, which is a rate-independent measure of drug availability to the systemic circulation. The value of this parameter increased 2.2-fold between the 0.5- and 1.0-g doses, but only 1.5-fold between the 1.0- and 2.0-g doses. Normalizing this value for the administered dose resulted in a somewhat lower F/V_d value for the 2.0-g dose compared with the other two doses, but only the difference between the 2.0- and 1.0-g doses reached the 95% significance level.

No significant differences were observed in the lag times or in the absorption rate constants between doses. The elimination rate constant was

similar for the 0.5- and 1.0-g doses, yielding mean plasma half-lives of 4.2 and 4.9 hr, respectively. Following the 2.0-g dose, however, the elimination half-life of probenecid was significantly increased to a mean value of 8.5 hr. The coefficient of determination for nonlinear regression analysis of data using Eq. 1 was slightly lower following the 2.0-g dose compared to the other two doses, but differences in this value between treatments were not significant.

The discrepancies observed in the $AUC^{0\rightarrow 48}/D$ values between the 2.0-g dose and the 0.5- and 1.0-g doses, and the similar dose-dependency of the value of k_{el} and $t_{1/2(el)}$, prompted the analysis of the 2.0-g data in terms of Eq. 2. The results obtained using this equation are compared with those using Eq. 1 in Table III. No significant differences were observed in the values of k_a, t_0, or FD/V_d calculated by the two methods. The coefficient of determination r^2 was also similar, indicating that neither equation described the overall data significantly better than the other. However the ratio V_m/K_m, which is analogous to k_{el} at low drug concentrations, of 0.20 yielded a drug half-life in plasma of 3.8 hr. This value is less than one-half the value calculated using Eq.1, and is not statistically different from the values obtained from the two lower doses.

Although the saturable elimination model did not provide a better fit to the 2.0-g data compared to the first-order elimination model, it did provide a superior fit to the elimination phase of each drug profile. This is clearly demonstrated for two subjects in Fig. 2. The mean coefficient of determination between observed and predicted probenecid levels for all subjects during the 12–48-hr sampling period was 0.973 when Eq. 2 was used for nonlinear regression analysis. This value was significantly higher than the value of 0.953 obtained when the data were fitted by means of Eq. 1.

To determine whether Eq. 2 also described the probenecid data resulting from the 0.5- and 1.0-g doses, nonlinear regressions were carried out on these data using k_a, t_0, and FD/V_d values obtained from the analysis using Eq. 1, and V_m and K_m values obtained from the 2.0-g dose data for each individual. The mean coefficients obtained by this method were 0.96 and 0.97 for the 0.5- and 1.0-g doses, respectively. These values were not significantly different from those obtained when these data were fitted to Eq. 1.

DISCUSSION

The data generated in this study provide evidence that the elimination of probenecid from plasma is saturable at therapeutic dose levels. Following the 0.5- and 1.0-g oral doses of probenecid, plasma curves could be described adequately by a kinetic model incorporating first-order absorption and elimination. Although a satisfactory description of probenecid plasma levels following the 2.0-g dose was obtained also with this model, the probenecid elimination half-life obtained was twice that following the lower doses. When saturable elimination of probenecid from the 2.0-g dose was assumed, however, the terminal elimination half-life of probenecid was similar for all three dosages. Also a more satisfactory description of the elimination phase of probenecid plasma levels following the 2.0-g dose was obtained by incorporating a saturable component in the kinetic model.

These observations are consistent with previous suggestions that the elimination of probenecid is dose-dependent (3, 8), the drug half-life increasing with increasing dose size. In this respect probenecid behaves similarly to salicylate (16, 17), phenytoin (18), theophylline (19, 20), and alcohol (21); all of which exhibit saturable elimination kinetics at drug levels below those considered to be toxic.

An alternative explanation for the prolonged probenecid profiles in plasma following the 2.0-g dose, and to a smaller extent following the 1.0-g dose (Fig. 1), involves drug absorption rather than elimination. This argument, which was used previously in this context (6), is based on the low aqueous solubility of probenecid, and proposes that slow dissolution of the drug in the GI tract from larger doses may give rise to prolonged absorption. While the data obtained in the present study do not preclude this possibility, the similar absorption rate constants, absorption lag times, and times that maximum drug concentrations occur in plasma from the three dosages make it unlikely. Also, dose-dependent elimination of probenecid was previously demonstrated in humans following intravenous doses (3). Although the value of F/V_d from the 2.0-g dose was statistically smaller than that from the 1.0-g dose (Table II), it cannot be determined from the present data whether this is due to a change in drug absorption or in drug distribution at the higher dose.

The plasma levels at which Michaelis–Menten-type elimination became evident in this study, 40–50 μg/ml, are in excellent agreement with the observation (3) that probenecid levels measured in the 50–220-μg/ml range declined at slower rates than levels in the 10–55-μg/ml range. The higher levels in that study were obtained following a 2.0-g iv dose, while the lower levels were obtained following a 0.5-g dose. Unfortunately, the plasma sampling in that study was not extended for a sufficient time following the 2.0-g dose to monitor plasma levels in the lower concentration range.

The mechanism causing slower elimination of probenecid at high circulating levels is uncertain. Altered renal clearance is unlikely. Probenecid is actively secreted into the kidney tubules, but it is efficiently reabsorbed into the peritubular capillaries, and only a small fraction of the drug is cleared by this route. Probenecid is extensively metabolized, the principal metabolites involving side-chain oxidation (~70%) and glucuronide conjugation (~20%) (1). The dose-dependency of the amounts of these metabolites formed in two individuals has been investigated (6), and relative urinary recoveries of unchanged drug, probenecid acyl glucuronide, and the mono-*N*-propyl, carboxylic acid, and secondary alcohol metabolites from 0.5-, 1.0-, and 2.0-g doses were obtained. The investigators concluded that no individual metabolic pathway was saturated at high probenecid levels as this would have altered the relative amounts of the different metabolites that were formed. However, some of the side-chain oxidation metabolites of probenecid may be formed through a single intermediate, *i.e.*, an epoxide. Saturation of this common step may lead to a reduced rate of metabolism without affecting the ratio of formation of some of the major metabolites (22). One also cannot exclude product inhibition by one or more of the metabolites formed or saturation of a transport step governing access of probenecid to the site(s) of metabolism, causing clearance of drug from plasma to be delayed. More studies are needed to identify the rate-limiting process(es), and also the possible influence of other drugs on the saturable elimination of probenecid.

While attempting to describe plasma probenecid data in terms of Eq. 2, the authors are aware of the problems inherent in applying this type of equation to drug–concentration profiles, and also to the uncertainty regarding the numerical values assigned to the parameters V_m and K_m (23). However, notwithstanding these limitations, use of the saturable model is felt to be justified in terms of the similarity of the pharmacokinetic parameters which were common to the saturable and first-order models from the different doses, the realistic values of V_m and K_m obtained, and also the close agreement between the drug elimination half-lives obtained from the V_m/K_m ratios and from k_{el}.

The clinical significance of saturable probenecid elimination is uncertain. In view of the profound effect that saturable elimination may have on the degree of drug accumulation with repeated doses (24), it is likely that probenecid levels may increase with repeated dosing to an extent greater than might be predicted if first-order elimination is assumed. The clinical implication of saturable probenecid elimination may be further complicated, as the uricosuric potency of the oxidized metabolites has been shown to be approximately the same as the parent drug in experimental animals (25, 26).

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Purity Profiles of Pteroylglutamate Reference Substances by High-Performance Liquid Chromatography

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Abstract □ High-performance liquid chromatography (HPLC) in the reversed-phase mode was used for the purity analysis of three pteroylglutamic acid-type reference substances (folic acid, leucovorin calcium, and methotrexate). The influence of the pH of the mobile phase on the separation of an artificial mixture of six pteroylglutamic acid derivatives and three potential impurities was studied. Results of purity analysis of current lots of USP reference standards are reported. A better separation of methotrexate from its major impurities was achieved by using a standard buffer, rather than an ion-pairing mobile phase. A separation of methotrexate and its biologically inactive 7-isomer is reported.

Keyphrases □ Pteroylglutamate—purity profiles, reference substances, high-performance liquid chromatography, methotrexate □ High-performance liquid chromatography—pteroylglutamate, purity profiles, reference substances, methotrexate □ Methotrexate—pteroylglutamate, purity profiles, reference substances, high-performance liquid chromatography

Three USP drug substances, available as USP reference standards, belong to the class of pteroylglutamic acid derivatives: folic acid (I), methotrexate (II), and leucovorin calcium (III) (authentic substance as calcium formyltetrahydrofolate). This class of compounds exhibits high hydrolytic and oxidative reactivity. High-performance liquid chromatography (HPLC) has proved to be the method of choice for purity analysis in this laboratory because of similarities of structure within this series. Additional purity profile data were gained from 5 to 8 collateral determinations, but are not reported here as these results are independent of chromatographic purity.

Almost all the HPLC techniques have been used for the separation and analysis of this class of compounds. Anion-exchange columns were used first in 1973 for the separation of folic acid from a mixture of water-soluble vitamins (1) and then for the separation of folic acid and its reduced and N^5- and N^{10}-substituted derivatives (2). Anion-exchange chromatography alone, or coupled with an amine column operated in the reversed-phase mode, has also been used for the analysis and quantitative determination of methotrexate (3).

An extensive study (4) resulted in the development of a reversed-phase HPLC assay and purity analysis method for folic acid. The assay was made the object of a collaborative study (5), and it has been adopted into the folic acid monograph in the USP (6).

For the reversed-phase chromatographic analysis of methotrexate, mixtures of methanol or acetonitrile with the following buffers have been suggested as mobile phases: pH 3.5, 0.005 *M* ammonium formate (7); pH 5, 0.005 *M* ammonium acetate (7); pH 6.7, 0.1 *M* KH_2PO_4 (8); pH 6.8, 2-amino-2-hydroxymethyl-1,3-propanediol (tris) (9); pH 7.2, 0.05 *M* KH_2PO_4[1]; pH 6, 0.1 *M* citric acid–0.2 *M* Na_2HPO_4[2]. The latter is used in the assay of methotrexate in USP XX–NF XV (10). Mobile phases consisting of pH 5 phosphate buffer–acetonitrile (9) and pH 4 citrate buffer–dioxane (11) were recommended for the analysis and assay of leucovorin calcium. The effect of pH on the retention behavior of pteroyloligo-glutamates in reversed-phase HPLC has also been reported (12).

A third technique, ion-pair chromatography, separated 21 UV-absorbing impurities in a clinical sample of methotrexate (13) and has also been utilized in the separation of folic acid and its dihydro- and tetrahydro derivatives (14).

The object of this investigation was to study the influence of the pH of the mobile phase on the chromatographic separation of some pteryolglutamic acid derivatives and to determine optimum conditions for the purity analysis of the USP reference standards by HPLC.

EXPERIMENTAL

Apparatus—An isocratic high-performance liquid chromatograph[3] with a 254-nm detector and a gradient chromatograph[4] equipped with a variable wavelength UV detector[5] were used. The instruments were

[1] United States Pharmacopeia, Drug Research and Testing Laboratory, unpublished research.
[2] Lederle Laboratories, private communication.
[3] Altex Model 110, Altex Scientific Inc., Berkeley, Calif.
[4] Model 3500B, SpectraPhysics, Santa Clara, Calif.
[5] Model 770 Spectrophotometric detector, SpectraPhysics, Santa Clara, Calif.

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