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Stabilization of the dipropionate ester of DHPG, 9-[(1,3-dihydroxy-2-propoxy)-methyl]guanine against enzymatic hydrolysis using complexation

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Summary

The diester of 9-[(1,3-dihydroxy-2-propoxy)-methyl]guanine (DHPG) undergoes enzymatic hydrolysis in the presence of rat and monkey intestinal homogenate forming the more polar compound DHPG which has diminished membrane permeability. The effects of various complexing agents on the enzymatic hydrolysis of the diester in the presence of rat and monkey intestinal homogenate were evaluated. Benzyl alcohol and salicylic acid derivatives caused a 2.8–7.2-fold enhancement in the stability of the diester. Solubility studies in the presence of these ligands indicated formation of 1:1 and 1:2 complexes with improved solubilities. Inhibition kinetic experiments were conducted using purified porcine liver esterase. The inhibition type, and the values of the kinetic parameters for benzyl alcohol and gentisic acid were determined from Lineweaver-Burk plots. These plots indicated competitive and mixed (combination of competitive and non competitive) inhibition for gentisic acid and benzyl alcohol, respectively. The competitive inhibition has been explained as complex formation between the inhibitor and the substrate. Thus co-administration of the diester and the complexing agent would protect the diester against enzymatic degradation during its residence time in GI tract.

Introduction

Many medicinal agents such as penicillins, peptides and nucleoside derivatives undergo a certain degree of chemical or enzymatic degradation in the gastrointestinal (GI) tract, which may result in partial or complete loss of biological activity. Water-soluble and polar compounds are often derivatized with bioreversible lipophilic groups in order to improve their transport across the intestinal membrane (Hoeksema et al., 1961; Glayton et al., 1976). The majority of these derivatives are ester type. Once in the general circulation these derivatives undergo chemical or enzymatic conversion to the biologically active parent molecule. However, during their transit through the GI tract the derivatives are exposed to the hostile environment of enzymes and chemical catalysts. A rapid conversion to the polar parent drug would result in diminished permeability. Therefore, increasing the stability of the derivative or labile drug substances towards enzymatic degradation in the GI tract would allow more of the intact molecule, with superior permeability or activity, to be absorbed.

Various metabolic inhibitors have been used to stabilize insulin against enzymatic degradation in

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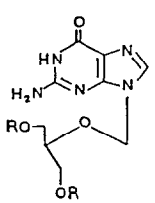
the GI tract. These include the inhibitor diisopropylfluorophosphate (Danforth and Moore, 1959) and specific proteolytic enzyme inhibitors, chymostatin and FK-448 (Fujii et al., 1985). Hori et al. (1983) found that certain peptides inhibited insulin degradation in percutaneous tissue after injection.

Alternative approaches are to protect the drug substance by entrapment within a bilayer or by complexation with a suitable ligand. The liposome entrapment has been used for the protection of insulin during oral absorption (Weingarten et al., 1981) and for nitrocefin and cephaloridine against in vitro hydrolysis by β-lactamase (Pourkavoos et al., 1984). However, the use of complexation to protect the drug against enzymatic degradation has not been explored in drug delivery.

The present study was undertaken to evaluate the feasibility of complexation as a means for stabilization of a susceptible molecule against enzymatic degradation. The diproprionate ester (I) of an antiviral agent DHPG (II) was selected as the model compound. From studies described previously (Benjamin et al., 1986) it was known that the diester I undergoes enzymatic hydrolysis to mono ester and DHPG in the presence of intestinal and liver homogenates.



II R=H DHPG

I $R=CH_3CH_2CO$ DIPROPIONYL DHPG

Experimental

Materials

The dipropionate ester of DHPG (Martin et al., 1986) was obtained from the Institute of Organic Chemistry (Syntex Research). The partially purified porcine liver esterase (1500 units/ml in 3.2 M ammonium sulfate buffer), gentisic acid ethanolamide, 5-methoxysalicylic acid, sodium glycocholate, gentisic acid and cyclodextrins were purchased from Sigma Chemical Co. (St. Louis, MO). Other compounds or reagents were obtained from J.T. Baker Chemical Co. (Phillipsberg, NJ).

Analytical methodology

A previously described (Benjamin et al., 1985) cation exchange reverse-phase dual-column HPLC method capable of simultaneous quantitation of Compound I and its hydrolysis products was used for all analyses.

Solubility studies

Excess Compound I was suspended in 2 ml of aqueous solution containing various amounts of the ligands in 2 ml glass vials. The pH of the solution was adjusted to 5.5 with sodium hydroxide or hydrochloric acid. The vials were stoppered with rubber closures and sealed with aluminum caps. The vials were rotated in a water bath at 25°C for 70 h. At the end of the equilibrium time, the suspension was filtered using syringe filter fitted with a 2 μm polyvic filter membrane (Millipore Corp., Bedford, MA). An aliquot of the filtrate was diluted with the mobile phase and injected onto the HPLC for quantitation.

The solubility data obtained were curve-fitted according to the method of Higuchi and Kristiansen (1970) to obtain the formation constants.

Intestinal homogenate stability studies

Preparation of rat and monkey intestinal homogenate and the incubation studies were carried out as previously described (Benjamin et al., 1986) except that the various additives were added to the incubation mixtures as aqueous solutions in buffered saline (0.5 M phosphate, 0.15 M sodium chloride pH 7.4). The rat intestinal homogenate incubations contained 4 ml of methanolic solution of Compound I (120 μg/ml), with 1, 3 or 5 ml of 10% w/v homogenate stock solution (to get 0.55%, 1.7% or 2.7% w/v, respectively, homogenate incubations). The volume was made up to 24 ml with the buffer containing the appropriate additive. The monkey intestinal homogenate incubations contained 4 ml of methanolic solution of Compound I (80 μg/ml), 4 ml of 10% w/v homogenate and 16 ml of buffered saline containing the

additive. The final homogenate concentration was 1.7% w/v.

Kinetics of inhibition using purified esterase

An enzyme stock solution was prepared according to literature references (Kawaguchi et al., 1985). An aliquot of the porcine liver esterase suspension was diluted to 35 units/ml in 0.025 M potassium phosphate pH 7.4 buffer. The solution was then filtered through a 0.8 μm membrane filter (polycarbonate Nuclepore, Pleasanton, CA) and stored at 4°C before use. The stock solution was used within 48 h. Solutions of Compound I in 0.025 M potassium phosphate pH 7.4 buffer were prepared to obtain 1.0, 1.3, 2.0, 2.5 and 5.0 mM drug solutions. Ten ml aliquots of drug solutions with and without inhibitor were equilibrated at 37°C in a water bath. The reaction was initiated by addition of 100 μl of the enzyme stock solution into the drug solution using a glass syringe. The solutions were mixed by agitation and immediately returned to the water bath. The reaction mixture was sampled at 0.5, 1, 1.5, 2, 3 and 4 min intervals. At each time point 375–625 μl aliquots of the reaction mixture were pipetted out using a micropipetter (Gilson Pipetman, Renin, Emmeryville, CA) and diluted with ice-cold mobile phase (methanol/0.001 M ammonium phosphate, pH 2.5, 30:70) to a final concentration of 0.05 mM. The samples were quantified using HPLC. For the inhibition kinetics experiments the final inhibitor concentration was 5 mM. The concentration of Compound I was plotted against time and the initial velocities were obtained by the slope of the least-squares fitted lines.

Results and Discussion

Stability in the intestinal homogenate

It has been shown previously (Benjamin et al., 1986) that the diester I undergoes enzymatic hydrolysis in the presence of intestinal homogenates. The additives which were initially screened for the stabilization of I against enzymatic hydrolysis are listed in Table 1. Except trichlorfon, (2,2,2-trichloro-1-hydroxyethyl)phosphonic acid dimethyl ester), these ligands are known to form complexes with certain compounds. Trichlorfon is a known cholinesterase inhibitor, which was included as a positive control. Each additive was initially screened at the two intestinal homogenate concentrations of 0.55% w/v (rat) and 1.7% w/v (monkey). The additives which appeared to produce the greatest stabilization of I at these homogenate concentrations were then challenged at a higher rat intestinal homogenate concentration of 2.7% w/v.

TABLE 1

Additives used in stability screening studies in the presence of intestinal homogenates

Additive	Conc. in incubation mixture	
	Rhesus monkey	Rat
PVP (K90)	5%	–
α-Cyclodextrin	5%	–
β-Cyclodextrin	1%	–
γ-Cyclodextrin	1%	–
Benzyl alcohol	2%	–
PEG 6000	5%	–
PEG 400	–	1%
Disodium EDTA	0.5%	–
Gentisic acid	5%	1%
Gentisic acid ethanolamide	1%	1%
Dextran T70	5%	–
Trichlorfon	1%	–
Dimethylacetamide	–	1%
5-Methoxysalicylic acid	–	1%
Sodium glycocholate	–	1%
Sol ketal	–	1%
Propylene glycol	–	1%

Table 2 and Figs. 1 and 2 show the effect of those additives which provided stabilization against enzymatic hydrolysis in rat and monkey intestinal homogenates compared to the reaction without additive (control). These results indicate that the rate of enzymatic hydrolysis is reduced significantly in the presence of benzyl alcohol, gentisic acid, gentisic acid ethanolamide and 5-methoxysalicylic acid compared with the control. At the highest rat intestinal homogenate concentration of 2.7% w/v, the increase in stabilization of Compound I over that of control is 7.2-fold for benzyl alcohol, 2.8-fold for gentisic acid and 5.8-fold for gentisic acid ethanolamide. The quan-

TABLE 2

The effect of complexing agents on the stability of dipropionyl DHPG in 2.7% w/v rat intestinal homogenate at 37°C

Additive	Conc. in incubation mixture	Rate constant (min^{-1})	$t_{1/2}$ (min)
Control	–	3.25×10^{-2}	21
Sol ketal	1%	1.91×10^{-2}	36
Gentisic acid	1%	1.12×10^{-2}	62
Gentisic acid ethanolamide	1%	5.46×10^{-3}	127
Benzyl alcohol	1%	4.44×10^{-3}	156
Methoxysalicylic acid	1%	a	

[a] Essentially no reaction

titation of I in the presence of 5-methoxy-salicylic acid was complicated by the coelution of both compounds. However, the rate of appearance of the degradation products peaks was very slow, indicating essentially no reaction. Other additives included in Table 1 do not show any significant stabilizing effect.

The additives that have been identified as providing the greatest stabilizing effect on I, benzyl alcohol, gentisic acid, gentisic acid ethanolamide and methoxy-salicylic acid, have similar structural

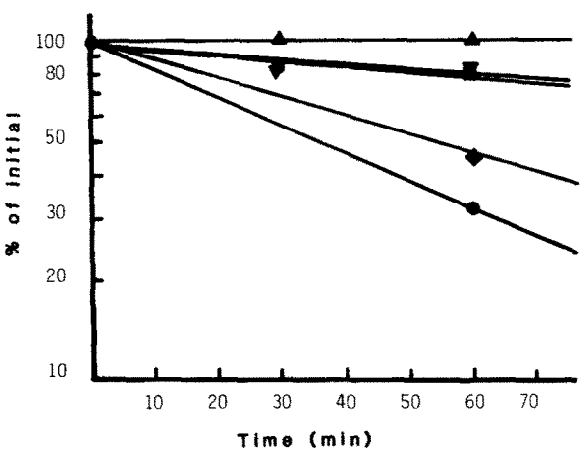


Fig. 1. First-order plots for the hydrolysis of dipropionyl DHPG (I) in 1.7% w/v rat intestinal homogenate at 37°C in the absence (●) and presence of benzyl alcohol (▲), gentisic acid ethanolamide (▼), trichlorfon (■) and PVP (◆).

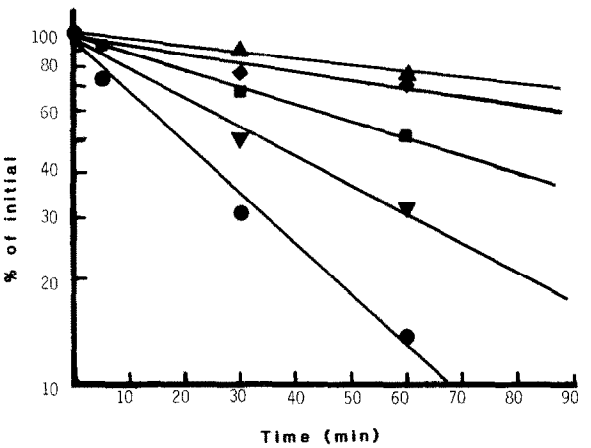


Fig. 2. First-order plots for the hydrolysis of dipropionyl DHPG (I) in 2.7% w/v rat intestinal homogenate at 37°C in the absence (●) and presence of benzyl alcohol (▲), gentisic acid ethanolamide (◆), gentisic acid (■) and sol ketal (▼).

features. They each contain an aryl ring with one or more hydroxy groups attached directly or α to the ring.

Solubility studies (Fig. 3) indicate that these additives increase the solubility of I by complex

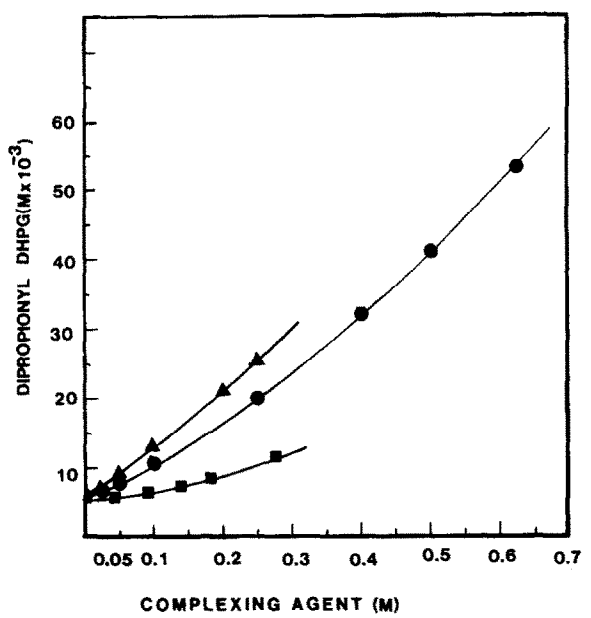


Fig. 3. Increase in solubility of dipropionyl DHPG (I) produced by addition of gentisic acid (●), gentisic acid ethanolamide (▲) and benzyl alcohol (■) in aqueous solution at pH 5.5 and 25°C.

formation. The non-linearity of the plot of solubility of I versus ligand concentration indicate formation of 1 : 1 and 1 : 2 complexes. The data in Fig. 3 were curve-fitted according to Higuchi and Kristiansen (1970) to obtain the formation constants $K_{1:1}$ and $K_{1:2}$ (Table 3). The solid lines in Fig. 3 are the theoretical lines and fit the experimental data very well.

Salicylic acid derivatives have been known to form similar molecular complexes with chartreusin (Poochikian and Cradock, 1979), acronine (Repta and Hincal, 1980), benzodiazepine (Badwan et al., 1983) and caffeine (Reuning and Levy, 1968). Although nature of such interactions has not been established, it is suggested that combination of electrostatic force of the donor–acceptor type (for gentisic acid) and hydrophobic forces play an important role in the stabilization of these complexes (Badwan et al., 1983; Poochikian and Cradock, 1979).

Mechanism of stabilization

Based on the solubility studies and the stability data in the presence of intestinal homogenate it is concluded that those ligands which stabilize Compound I against enzymatic hydrolysis form complexes with it. This suggests that the formation of a complex between the substrate (I) and the inhibitor (ligand) is an important step in the inhibition of the enzyme. To elucidate the nature of the inhibition, enzyme kinetic experiments were conducted using purified porcine liver esterase. These experiments were initiated with rat intestinal homogenate. However, measurements of the initial velocities requires use of very dilute homogenate suspension and it was not possible to keep various factors, effecting the esterase activity, constant during different experiments resulting in large scatter in the data. Use of purified esterase enzyme proved to be very convenient and the data was very reproducible. The initial velocities for various substrate concentrations, with and without inhibitors are listed in Table 4. The relationship of substrate concentration and initial velocity is non-linear and follows Michaelis-Menten kinetics. The double reciprocal plots of the data according to Lineweaver-Burk method (1934) are shown in Fig. 4. The solid lines are the best least-square fitted lines. The shapes of the lines indicate (Webb, 1963) that inhibition by gentisic acid is essentially a competitive inhibition (increase in K_m, decrease in affinity for the enzyme) and that by benzyl alcohol is a mixed inhibition (increase in K_m and decrease in V_m), a combination of competitive and non-competitive inhibition.

TABLE 3

Association constants for formation of complexes between dipropionate ester of DHPG (I) and various ligands in aqueous solution at pH 5.5 and 25°C

Determined by the solubility method.

Ligand	$K_{1:1}$ (M^{-1})	$K_{1:2}$ (M^{-1})
Benzyl alcohol	3.49	1.37
Gentisic acid	6.29	2.47
Gentisic acid ethanolamide	9.98	2.51

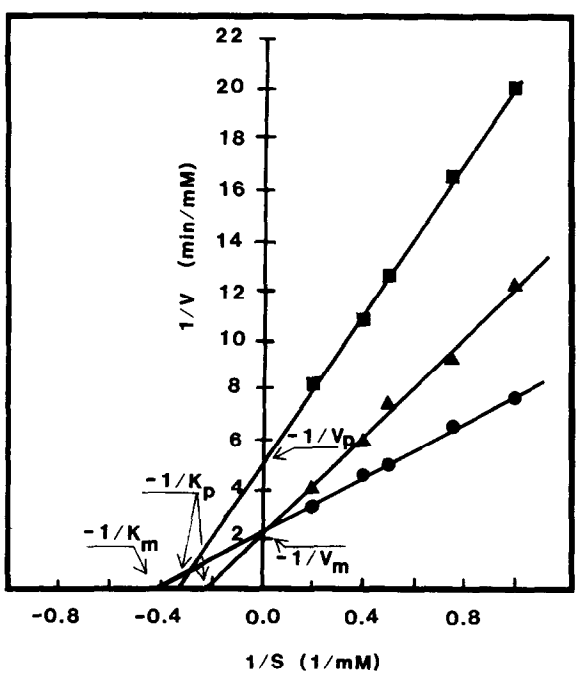


Fig. 4. Lineweaver-Burk plots for the inhibition of porcine liver esterase hydrolysis of dipropionyl DHPG in 0.025 M phosphate buffer pH 7.4 at 37°C: (●) no inhibitor, (▲) gentisic acid 5 mM, (■) benzyl alcohol 5 mM. Each point is an average of 3–4 experiments.

TABLE 4

The effect of gentisic acid and benzyl alcohol (inhibitors) on the kinetics of the hydrolysis of dipropionyl DHPG (substrate) by porcine liver esterase in 0.025 M potassium phosphate buffer pH 7.4 at 37°C

Inhibitor	Substrate conc. (mM)	Initial velocities [a] (mM, min^{-1})	Inhibitor type [b]	Kinetic parameters [c]
None	5.0	0.301 ± 0.015		$K_m = 2.32$ mM
	2.5	0.216 ± 0.010		$V_m = 0.427$ mM · min^{-1}
	2.0	0.199 ± 0.019		
	1.3	0.151 ± 0.012		
	1.0	0.131 ± 0.006		
Benzyl alcohol (5 mM)	5.0	0.122 ± 0.017	Mixed inhibition	$K_p = 2.94$ mM
	2.5	0.093 ± 0.009		
	2.0	0.080 ± 0.003		$V_p = 0.198$ mM · min^{-1}
	1.3	0.061 ± 0.004		$K_i = 2.84$ mM
	1.0	0.050 ± 0.006		
Gentisic acid (5 mM)	5.0	0.241 ± 0.025	Competitive inhibition	$K_p = 4.56$ mM
	2.5	0.166 ± 0.008		
	2.0	0.135 ± 0.005		$V_p = 0.462$ mM · min^{-1}
	1.3	0.109 ± 0.008		$K_i = 7.72$ mM
	1.0	0.082 ± 0.005		

[a] Average ± S.D. of 3–4 experiments.
[b] From the pattern of Lineweaver-Burk plots.
[c] Calculated from the Lineweaver-Burk plots.

K_m = Michaelis constant; V_m = maximum rate of hydrolysis in the absence of inhibitor; V_p = maximum rate of hydrolysis in the presence of inhibitor; K_p = effective Michaelis constant in the presence of inhibitor; and K_i = inhibition constant.

The kinetic parameters were calculated from these plots and are included in Table 4. The Michaelis and apparent Michaelis constants K_m and K_p are obtained from the X-intercept, while the maximum velocities V_m and V_p are obtained from the Y-intercept. The inhibition constants K_i are calculated from the ratio of the slopes of the lines with and without inhibitor (Webb, 1963).

Generally, the competition between the substrate and the inhibitor for the enzyme results in competitive inhibition. However, it has been suggested (Cavanaugh et al., 1955; Segal, 1975; Sluyterman and Wijdenes, 1973; Webb, 1963) that complexation between substrate and inhibitor yields plots that superficially resemble competitive inhibition. In this case, the inhibition is due either to competition of enzyme and substrate for the inhibitor (Eqns. 1–3) or competition between the substrate–inhibitor complex and the substrate for the enzyme (Eqns. 1, 2 and 4).

$$E + S \rightleftharpoons ES \rightarrow E + P \quad (1)$$

$$S + I \rightleftharpoons SI \quad (2)$$

$$E + I \rightleftharpoons EI \quad (3)$$

$$E + SI \rightleftharpoons ESI \quad (4)$$

We have not established which of the above mechanisms is applicable here. In either case, complex formation between the substrate and inhibitor is the primary reason for the inhibition by gentisic acid. The value of formation constant (Table 3) for the complexation of gentisic acid and compound I indicates strong interaction between the two compounds and supports complexation as a probable explanation for the inhibition by gentisic acid. Although salicylates have been reported

(Smith and Dawkins, 1970) to competitively inhibit the activities of certain enzymes, these do not include esterases. Therefore it is unlikely that part of inhibition in the present study is due to classical competitive inhibition.

The mixed inhibition by benzyl alcohol described here is a combination of competitive and non-competitive inhibition. The complexation between benzyl alcohol and the diester could be responsible for the competitive inhibition. In non-competitive inhibition, the substrate and inhibitor bind to the enzyme, at different sites, independent of each other. However, the resulting ESI complex is inactive. The binding of benzyl alcohol to any other site on esterase could induce changes in the catalytic site and result in non-competitive inhibition. Benzyl alcohol is reported to inhibit the activity of monoamine oxidase (Fowler and Callingham, 1980; McEwen et al., 1968) and esterase activity of carboxypeptidase A (Davies et al., 1968) enzymes.

The complexation and kinetic data suggest that the mixed inhibition by benzyl alcohol is predominantly non-competitive. The value of formation constant (Table 3) is small, indicating weak interaction between benzyl alcohol and the diester I. On the other hand, the small value of K_i (2.84 mM) means that benzyl alcohol has strong affinity for the enzyme. The affinity of the diester for the enzyme is also strong ($K_m = 2.32$ mM). Thus, binding of both benzyl alcohol and diester to different sites on the enzyme and formation of small amount of complex results in predominantly non-competitive, mixed inhibition.

Conclusion

The work presented here illustrates that complexation can be effectively utilized to enhance the stability of appropriate drug substances against enzymatic hydrolysis. This could be a useful tool in optimization of oral delivery of labile drug molecules or their lipophilic prodrugs. The salicylic acid derivatives are of particular interest since they appear to act predominantly by complexation with the substrate without direct interaction with the enzyme. The optimum dosage form should be designed to deliver the complex directly in small intestine avoiding its dissolution and extensive dilution by the stomach contents. The dilution in stomach contents would tend to dissociate the complex and diminish protection against enzymes. An Enteric coated dosage form, capsule or tablet could be the most effective means of delivering high local concentrations of drug and complexing agents in the small intestine, where the dilution is moderate. The transport properties of the complexed drug, such as dissolution rate and permeability would also be important in overall absorption of the drug from the GI tract. The complexes described in the present study are expected to show faster dissolution rates due to higher solubility. Salicylic acid derivatives are especially useful because of their ability to enhance the intestinal absorption of antibiotics and other polar compounds (Nishihata et al., 1982; Suzuka et al., 1985). The combined effect of rapid dissolution rate, improved stability in the GI tract and enhanced permeability could result in a marked enhancement in oral absorption of the diester. Studies are in progress to formulate and test this delivery system in in vivo absorption studies.

References

Badwan, A., El-Khardagui, L.K., Saleh, A.M. and Khalil, S.A., The solubility of benzodiazepines in sodium salicylate solution and a proposed mechanism for hydrotropic solubilization. *Int. J. Pharm.*, 13 (1983) 67–74.

Benjamin, E.J., Firestone, B.A. and Schneider, J.A., A dual-column HPLC method for the simultaneous determination of DHPG (9-[(1,3-dihydroxy-2-propoxy)methyl]guanine) and its mono and diesters in biological samples. *J. Chromatogr. Sci.*, 23 (1985) 168–170.

Benjamin, E.J., Firestone, B.A., Bergstrom, R., Lin, Y.T., Fass, M., Massey, I. and Tsina, I., Selection of a derivative of antiviral agent DHPG, 9-[(1,3-dihydroxy-2-propoxy)-methyl]guanine, with improved oral absorption. In-vitro screening studies. *Pharm. Res.*, submitted.

Cavanaugh, D.J., Harris, J. and Hearon, J.Z., Enzyme inhibition by complexing of substrates: inhibition of tyrosinase by titanium compounds. *J. Am. Chem. Soc.*, 77 (1955) 1531–1535.

Clayton, J.P., Cole, M., Elson, W., Ferres, H., Hanson, J.C., Mizen, L.W. and Sutherland, R., Preparation, hydrolysis, and oral absorption of lactonyl ester of penicillins. *J. Med. Chem.*, 19 (1976) 1385–1391.

Danforth, E., Jr. and Moore, R.O., Intestinal absorption of insulin in the rats. *Endocrinology*, 65 (1959) 118–123.

Davies, R.C., Auld, D.S. and Vallee, B.L., The effect of modifiers on the hydrolysis of esters and peptides by carboxypeptidase A. *Biochem. Biophys. Res. Commun.*, 31 (1968) 628–633.

Fowler, C.J. and Callingham, B.A., The effect of lipophilic compounds upon the activity of rat liver mitochondrial monoamine oxidase-A and -B. *Biochem. Pharmacol.*, 29 (1980) 1177–1183.

Fujii, S., Yokoyama, T., Ikegaya, K., Sato, F. and Yokoo, N., Promoting effect of the new chymotrypsin inhibitor FK-448 on the intestinal absorption of insulin in rats and dogs. *J. Pharm. Pharmacol.*, 37 (1985) 545–549.

Higuchi, T. and Kristiansen, H., Binding specificity between small organic solutes in aqueous solution. Classification of some solutes into two groups according to binding tendencies. *J. Pharm. Sci.*, 59 (1970) 1601–1608.

Hoeksema, H., Whitfield, G.B. and Rhuland, L.E., Effect of selective acylation on the oral absorption of a nucleoside by humans. *Biochem. Biophys. Res. Commun.*, 6 (1961) 213–216.

Hori, R., Komada, F. and Okumura, K., Pharmaceutical approach to subcutaneous dosage forms of insulin. *J. Pharm. Sci.*, 72 (1983) 435–440.

Kawaguchi, T., Suzuki, Y., Nakahara, Y., Nambu, N. and Nagai, T., Activity of esterase in the hydrolysis of 3,5-diesters of 5-flouro-2-deoxyuridine in relation to the structure of the diester prodrugs. *Chem. Pharm. Bull.*, 33 (1985) 301–307.

Lineweaver, H., Burk, D., The determination of enzyme dissociation constants. *J. Am. Chem. Soc.*, 56 (1934) 658–666.

Martin, J., Tippie, M., McGee, D., Verheyden, J., Synthesis and antiviral activity of various esters of DHPG (9-((1,3-dihydroxy-2-propoxy)methyl) guanine). *J. Pharm. Sci.*, (1986) submitted.

McEwen, C.M., Jr., Sasaki, G. and Lenz, W.R., Jr., Human liver mitochondrial monoamine oxidase. I. Kinetic studies of model interactions. *J. Biol. Chem.*, 243 (1968) 5217–5225.

Nishihata, T., Takahagi, H. and Higuchi, T., Enhanced small intestinal absorption of cefmetazole and cefoxilin in rats in the presence of non-surfactant adjuvants. *J. Pharm. Pharmacol.*, 35 (1982) 124–125.

Poochikian, G.K. and Cradock, J.C., Enhanced Chartreusin solubility by hydroxybenzoate hydrotropy. *J. Pharm. Sci.*, 68 (1979) 728–732.

Pourkavoos, N., Hodges, N.A., Olliff, C.J., Newhall, C. and Sharratt, P., The protection of β-lactam antibiotics from enzymic inactivation by entrapment within liposomes. In Aiache J.M. and Hintz, J.L. (Ed.), *Biopharm. Pharmacokinet. Eur. Congr. 2nd, Vol. 1*, 1984, pp. 547–554.

Repta, A.J. and Hincal, A.A., Complexation and solubilization of acronine with alkylgentisates. *Int. J. Pharm.*, 5 (1980) 149–155.

Reuning, R.H. and Levy, G., Effect of complex formation on drug absorption VII. Effect of complexation and self association. *J. Pharm. Sci.*, 57 (1968) 1335–1346.

Segel, I.H., *Enzyme Kinetics*, New York, 1975, 206 pp.

Sluyterman, L.A.A.E. and Wijdenes, J., An unusual type of enzyme inhibition. *Biochim. Biophys. Acta*, 321 (1973) 697–699.

Smith, M.J.H. and Dawkins, P.D., Review: Salicylates and enzymes. *J. Pharm. Pharmacol.*, 23 (1971) 729–744.

Suzuka, T., Nishihata, T., Yamazaki, M. and Kamada, A., Effect of salicylate and disodium ethylene-diaminetetraacetic acid on the rat intestinal absorption of cefmetazole. *Chem. Pharm. Bull.*, 33 (1985) 4600–4605.

Webb, J.L., *Enzyme and Metabolic Inhibitors, Vol. I*, Academic Press, New York, 1963, 149 pp.

Weingarten, C., Moufti, A., Desjeux, J.F., Luong, T.T., Durand, G. Devissagnet, J.P. and Puiseux, F., Oral ingestion of insulin liposomes: effects of the administration route. *Life Sciences*, 28 (1981) 2747–2752.