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Absorption enhancement of growth hormone from the gastrointestinal tract of rats

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Summary

An increased interest in non-injectable dosage forms for polypeptides has led to identification of some compounds which can increase absorption of these drugs. Sodium salicylate administered in rectal suppositories with recombinant methionyl human growth hormone (met-hGH) lead to measurable serum concentrations and demonstratable biological activity of the polypeptide. Sodium salicylate and mineral oil were tested in the ligated stomach, duodenum, ileum and colon of rats for their ability to enhance absorption of met-hGH. Rats were injected in one of the segments of the gastrointestinal tract with 3 mg/kg of met-hGH with or without sodium salicylate in aqueous buffer or mineral oil vehicle. Blood samples were collected at various time points and assayed for hGH. Absolute bioavailabilities calculated for each formulation in each intestinal segment indicated that the only treatment with greater than 3% bioavailability was injection of sodium salicylate in mineral oil into the ileum (7%) and colon (9.5%). Statistical analysis proved the surprising interaction of salicylate and mineral oil to be synergistic in absorption enhancement. This is a promising finding but several issues must be addressed and pharmaceutical formulations must be optimized before it can be developed into an oral dosage form for polypeptide drugs.

Introduction

The development of recombinant DNA technology has made it possible to produce large amounts of polypeptides for use as therapeutic agents. The use of drugs such as insulin, interferon, and growth hormone will greatly increase as a result of their availability and stimulate research into new clinical indications.

As new indications are found for these drugs and the markets for current indications are developed, the need for convenient, effective dosage forms will increase. Currently polypeptide drugs are administered by injection. Injections are painful and sometimes difficult to administer relative to other dosage forms. Patient compliance is an important consideration since some of these drugs may require frequent administration or administration to juvenile or geriatric patients. For example, methionyl-human growth hormone (met-hGH) produced by recombinant DNA methods (Goeddel et al., 1979) is administered by intramuscular injection 3 times a week to young children suffering from hypopituitary dwarfism. It is a large polypeptide with a molcular weight of approxi-

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mately 22 500 Da. The exact mechanism of action of growth hormone is not fully elucidated but there is evidence that it acts to release somatomedins which produce somatic growth (Phillips and Vassilopoulou-Sellin, 1980a and b).

An oral dosage form would be far superior to injections in terms of patient acceptance. However, several factors contribute to make met-hGH and polypeptide drugs in general appear to be unlikely candidates for oral delivery. Low gastric pH, presence of proteolytic enzymes in the intestine, and poor mucosal permeability to large molecules are all potential barriers to absorption following oral administration of polypeptides.

Until recently the limited supply and extremely high cost of most polypeptides made production of a dosage form with less than 90–100% bioavailability unacceptable. In fact, sufficient quantities of active ingredients did not exist to support pharmaceutical research and development programs. The advent of recombinant DNA technology is easing some of these restrictions and making it possible to address some important issues in polypeptide drug delivery.

While attempts to administer polypeptides orally date from the discovery of insulin (Banting and Best, 1922), recent efforts have focused on the use of permeation enhancers. This line of research has lead to the discovery that a variety of substances can enhance the absorption of normally non-absorbed molecules from the gastrointestinal tract. Salicylates (Nishihata et al., 1982), enamine derivatives (Kim et al., 1983), bile salts (Ziv et al., 1981), and lipid–bile salt mixed micelles (Muranishi et al., 1977), have been shown to increase absorption of insulin and heparin from the GI tract or rectum.

The current study examines the ability of some of these substances to enhance absorption of met-hGH administered in rectal suppositories in rats. The best enhancer is then chosen to study growth-promoting ability of the hormone and absorption from higher levels of the gastrointestinal tract.

Materials and Methods

Absorption enhancement of met-hGH was studied by measuring serum concentrations following administration of rectal suppositories containing met-hGH and an enhancer in a cocoa butter base. Enhancers tested were sodium salicylate (Sigma), acetyl salicylate (Sigma), and an enamine derivative of phenylalanine and ethyl acetoacetate. The enamine was synthesized by the method of Dane and Docker (1965). Methionyl human growth hormone was produced by recombinant DNA methods and lyophilized in mannitol phosphate excipient. Suppositories were prepared by adding 2 g of cocoa butter to a vial of lyophilized powder containing 10 mg of recombinant met-hGH and 8, 20, or 80 mg of enhancer. The mixture was warmed and mixed constantly while aliquots were pipetted into a chilled cone-shaped mold. The cocoa butter solidified before particles could settle. The resulting suppositories weighed approximately 120 mg and contained 600 μg met-hGH and 480 μg, 1.2 mg or 4.8 mg of enhancer. The suppositories were refrigerated and used on the following day. Animals used in this study were male CD rats weighing approximately 300 g from Charles River Breeding Laboratory (Wilmington, MA). The rats were fasted for 12–18 h. Anesthesia was induced with an intramuscular injection of xylazine (Rompun) at a dose of 3 mg/kg followed 20 min later by an intramuscular injection of a combination of ketamine hydrochloride (Vetalar, 40 mg/kg) and acepromazine maleate (Acepromazine, 0.75 mg/kg). The ketamine hydrochloride (20 mg/kg) and xylazine (1.5 mg/kg) combination was administered as needed to maintain anesthesia for the duration of the experiment. The rats were kept on a heated pad at 37°C while the experiment was in progress. Three rats were used to test each of the three enhancers at each of the three concentrations. Blood samples were collected by cardiac puncture 5, 10, 30, 45, 60, and 90 min after administration of a single suppository.

Serum concentrations of methionyl human growth hormone were determined using a commercial immunoradiometric assay (Tandem-R-HGH, Hybritech, San Diego, CA). The assay uses

two monoclonal antibodies directed against different antigenic sites on the hGH molecule. One is coated on a plastic bead and the other is labeled with ^{125}I. Serum samples containing hGH are mixed with both antibodies and excess labeled antibody is washed away. Radioactivity measured in a gamma counter is proportional to serum hGH concentration. Sample concentrations are computed by comparison to the linear regression of counts per minute versus growth hormone concentrations for a set of standards.

The assay is highly specific for human growth hormone with negligible cross-reactivity from rat growth hormone or other pituitary hormones. In this laboratory the assay has proven to be a good predictor of biological activity of methionyl human growth hormone.

The growth promoting activity of met-hGH administered in rectal suppositories was tested using a bioassay described by Wilhelmi (1966). Hypophysectomized female CD rats were obtained from Charles River Breeding Laboratory. Body weights were measured every 2 or 3 days for 10 days prior to treatment. Animals which gained more than 6 g during this pretreatment period were assumed to have residual pituitary tissue and were excluded from the study. The animals with stable weights ranged from 96 to 116 g and were assigned to treatment or control groups so that there was no difference in body weights between the two groups at the commencement of treatment. Once daily for 10 days each animal was given two rectal suppositories each containing approximately 170 μg met-hGH and 7 mg sodium salicylate or mannitol phosphate excipient and sodium salicylate. Body weights were recorded daily and on the day following the tenth treatment. Total weight gains in the two groups were compared by a t-test.

Absorption of met-hGH from higher levels of the GI tract was studied in an animal model similar to the one described previously. Male CD rats weighing approximately 300 g were anesthetized and a midline incision was made to expose the proper portion of the GI tract. The formulations (0.2 ml) were injected with a 21-gauge, one inch needle into the cardiac portion of the stomach, the duodenum 5 mm distal to the pylorus, the ileum 5 mm proximal to the cecum, or the colon 5 mm distal to the cecum. Sutures were tied above and below the deposit of drug in the intestine to prevent migration of the injected formulation. In order to prevent leakage the lower suture was placed between the point where the needle entered the intestine and the site where the drug was deposited by the needle. Care was taken not to ligate blood vessels. The abdominal incision was closed with wound clips. Blood samples were collected from the lateral aspect of the ophthalmic venous plexus at 5, 10, 20, 30, 45, 60, 90, and 180 min after administration of the drug. Animals were killed by lethal injection following collection of the final sample.

The following four formulations were tested for absorption from the gastrointestinal tract:

met-hGH (5 mg/ml) in aqueous buffer
met-hGH (5 mg/ml) + sodium salicylate (40 mg/ml) in aqueous buffer
met-hGH (5 mg/ml) in mineral oil
met-hGH (5 mg/ml) + sodium salicylate (40 mg/ml) in mineral oil

Aqueous formulations were prepared by reconstitution with water for injection and addition of sodium salicylate. These preparations were gently mixed and all components completely dissolved. The non-aqueous formulations were prepared by adding mineral oil (Sigma) to the lyophilized powder and sodium salicylate and mixing by drawing up and expelling from a glass pipet. Mineral oil was chosen for this study because it is a commonly used material which is compatible with biological tissue as well as other components of pharmaceutical dosage forms. Fig. 1 shows photographs of the suspensions obtained. Particles of met-hGH and excipients appear as rods up to 25 μm long and flakes up to 15 μm in diameter. Sodium salicylate particles appear more spherical and about 5–15 μm in diameter. The non-spherical nature of the growth hormone and excipient particles renders traditional particle sizing and counting procedures inappropriate.

Six animals were used for each formulation at each injection site. An additional group of 6 animals was injected intravenously in the tail vein with 0.2 ml growth hormone (0.25 mg/ml) in aqueous mannitol phosphate buffer. Blood sam-

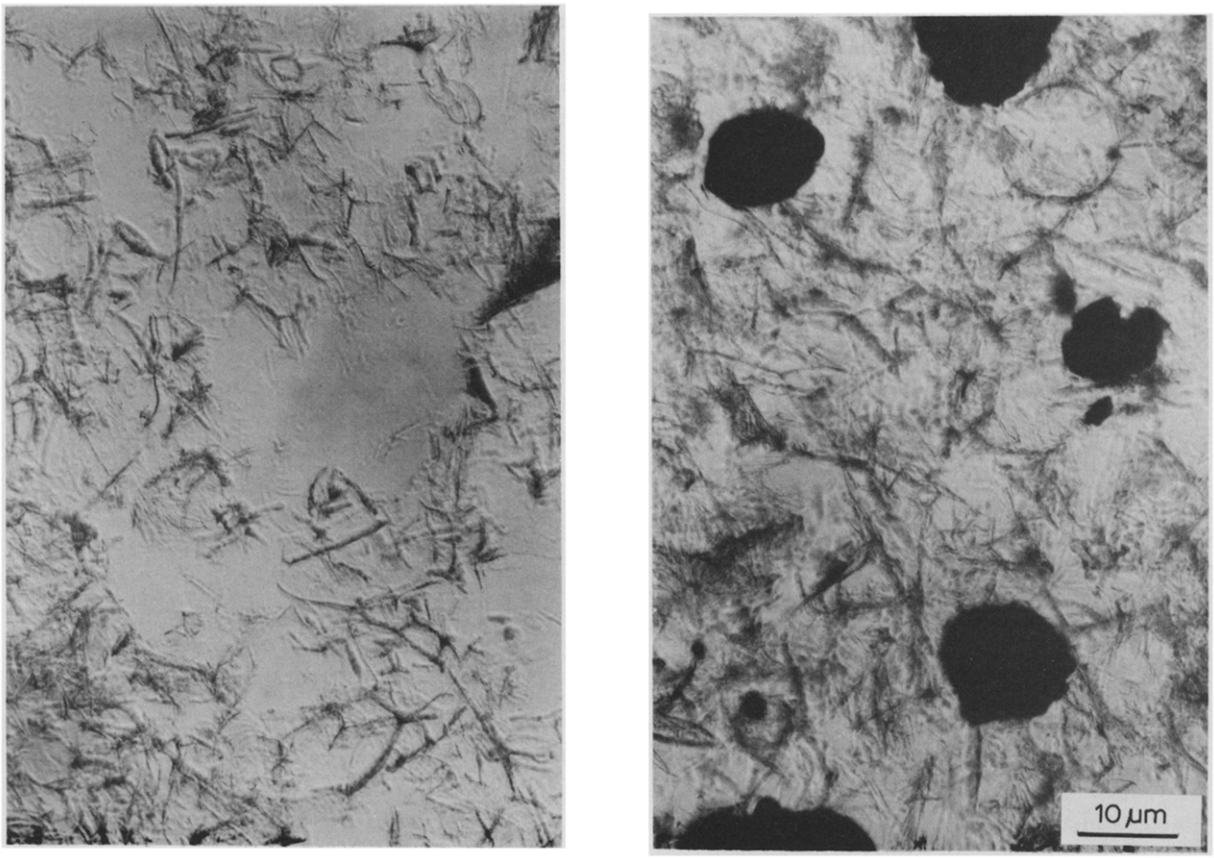

Fig. 1. Photomicrographs of suspensions of met-hGH in mineral oil with (right) and without (left) sodium salicylate.

ples were collected at 2.5 min after injection as well as the same time points as above.

The area under the curve for the serum concentration vs time plots was calculated using the trapezoid method (Notari, 1980). Bioavailability was computed by comparing the area under the curve for each treatment group to that for the intravenously injected rats (Gibaldi and Perrier, 1982).

Results

Sodium salicylate appeared to be the best enhancer of met-hGH absorption in rectal suppositories. Fig. 2 shows mean serum concentrations versus time for the 3 enhancers at the 3 concentrations tested. The enamine derivative and sodium salicylate both produce higher serum met-hGH concentrations at higher enhancer concentrations. Sodium salicylate is a better enhancer at all 3 concentrations. Acetyl salicylate did not enhance absorption of met-hGH even at the highest concentration tested.

Significant growth was observed when sodium salicylate was used as a met-hGH enhancer in hypophysectomized rats. Fig. 3 shows daily changes in body weight in animals tested with rectal suppositories containing met-hGH and sodium salicylate or containing sodium salicylate and excipient. The weight gain of the met-hGH group through 10 days was significantly greater than controls ($t = 4.26$, df = 17, $P < 0.001$).

Surprisingly high serum concentrations of met-hGH were measured following administration with sodium salicylate in a mineral oil vehicle in the

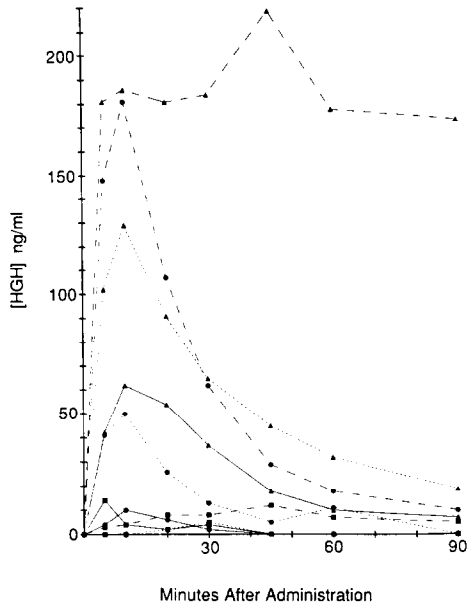

Fig. 2. Serum concentrations of met-hGH following administration with sodium salicylate (triangles), an enamine derivative (circles) or acetyl salicylate (squares) at concentrations of 4 (solid lines), 10 (dotted lines), or 40 mg/ml (dashed lines). $n = 3$ for each group.

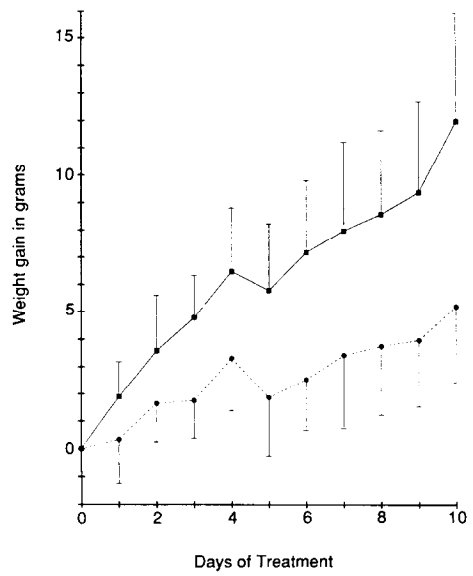

Fig. 3. Weight gain of hypophysectomized rats treated with rectal suppositories containing met-hGH and sodium salicylate (squares) ($n = 10$) and controls (circles) ($n = 9$). Values are mean and S.D..

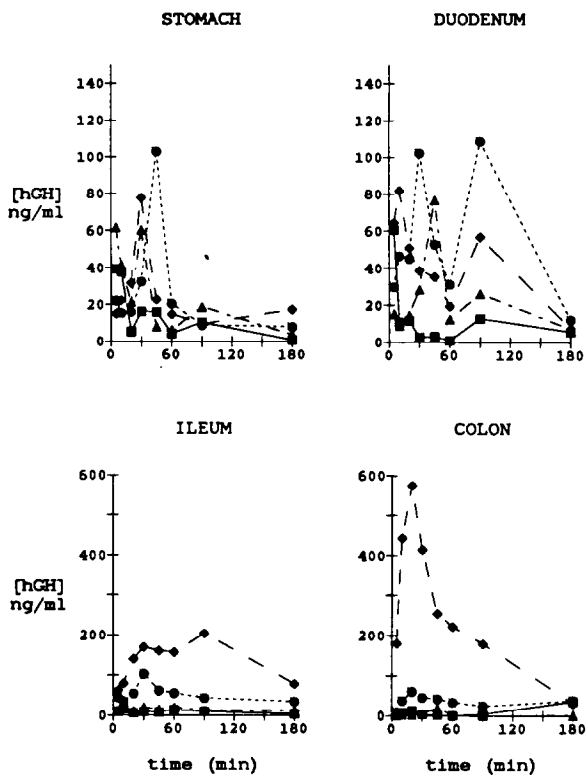

Fig. 4. Serum concentrations of met-hGH versus time following administration of four preparations of met-hGH into various segments of the GI tract. Values represent the mean of 5 or 6 rats. Symbols for the preparations are: triangles, aqueous buffer; squares, aqueous buffer plus salicylate; circles, mineral oil; diamonds, mineral oil plus salicylate.

ileum (over 200 ng/ml) and colon (nearly 600 ng/ml). Fig. 4 shows the mean serum concentration versus time curves for all 4 formulations tested in all 4 segments of the gastrointestinal tract.

Serum concentrations following administration to the stomach and duodenum did not reach much above 100 ng/ml regardless of the formulation. Following injection into the ileum or colon met-hGH serum concentrations are also below 100 ng/ml unless the formulation contains both sodium salicylate and mineral oil. The levels reached by using both substances together not only far exceed those obtained with either material alone but even exceed the sum of the concentrations obtained using the materials alone. The low

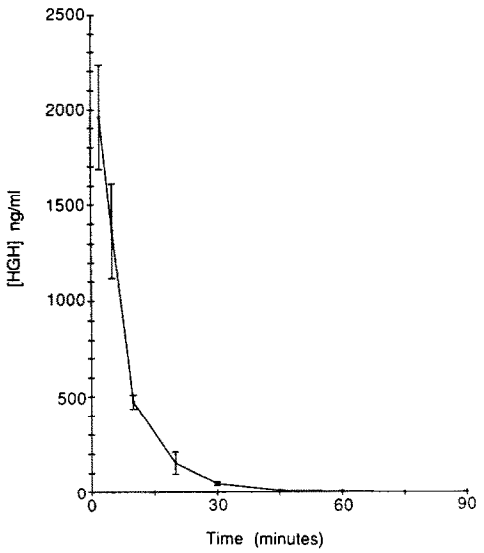

Fig. 5. Serum concentrations of met-hGH versus time following intravenous injection of 150 μg/kg. Values represent the mean + S.D. for 5 animals.

levels following administration of met-hGH in aqueous buffer indicate that surgically induced artifact is not responsible for the positive findings.

Absolute bioavailability for the different treatments computed by comparing the areas under the curves in Fig. 4 to the area under the serum concentration versus time curve following intravenous injection (Fig. 5) is shown in Table 1. The table demonstrates numerically the observations made above that the relationship between sodium salicylate and mineral oil is more than simply additive.

TABLE 1

BIOAVAILABILITY OF met-hGH FROM FOUR SECTIONS OF THE GI TRACT ADMINISTERED IN FOUR FORMULATIONS

Values are the mean percent ± S.D.

Formulation	Stomach	Duodenum	Ileum	Colon
Aqueous buffer	0.8 ± 0.7	1.0 ± 1.1	0.7 ± 0.4	0.2 ± 0.2
Salicylate and buffer	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	0.6 ± 0.9
Mineral oil	1.0 ± 0.5	2.9 ± 3.7	2.3 ± 2.4	1.6 ± 2.3
Salicylate and mineral oil	0.9 ± 0.4	1.7 ± 1.2	7.0 ± 4.7	9.5 ± 4.3

A two-way analysis of variance was performed on bioavailability in the 4 segments of the GI tract. The main effects tested were the presence or absence of salicylate and the presence or absence of mineral oil. This analysis produces a statistical significance value (P) for each main effect and a value for the interaction of the main effects. A statistically significant value ($P < 0.05$) for one of the main effects, for example, salicylate, would indicate that the presence of salicylate in the formulation had an influence on bioavailability. A significant interaction effect indicates either an interfering or synergistic relationship between salicylate and mineral oil (Sokal and Rohlf, 1981). Table 2 summarizes the P-values obtained in all 4 segments of the GI tract. These results indicate that there is an effect on bioavailability by salicylate in the colon and by mineral oil in the ileum and the colon. Furthermore, since the bioavailability in the presence of salicylate and mineral oil is greater than with either alone, the significant interaction effect in the ileum and colon indicates a synergistic relationship. Group-to-group comparisons were made by Student's t-tests on the bioavailability in the ileum and colon. Bioavailability following treatment with salicylate plus mineral oil was significantly greater than with any other treatment. None of the other group to group comparisons indicated significance. Therefore the significance seen in the analysis of variance arises from the effect of this single group.

The variation among animals in any treatment group is something which needs to be addressed. Table 3 shows the mean and standard deviation of serum met-hGH concentrations at each time point for every condition tested in the GI tract study as well as the area under the curve computed by the

TABLE 2

SUMMARY OF RESULTS OF TWO-WAY ANALYSIS OF VARIANCE

Effect tested	Stomach	Duodenum	Ileum	Colon
Salicylate	NS	NS	NS	$P < 0.01$
Mineral oil	NS	NS	$P < 0.01$	$P < 0.001$
Interaction	NS	NS	$P < 0.05$	$P < 0.01$

NS = not significant ($P > 0.05$).

TABLE 3

SERUM CONCENTRATIONS (ng/ml) OF met-hGH FOLLOWING ADMINISTRATION OF FOUR FORMULATIONS TO THE GI TRACT OF RATS

Values are mean ± S.D. Units for area under the curve (AUC) are ng·min/ml.

Group	5 min	10 min	20 min	30 min	45 min	60 min	90 min	180 min	AUC
Stomach									
Buffer	61.6 ± 108	41.4 ± 50.5	21.1 ± 50.0	60.2 ± 111	8.1 ± 9.5	6.5 ± 8.4	18.6 ± 20.5	3.9 ± 4.8	2980 ± 2580
Buffer + Sal.	39.5 ± 56.8	37.9 ± 24.0	5.4 ± 6.3	16.3 ± 15.2	16.0 ± 26.3	4.3 ± 2.7	10.4 ± 15.7	1.0 ± 2.1	1540 ± 1090
Mineral oil	22.1 ± 18.5	15.5 ± 11.9	16.0 ± 14.1	32.4 ± 32.8	102 ± 145	20.4 ± 27.2	9.0 ± 6.4	8.0 ± 6.0	3630 ± 1870
M.O. + Sal.	15.1 ± 14.3	22.3 ± 19.9	31.7 ± 38.1	77.8 ± 152	22.7 ± 23.7	14.9 ± 8.3	9.5 ± 4.3	17.3 ± 13.3	3520 ± 1680
Duodenum									
Buffer	15.4 ± 10.6	9.2 ± 8.8	14.7 ± 21.2	28.5 ± 66.0	76.7 ± 175	12.4 ± 20.8	26.2 ± 37.8	7.0 ± 13.2	3930 ± 4120
Buffer + Sal.	60.7 ± 120	10.9 ± 6.9	11.8 ± 7.7	2.9 ± 3.4	3.3 ± 2.6	0.9 ± 2.3	12.9 ± 11.6	5.9 ± 11.6	1490 ± 1190
Mineral oil	29.8 ± 28.8	46.7 ± 41.2	45.3 ± 36.1	102 ± 185	53.1 ± 62.2	31.2 ± 39.7	108 ± 177	12.1 ± 11.0	10700 ± 13900
M.O. + Sal.	64.5 ± 68.6	82.0 ± 63.7	51.1 ± 39.0	38.8 ± 22.7	35.5 ± 15.0	19.5 ± 11.1	56.8 ± 82.8	7.5 ± 3.8	6030 ± 4640
Ileum									
Buffer	10.7 ± 9.3	37.4 ± 67.2	12.1 ± 11.3	20.8 ± 10.4	14.6 ± 11.5	20.1 ± 16.7	12.6 ± 10.9	12.6 ± 11.6	2680 ± 1530
Buffer + Sal.	10.3 ± 9.3	16.7 ± 16.2	6.9 ± 6.4	9.4 ± 8.6	8.3 ± 8.6	14.2 ± 10.9	10.5 ± 12.1	4.9 ± 6.0	1650 ± 1280
Mineral oil	44.1 ± 61.5	36.4 ± 32.8	54.2 ± 53.5	103 ± 146	62.2 ± 64.5	55.5 ± 50.4	43.1 ± 49.1	35.3 ± 38.0	8570 ± 9060
M.O. + Sal.	62.8 ± 65.8	79.6 ± 53.3	140 ± 150	169 ± 191	160 ± 137	157 ± 156	204 ± 133	79.0 ± 40.7	26300 ± 17500
Colon									
Buffer	1.7 ± 3.5	3.6 ± 4.8	2.9 ± 5.7	5.2 ± 6.2	20.1 ± 34.5	1.8 ± 3.6	1.1 ± 2.1	0.9 ± 1.8	571 ± 620
Buffer + Sal.	2.5 ± 4.1	9.4 ± 8.5	13.6 ± 9.0	4.4 ± 5.3	2.6 ± 2.9	1.3 ± 2.0	4.4 ± 4.5	35.9 ± 69.4	2210 ± 3260
Mineral oil	10.4 ± 17.8	38.1 ± 62.9	61.4 ± 91.4	46.2 ± 70.6	42.4 ± 64.4	34.3 ± 65.0	24.9 ± 41.9	38.8 ± 53.7	6150 ± 8600
M.O. + Sal.	180 ± 96.3	444 ± 279	576 ± 250	415 ± 129	255 ± 103	223 ± 178	179 ± 248	30.3 ± 37.1	35700 ± 16200

trapezoid method. It is clear that there was considerable variation particularly in the conditions in which bioavailability was low. The high variation typically resulted from a single animal with high values at most time points in a group with low bioavailability or one with generally low concentrations in a group with higher bioavailability. These extreme values have not been treated as statistical outliers in this analysis yet the analysis-of-variance still indicates a significant effect. The variations likely result from the severe surgical intervention characteristic of this animal model. Further development of such a dosage form will obviously require a less invasive test system.

Discussion

The observation that sodium salicylate enhances absorption of met-hGH from rectal suppositories naturally led to the investigation of the growth promoting potential of the hormone administered by this route. The studies described here demonstrate that the met-hGH absorbed is not only detectable by a double monoclonal antibody assay but that it promotes growth in hypophysectomized rats. This is convincing evidence that the polypeptide being absorbed is intact and biologically active. These rectal suppository studies were very encouraging but an oral dosage form requires absorption from higher levels of the GI tract.

The unexpected finding that there is a synergistic relationship between sodium salicylate and mineral oil in enhancing absorption of a large polypeptide from the GI tract suggests that an oral dosage form may be possible. Enteric coatings currently used for orally administered drugs could be employed to delay release of the drug and enhancer until it reached the lower levels of the GI tract where appreciable absorption may take place.

The lack of absorption of methionyl human growth hormone from the stomach and duodenum could be the result of a number of factors. The fact that it is absorbed from the lower GI tract suggests that high concentrations of proteolytic enzymes present in the upper regions degrade the growth hormone.

Muranishi et al. (1980) showed that mixed micelles could increase the absorption of a normally unabsorbable, large water-soluble drug, heparin. They found this increase to be due to an alteration in mucosal membrane permeability. It is interesting that in their studies the permeation enhancement was greater in the colon than in the ileum. This observation fits with the data presented here which implies that alteration of the mucosal barrier may be more likely to occur in the colon than in higher levels of the GI tract. Salicylates and enamine derivatives of amino acids have been shown to be effective absorption enhancers in rectal suppositories (Nishihata et al., 1982; Kim et al., 1982). It was speculated that the effect was related to their ability to interact with calcium (Kamada et al., 1981). It was also determined that the enhancing activity persisted only as long as the enhancing compound was available for absorption itself.

Ali-Hammani and Richards (1983) found that sodium salicylate administered in an oily vehicle was absorbed more slowly but more completely than when given in an aqueous solution. They suggested that the primary factor was the effect of the oil on gut transit time. They also speculated, however, that the oil may have been acting as a reservoir for the release of the salicylic acid. Since in the current study the gut segment was tied off and the rat was anesthetized, the transit time was not a factor.

The serum concentration versus time curve following injection in the colon shown in Fig. 4 indicates that the peak concentration occurs 30 min after injection. The decline in concentration is gradual compared to that seen following intravenous injection (Fig. 5). Fig. 5 shows that met-hGH disappears rapidly from the serum once it is absorbed. The late peak and relatively slow decline in concentration shown in Fig. 4 likely indicate a sustained absorption of met-hGH.

To understand the mechanism by which mineral oil and salicylate enhance growth hormone absorption detailed immunohistochemical studies must be performed. At this point we do not know whether the protein is absorbed primarily in aggregate (crystal) or monomer form. In this study mineral oil could be working to delay the release

of sodium salicylate and thus prolong the absorption enhancement period. It is not clear, however, how this phenomenon alone could account for the dramatic effects seen here. It may well be that the oil vehicle is serving an additional function such as protecting the met-hGH from degradation prior to absorption. Whether the promising results seen here with sodium salicylate plus mineral oil are an effect of either or both of these described mechanisms, sustained release of the enhancer and protection of the polypeptide are important characteristics of a successful oral dosage form. Current pharmaceutical manufacturing methods for producing oral sustained release dosage forms should be able to provide these important characteristics. It would seem, then, that although considerable development efforts remain, the fundamental elements exist to produce an oral delivery system for polypeptide drugs.

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References

Ali-Hammami, O.M.O. and Richards, J.H., Bioavailability studies on an orally administered, oily suspension of a water soluble drug. *Pharm. Acta Helv.*, 58 (1983) 237–240.

Banting, F.G. and Best, C.H., Pancreatic extracts. *J. Lab. Clin. Med.*, 7 (1922) 464–472.

Dane, E. and Docker, T., Sythese von 6-[Dα-Amino-phenlacetylamino]-penicillansaure unter Verwendung von B-Dicarbonylverbindungen als Aminoschutzgruppen. *Chem. Ber.*, 98 (1965) 789–797.

Gibaldi, M. and Perrier, D., *Pharmacokinetics*, Marcel Dekker Inc., New York, 1982, p. 169.

Goeddel, D.V., Heynecker, H.L., Hozumi, T., Arentzen, R., Itakura, K., Yansura, D.G., Ross, M.J., Miozzari, G., Crea, R. and Seeburg, P.H., Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. *Nature (London)*, 281 (1979) 544–548.

Kamada, A., Nishihata, T., Kim, S., Yamamoto, M. and Yata, N., Study of enamine derivatives of phenylglycine on the rectal absorption of insulin. *Chem. Pharm. Bull.*, 29 (1981) 2012–2019.

Kim, S., Kamada, A., Higuchi, T. and Nishihata, T., Effect of enamine derivatives on the rectal absorption of insulin in dogs and rabbits. *J. Pharm. Pharmacol.*, 35 (1982) 100–103.

Muranishi, N., Kinugawa, M., Nakajima, Y., Muranishi, S., and Sezaki, H., Mechanism for the inducement of intestinal absorption of poorly absorbed drugs by mixed micelles I. Effects of various lipid–bile salt mixed micelles on the intestinal absorption of streptomycin in rat. *Int. J. Pharm.*, 4 (1980) 271–279.

Muranishi, S., Tokunaga, Y., Taniguchi, K. and Sezaki, H., Potential absorption of heparin from the small intestine and the large intestine in the presence of monoolein mixed micelles. *Chem. Pharm. Bull.*, 25 (1977) 1159–1161.

Nishihata, T., Rytting, J., Kamada, A., Higuchi, T., Routh, M. and Caldwell, L., Enhancement of rectal absorption of insulin using salicylates in dogs. *J. Pharm. Pharmacol.*, 35 (1982) 148–151.

Notari, R.E., *Biopharmaceutics and Clinical Pharmacokinetics*, Marcel Dekker, New York, 1980, p. 86.

Phillips, L.S. and Vassilopoulou-Sellin, R., Somatomedins. *N. Engl. J. Med.*, 302 (1980a) 371–380.

Phillips, L.S. and Vassilopoulou-Sellin, R., Somatomedins. *N. Engl. J. Med.*, 302 (1980b) 438–446.

Sokal, R.R. and Rohlf, F.J., *Biometry*, W.H. Freeman and Co., San Francisco, 1981, p. 329.

Wilhelmi, A.E., Growth hormone-measurement-bioassay. In Berson, S.A., Yalow, R.S. (Eds.), *Methods in Investigative and Diagnostic Endocrinology*, Elsevier, Amsterdam, vol 2A (1973) 296–302.

Ziv, E., Kidron, M., Berry, E.M. and Bar-On, H., Bile salts promote the absorption of insulin from the rat colon. *Life Sci.*. 29 (1981) 803–809.