that this thiophene derivative might be transformed from the piperazinedione product.

To clarify the possible formation of II from IV*a* or V*a*, the product mixture IV*a* and V*a* was heated in an alkaline phosphate solution. Several secondary degradation products were detectable on TLC, but none had the same R_f value as II. The results suggest that product II does not form *via* IV*a* and V*a* but is directly formed from cefadroxil *via* a different pathway.

Product III was difficult to isolate in amounts sufficient to characterize it; its formation was confirmed in neutral and basic degradation solutions of cefadroxil.

A possible route to account for the observed formation of II, III, IV*a*, and V*a* is shown in Scheme IV.

Temperature Dependency—Rate constants for the overall disappearance of cefadroxil were obtained from 35 to 60° at pH 7.00 and 11.50. The pH of the reaction solution was maintained constant by the use of a pH-stat. The Arrhenius plots are shown in Fig. 9. From these data, the apparent activation energies at pH 7.00 and 11.50 was determined to be 21.4 and 23.8 kcal/mole, respectively.

In aqueous solution at pH 7.00 and 35°, cefadroxil degraded *via* intramolecularly catalyzed reaction (k_b reaction) to an extent of 83% and *via* a water-catalyzed β-lactam opening (k_0 reaction) to an extent of 17%.

At pH 11.50, the hydroxide-ion catalyzed degradation proceeded exclusively, the contribution of the k_b reaction being only 0.1%. The apparent activation energy of 23.8 kcal/mole at this alkaline pH may include the heat of ionization of water, 13.1 kcal/mole (13). The net activation enthalpy, $\Delta H^{\neq}$, of hydroxide-ion-catalyzed degradation of cefadroxil was calculated to be 10.5 kcal/mole at 35°.

Comparative Stability Among Aminocephalosporins—Table II lists various rate constants defined by Eq. 10 for the degradation of cephaloglycin, cephalexin, and cephradine determined previously under the same kinetic conditions. It is apparent that the stability pattern of cefadroxil resembles that of cephalexin and cephradine over all pH regions.

A comparison of the specific rate constants of k_0, k_b, and k_{OH} for the four aminocephalosporins shows that the β-lactam moiety of cephaloglycin, which has an acetoxymethyl group in the C-3 position, is about four to five times more susceptible to attack of both water and hydroxide ion and eight to 18 times more reactive to intramolecular attack by the C-7 side-chain amino group than that of cephalosporins possessing a methyl group at the C-3 position, *i.e.*, cefadroxil, cephalexin, and cephradine. The difference in reactivity between cephaloglycin and the other three antibiotics may be ascribed to the difference in the inductive ability of their C-3 substituents and/or leavability of the C-3 moiety, as suggested previously for cephalosporin degradations (1, 3, 15–18).

REFERENCES

(1) T. Yamana and A. Tsuji, *J. Pharm. Sci.*, **65**, 1563 (1976).

(2) T. Yamana, A. Tsuji, K. Kanayama, and O. Nakano, *J. Antibiot.*, **27**, 1000 (1974).

(3) H. Bundgaard, *Arch. Pharm. Chemi. Sci. Ed.*, **4**, 25 (1976).

(4) E. S. Rattie, D. E. Guttman, and L. J. Ravin, *Arzneim.-Forsch.*, **28**, 944 (1978).

(5) A. I. Cohen, P. T. Funke, and M. S. Puar, *J. Pharm. Sci.*, **62**, 1559 (1973).

(6) H. Bundgaard, *Arch. Pharm. Chemi Sci. Ed.*, **5**, 149 (1977).

(7) A. Dinner, *J. Med. Chem.*, **20**, 963 (1977).

(8) R. E. Buck and K. E. Price, *Antimicrob. Agents Chemother.*, **11**, 324 (1977).

(9) K. Satake, T. Okuyama, M. Ohashi, and T. Shinoda, *J. Biochem. (Tokyo)*, **47**, 654 (1960).

(10) A. Albert and E. P. Serjeant, "The Determination of Ionization Constants," Chapman and Hall, London, England, 1971.

(11) M. A. Schwartz, A. P. Granatek, and F. H. Buckwalter, *J. Pharm. Sci.*, **51**, 523 (1962).

(12) T. Yamana, A. Tsuji, E. Kiya, and E. Miyamoto, *ibid.*, **66**, 861 (1977).

(13) H. S. Harned and W. J. Hamer, *J. Am. Chem. Soc.*, **55**, 2194 (1933).

(14) E. A. Guggenheim, *Phil. Mag.*, **2**, 538 (1926).

(15) R. B. Hermann, *J. Antibiot.*, **26**, 223 (1973).

(16) J. M. Indelicato, T. T. Norvilas, R. R. Pfeiffer, W. J. Wheeler, and W. L. Wilham, *J. Med. Chem.*, **17**, 523 (1974).

(17) D. B. Boyd, R. B. Hermann, D. E. Presti, and M. M. Marsh, *ibid.*, **18**, 408 (1975).

(18) H. Bundgaard, *Arch. Pharm. Chemi. Sci. Ed.*, **3**, 94 (1975).

Alterations in Integrity of Goldfish Membrane Induced by Edetate Disodium

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Abstract □ The effect of the chelating agent edetate disodium on the integrity of the goldfish membrane was examined. The time to produce death in goldfish exposed to secobarbital sodium was used as a reflection of membrane integrity. Although a minimum edetate disodium concentration was necessary to induce alterations in integrity, no direct relationship between the effect and concentration of the chelating agent was evident. The chelating agent's effect appeared to be an enhancement of the transport of the ionized drug form. The change in membrane integrity existed at least 24 hr after theoretical exposure to edetate disodium, but cyclic alterations in integrity could not be ruled out. The effect on integrity was also demonstrated to be nonpermanent, and the apparent loss in integrity was partially restored by calcium but not by magnesium.

Keyphrases □ Permeability—effect of edetate disodium on goldfish membrane □ Edetate disodium—effect on membrane permeability, goldfish □ Chelating agents—edetate disodium, effect on membrane permeability, goldfish

Edetic acid is a chelating agent capable of changing transfer rates of certain substances across membrane barriers (1–4). It was reported (1) that the sodium salt of edetic acid was capable of enhancing the absorption of heparin and synthetic heparinoids from the GI tract of rats and dogs and that these results were consistent with another investigation (2) in the monkey, dog, cat, rabbit, and human. Feldman and Gibaldi (3) demonstrated that edetic acid was capable of affecting the transfer rate of salicylate, but not salicylamide, across the everted rat intestine. They concluded that edetic acid was capable of altering the barrier for water-soluble, but not lipid-soluble, compounds. It was also demonstrated (4) that the sodium salt of edetic acid was capable of increasing phenolsulfonphthalein absorption in rats.

Although edetic acid is capable of changing the permeability of membranes to various compounds, the exact nature of the mechanism involved is unknown. Regardless of the mechanism, numerous studies have been performed to see if the effects of the chelating agent can be reversed. Windsor and Cronheim (1) found that the magnesium and

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Table I—Effect of Recovery Time on Time of Death of Goldfish Exposed to Edetate Disodium and 0.1 m*M* Secobarbital Sodium Solution

Recovery	Treatment			Control			Significance
	Mean Time	*SD*	*n*[a]	Mean Time	*SD*	*n*	
Zero	14.86	4.26	10	26.93	5.20	8	$p < 0.05$
30 min	15.19	2.78	9	18.96	4.60	9	NS[b]
24 hr	19.17	2.32	8	24.63	4.09	8	$p < 0.05$
48 hr	21.80	6.19	10	22.07	4.73	8	NS
72 hr	37.60	4.83	9	40.23	5.55	9	NS

[a] *n* = number of fish. [b] Not significant ($p > 0.05$).

Table II—Effect of Concentration of Edetate Disodium on Time of Death of Goldfish in 0.1 m*M* Secobarbital Sodium Solution

Concentration of Edetate Disodium, mg/ml	Treatment			Control			Significance
	Mean Time of Death, min	*SD*	*n*[a]	Mean Time of Death min	*SD*	*n*	
0.05	26.29	4.36	10	25.72	4.10	9	NS[b]
0.10	26.29	3.97	10	32.73	7.49	10	$p < 0.05$
0.15	19.51	2.84	10	26.32	2.62	10	$p < 0.05$
0.20	23.03	1.79	10	32.27	8.07	10	$p < 0.05$
0.25	15.19	1.10	7	19.80	4.76	8	$p < 0.05$
0.30	17.29	2.11	7	20.15	2.49	7	$p < 0.05$
0.40	18.25	4.61	10	22.62	4.61	10	$p < 0.05$

[a] *n* = number of fish. [b] Not significant ($p > 0.05$).

calcium salts of edetic acid were incapable of increasing absorption, and Tidball (4) reported that rinsing with magnesium salts fully restored normal permeability. It was also reported (3) that neither calcium nor magnesium was capable of restoring normal permeability of the rat intestinal membrane.

A previous report (5) presented preliminary findings of the effect of edetate disodium on the goldfish membrane. It was considered of interest to examine the effects of edetic acid on the goldfish since they have been used in a number of studies (6–9) for the assessment of absorption and toxicity of numerous compounds. Goldfish offer an advantage over other experimental animals in that they can be exposed to chelating agents for relatively long periods of time. It also is reasonably easy to assess the effects on the membrane at different times after exposure. This report is a followup of our preliminary findings.

EXPERIMENTAL

Recovery Time Studies—Goldfish (*Carassius auratus*), 1–3 g, were placed in groups of five in tanks containing 2 liters of edetate disodium[1] (0.2 mg/ml). The solutions were made with glass-distilled[2] water and were adjusted[3] to pH 7.4 using 0.1 *N* NaOH or HCl. The fish were left in the solutions for 24 hr and then were placed for varying periods in recovery tanks containing 2 liters of glass-distilled water adjusted to pH 7.4. Individual fish were then placed into beakers containing 200 ml of 0.1 m*M* secobarbital sodium[4] in a 0.05 *M* phosphate buffer, pH 7.4. Cessation of gill and mouth movements was noted, and the time to death was computed.

Controls were run for each group following the procedure for the edetate disodium-treated fish, except that the chelating agent was omitted from the treatment tank.

All investigations were performed at (20 ± 2°).

Concentration Studies—Five fish (1–3 g) were placed in tanks containing 2 liters of edetate disodium (varying concentrations) in glass-distilled water adjusted to pH 7.4 using 0.1 *N* NaOH or HCl. The fish were left in the tanks for 24 hr and then placed for 24 hr in a recovery tank containing 2 liters of glass-distilled water adjusted to pH 7.4. Individual fish were then placed in beakers containing 200 ml of 0.1 m*M* secobarbital sodium in 0.05 *M* phosphate buffer, pH 7.4. Cessation of gill and mouth movements was noted, and the time to death was computed.

Controls were run in the same manner as described previously. All experiments were performed at 20 ± 2°.

pH Study—Five fish (1–3 g) were placed in tanks containing 2 liters of 0.2 mg of edetate disodium/ml (adjusted to pH 7.4) in glass-distilled water for 24 hr. Individual fish were then immediately placed (zero recovery) in beakers containing 200 ml of 0.1 m*M* secobarbital sodium solution buffered to pH 6.4, 7.4, or 8.4 with 0.05 *M* phosphate buffer. Again, time to death was calculated.

Controls were run in the same manner as already described and all experiments were performed at 20 ± 2°.

Calcium and Magnesium Study—Five fish (1–3 g) were placed in tanks containing 2 liters of 0.2 mg of edetate disodium/ml (adjusted to pH 7.4) in glass-distilled water for 24 hr. Fish were then placed in recovery tanks for 24 hr. The recovery tanks contained either 2 liters of glass-distilled water or 2 liters of a 1 m*M* solution of calcium chloride or magnesium chloride. All tanks were adjusted to pH 7.4. After 24 hr, individual fish were placed in beakers containing 200 ml of a 0.1 m*M* secobarbital sodium solution buffered to pH 7.4 with 0.05 *M* phosphate buffer. Time to death was computed.

Controls were run by placing fish in glass-distilled water for 48 hr (water was changed at the end of 24 hr) and then placing individual fish in secobarbital solution as described.

All experiments were performed at 20 ± 2°.

RESULTS AND DISCUSSION

The results of the recovery time study are summarized in Table I. As is evident from the control data, there appears to be a large amount of day-to-day variation in response. This is evident from the control segment of the table, since the controls are essentially the same procedure, one should expect similar results. However, the mean time of deaths, ranged from 18.96 to 40.23 min.

Comparisons between the treated and control fish (which were performed at the same time) were made within each block of the table using a Student *t* test. A significant difference ($p < 0.05$) existed between the treated and control fish for the 0- and 24-hr recovery studies. There was no significant difference for the 30-min and 48- and 72-hr recovery studies. Although the reason for the lack of a significant difference at the 30-min recovery study is unknown, it is consistent with previous results (5). If it is assumed that the amount of secobarbital necessary to produce death is the same between treated and control fish, then the significant difference for the 24-hr recovery study implies that alterations in membrane permeability induced by edetate disodium may exist for a substantial time after theoretical exposure of the membrane to the chelating agent, at least in the described system. The effect of the chelating agent on membrane permeability is not permanent, however, as evident from the 48- and 72-hr recovery treatments.

[1] Fisher Scientific Co., Fair Lawn, N.J.
[2] Corning Model AG-1B.
[3] Heath Model EU-302A.
[4] Robinson Laboratory, San Francisco, Calif.

Table III—Difference between the Mean of Each Control and Individual Observations of Each Treatment

Observation	Edetate Disodium Concentration, mg/ml					
	0.10	0.15	0.20	0.25	0.30	0.40
1	6.44	10.49	7.69	5.49	4.85	6.44
2	−0.29	9.75	9.55	3.35	0.40	−3.18
3	10.38	9.57	10.35	4.02	6.27	4.82
4	9.51	7.47	11.10	3.85	1.59	6.14
5	11.00	7.04	7.32	4.48	1.97	5.00
6	3.68	5.22	11.97	6.62	3.57	5.42
7	1.88	1.67	8.47	4.43	1.34	5.42
8	4.85	3.10	6.70	—	—	4.09
9	10.58	6.80	8.34	—	—	3.49
10	5.11	6.97	10.87	—	—	6.10
Mean	6.31	6.81	9.24	4.61	2.86	4.27
SD	3.95	2.85	1.79	1.11	2.12	2.81

Table IV—Effect of Calcium and Magnesium on Enhanced Permability Induced by Edetate Disodium

Group	Treatment[a] A			Treatment[b] B		
	Mean Time of Death	*n*[c]	*SD*	Mean Time of Death	*n*	*SD*
I	29.23	10	4.43	29.95	10	5.87
II	19.70	9	4.63	19.15	10	1.45
III	19.63	10	3.76	23.24	10	4.06

[a] Group III contained a 1 m*M* magnesium chloride solution. [b] Group III contained a 1 m*M* calcium chloride solution. [c] *n* = number of fish.

The results of the concentration of edetate disodium on time of death study are listed in Table II. A significant difference ($p < 0.05$) existed between each treatment and control, except for the lowest concentration of the chelating agent. To determine the effect of chelating agent concentration on time of death, the effect of day-to-day variation should be eliminated since the studies were performed on different days. This correction was made for the data in which the treatments were significantly different from the controls, *i.e.*, all but the lowest concentration of edetate disodium, by subtracting each individual observation of the treatments from the mean of each control performed at the same time as the treatment (Table III).

An analysis of variance for unequal data sets was performed on the differences for each concentration, and the result indicated a significant difference ($p < 0.05$) between the means. The means were analyzed using the Newman–Keuls method with a harmonic mean of the sample size to compensate for unequal data sets. These results indicated a difference between the treatment concentrations of 0.10, 0.15, and 0.20 mg/ml with the 0.30-mg/ml treatment. There was also a difference between the 0.2-mg/ml concentration and the 0.25- and 0.40-mg/ml treatments. A plot of the mean differences *versus* concentration of treatment (Fig. 1) shows that despite significant differences between the treatments, no direct relationship between concentration of edetate disodium and effect was apparent.

Experiments were also performed to determine if the effect of edetate disodium on membrane permeability could be reversed by exposing the fish to calcium or magnesium chloride solutions. In each experiment, the times of death of three groups of fish in secobarbital sodium were determined. The first group was the control, the second group was treated with edetate disodium and placed in a recovery tank for 24 hr, and the third group was exposed to the chelating agent and then placed in a recovery tank containing calcium or magnesium chloride for 24 hr before being placed in the secobarbital sodium solution. All groups were studied at the same time, and the results are summarized in Table IV.

An analysis of variance was performed, and the results indicated a significant difference between the means for both studies. Analysis of the means using the Newman–Keuls method indicated that, in the magnesium chloride study, the control was significantly different from the edetate disodium group and the edetate disodium/magnesium chloride group. No difference existed between the edetate disodium and the edetate disodium/magnesium chloride group. Thus, it appears that magnesium is incapable of restoring membrane integrity under these experimental conditions. In the calcium chloride study, a significant difference ($p < 0.05$) existed between all three treatments. Thus, it appears that calcium is able to restore, at least partially, the apparent loss of membrane integrity induced by edetate disodium.

In previous experiments (5) fish also were treated with edetate disodium and then exposed to secobarbital sodium buffered to varying pH values. While the treated fish seemed to fit the model proposed by Levy and Gucinski (10) for the absorption of secobarbital sodium, the controls did not. It was decided to repeat the pH study using a 24-hr treatment, zero recovery. The results of both studies are listed in Table V. Comparisons were made within each block using a Student *t* test, and there was no significant difference between treatments and control at pH 6.4 regardless of the treatment. In the 24-hr treatment, 30-min recovery, there was no significant difference at any pH. In the 24-hr treatment, zero recovery and the 24-hr treatment, 24-hr recovery, there were significant differences at pH 7.4 and 8.4.

Since secobarbital is a weak acid with a pKa of 7.9, it appears that the chelating agent changes membrane permeability to the ionized drug form since there was a significant difference between treated and control animals only at pH values (7.4 and 8.4) where the drug appreciably existed as the ionized form. The apparent increase in membrane permeability to the ionized drug form is not enough to compensate for the reduction in the absorption of the unionized form since the times to produce death increased with increasing buffer pH. Linear regression was performed on the reciprocal of the mean time of death *versus* the fraction of drug unionized for the 24-hr treatment zero recovery study and its controls.

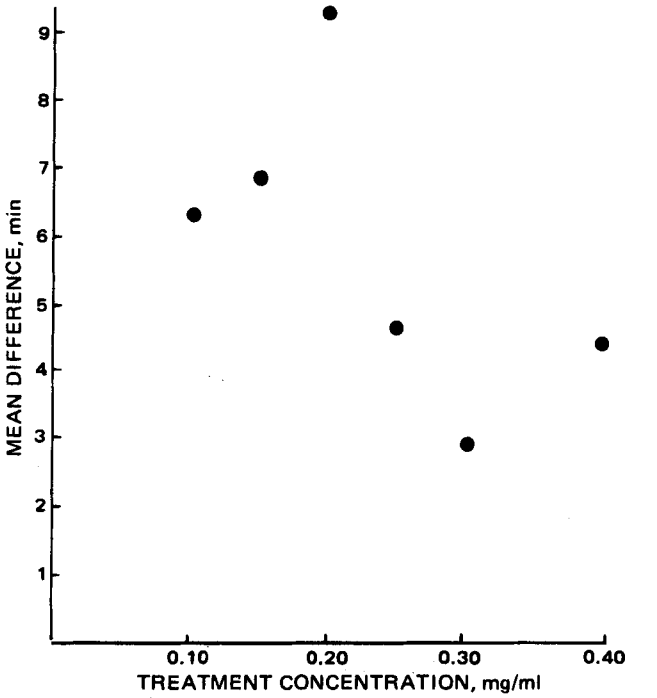

Figure 1—*Plot of the mean differences* versus *concentration of the chelating agent treatments.*

Table V—Effect of pH and Recovery Time on Time of Death of Goldfish Treated with Edetate Disodium and Exposed to 0.1 m*M* Secobarbital Sodium

	pH 6.4			pH 7.4			pH 8.4		
	Mean Time ± *SD*, min	*n*[a]	Significance	Mean Time ± *SD*, min	*n*	Significance	Mean Time ± *SD*, min	*n*	Significance
Treatment[b]	18.58 ± 3.54	5	NS[c]	18.74 ± 0.98	7	$p < 0.05$	39.11 ± 5.93	3	$p < 0.05$
Control[d]	19.76 ± 1.95	5		21.88 ± 2.87	10		51.77 ± 5.05	4	
Treatment[e]	14.88 ± 0.99	10	NS	20.21 ± 3.74	10	NS	52.29 ± 9.70	10	NS
Control[f]	13.49 ± 2.51	10		23.89 ± 5.80	10		62.30 ± 16.93	10	
Treatment[g]	18.73 ± 5.92	10	NS	23.03 ± 1.79	10	$p < 0.05$	58.83 ± 17.31	10	$p < 0.05$
Control[h]	15.80 ± 3.51	5		32.27 ± 8.07	10		97.84 ± 22.19	10	

[a] *n* = number of fish. [b] 24-hr treatment, zero recovery. [c] Not significant ($p > 0.05$). [d] 24-hr blank, zero recovery. [e] 24-hr treatment, 30-min recovery. [f] 24-hr blank, 30-min recovery. [g] 24-hr treatment, 24-hr recovery. [h] 24-hr blank, 24-hr recovery.

The predicted least-squares equations are, respectively:

$$1/t = 0.0413f_u + 0.0170 \quad (r = 0.9660) \quad \text{(Eq. 1}a\text{)}$$

and:

$$1/t = 0.0443f_u + 0.0093 \quad (r = 0.9906) \quad \text{(Eq. 1}b\text{)}$$

According to the Levy–Gucinski model (10) concerning the absorption of the ionized and unionized forms of drug in goldfish, a plot of reciprocal time of response ($1/t$) as a function of the fraction of drug unionized should be linear with a slope equal to $K_b(K_u - K_i)$ and an intercept equal to K_bK_i.

$$1/t = K_bK_i + K_b(K_u - K_i)f_u \quad \text{(Eq. 2)}$$

where K_b is a constant equal to the quotient of the secobarbital concentration to which the fish were exposed divided by the amount of barbiturate in the fish at death; K_i and K_u are the first-order rate constants for the ionized and unionized drug forms, respectively. Upon inspection of the data, two discrepancies appear. The controls fit the model reasonably well while the treated animals do so to a lesser degree. In both cases, however, curvature of a form representing an inverted exponential cannot be ruled out. The intercepts of both plots are also not significantly different ($p > 0.05$) from zero. Whether these discrepancies represent a breakdown of the model or assumptions of the model as specified by Levy and Gucinski or simply a large amount of statistical variation cannot be discerned at this time. However, due to these discrepancies, the analysis as performed earlier (5), using the ratio of slope to intercept to evaluate the ratios of K_u:K_i was not performed. Regardless of the model that characterizes the absorption of secobarbital sodium under these experimental conditions, it appears from Table V that edetate disodium changes membrane permeability to the ionized form of the drug and that the effect can be present 24 hr after theoretical exposure to the chelating agent.

REFERENCES

(1) E. Windsor and G. E. Cronheim, *Nature*, **190**, 263 (1961).

(2) M. A. Seidell, E. Windsor, and A. Surtshin, *Clin. Res.*, **8**, 246 (1960).

(3) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **58**, 967 (1969).

(4) C. S. Tidball, *Am. J. Physiol.*, **206**, 243 (1964).

(5) P. J. Cascella and P. D. Kindelspire, *J. Pharm. Sci.*, **69**, 972 (1980).

(6) G. Levy and J. A. Anello, *ibid.*, **57**, 101 (1968).

(7) G. Levy, K. E. Miller, and R. H. Reuning, *ibid.*, **55**, 394 (1966).

(8) C. H. Nightingale, M. Tse, and E. I. Stupak, *ibid.*, **61**, 1948 (1972).

(9) S. Feldman, M. DeFrancisco, and P. J. Cascella, *ibid.*, **64**, 1713 (1975).

(10) G. Levy and S. P. Gucinski, *J. Pharmacol. Exp. Ther.*, **146**, 80 (1964).

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Effect of Various Vehicles and Vehicle Volumes on Oral Absorption of Triamterene in Rats

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Abstract □ The oral bioavailability of triamterene in rats was investigated after its administration as a suspension in lipid and aqueous vehicles in addition to a lactic acid solution. Triamterene is poorly soluble in both aqueous and lipid vehicles. A 1-ml oral dose volume showed that lipid vehicles may provide some enhancement of oral availability compared with aqueous suspensions. However, when the vehicle volume was reduced to a realistic dosage form volume for the rat, peanut oil and aqueous suspensions were indistinguishable from each other with respect to peak height, peak time, and overall bioavailability. For a given vehicle small vehicle volumes resulted in a better relative oral availability than did large vehicle volumes.

Keyphrases □ Triamterene—poor solubility in lipid and aqueous vehicles □ Vehicle volume—small *versus* large vehicle volumes in oral availability in rats □ Oral bioavailability—parameters of triamterene administered as suspensions in lipid and aqueous vehicles and lactic acid solution

The rate and extent of absorption of orally administered drugs are influenced by factors within the GI environment, such as the solubility and extent of drug ionization, gastric emptying, intestinal motility, and stimulation of bile flow. The relative contribution of each factor to oral availability is often difficult to distinguish. The vehicle into which a