International Journal of Pharmaceutics, 101 (1994) 115–120

IJP 03323

Enhancement of human calcitonin absorption across the rat colon in vivo

John Hastewell [a], Susan Lynch [a], Roy Fox [a], Ian Williamson [a], Paul Skelton-Stroud [b] and Martin Mackay [a]

[a] *Drug Preformulation and Delivery, Ciba Pharmaceuticals, Wimblehurst Road, Horsham RH12 4AB (UK) and* [b] *Preclinical Safety, Ciba Pharmaceuticals, Stamford Lodge, Altrincham Road, Morley SK9 4LY (UK)*

(Received 5 April 1993)
(Accepted 3 May 1993)

Key words: Human calcitonin; Absorption enhancer; Oral drug delivery; Colon; Hypocalcaemic response; Mixed micelles

Summary

We have studied the influence of equimolar monoolein: sodium taurocholate enhancer formulations on the absorption of human calcitonin (hCT) and two markers of intestinal permeability, horseradish peroxidase (HRP) and polyethylene glycol, molecular weight 4000 (PEG 4000). hCT, HRP and PEG 4000 were all absorbed across the colonic mucosa to a limited extent. The use of 40 mM monoolein : 40 mM sodium taurocholate significantly ($p < 0.001$) enhanced (9.0 ± 1.0-fold) the absorption of all three molecules with no damage to the mucosal tissue at the light microscopy level. At concentrations of 20 mM and below, the monoolein:sodium taurocholate formulation did not enhance the absorption of hCT. HRP immunohistochemistry showed an intracellular localisation suggesting that the transcellular pathway was involved in the absorption process. The increased absorption of hCT in the presence of the 40 mM enhancer formulation was able to elicit a maximal hypocalcaemic response, whereas no significant effect was observed in the absence of the enhancer. We conclude that the absorption enhancer used in this study, can increase intestinal absorption of a range of molecules without causing major tissue damage. Such formulations may offer advantages as they enable pharmacodynamic responses to be elicited from smaller doses of therapeutic peptides and proteins.

Introduction

Human calcitonin (hCT) is a 32 amino acid hormone produced by C-cells of the thyroid gland. It lowers blood calcium levels by inhibition of bone resorption and increasing urinary calcium excretion (Austin and Heath, 1981). Calcitonins (CTs) from several sources are used in the treatment of disease associated with accelerated bone resorption. These disorders include Paget's disease (Greenberg et al., 1974; Horwith et al., 1977) and postmenopausal osteoporosis (McDermott and Kidd, 1987).

The problems with current therapy are the route of administration and side-effects. The usual routes of administration are subcutaneous and intramuscular. Recently, intranasal administration of hCT and salmon CT (sCT) has proved effective in the management of Paget's disease (Ziegler et al., 1979; Reginster et al., 1985, 1992; Muff et al., 1990) and postmenopausal osteoporosis (Overgaard et al., 1989, 1992; Vega et al., 1989). Side-effects (nausea and facial flushing)

Correspondence to: M. Mackay, Drug Preformulation and Delivery, Ciba Pharmaceuticals, Wimblehurst Road, Horsham, West Sussex RH12 4AB, U.K.

are common following injection of sCT and hCT (Gennari et al., 1983). Tolerance is excellent after intranasal CT compared with administration by injection (Reginster and Franchimont, 1985; Reginster et al., 1992).

Whilst nasal sCT is now a successful product, oral administration would be the favoured mode of delivery for CT. Unfortunately, the properties of CT make it a poor candidate for oral delivery. To date, there are few data which demonstrate the potential availability of CT following oral or intra-intestinal administration. We have reported the absorption of hCT from rat colon (Hastewell et al., 1992) and human colon (Antonin et al., 1992; Beglinger et al., 1992). In both rat and man the bioavailability of intra-colonically administered hCT was less than 1%. This bioavailability needs to be increased before oral hCT can be used in the clinic. In this study we have used a lipidic enhancer system based on the approach of Muranishi (1985) to increase the bioavailability and have determined the acute toxicity of the enhancement system on the colon of rat.

Materials and Methods

Materials

All chemicals were of analytical grade. hCT (Cibacalcin®) was supplied by Ciba, Basle, Switzerland. HRP antisera was purchased from Nordic (Tilburg, The Netherlands).

Surgery

Female Wistar rats (90–120 g) were used throughout. The animals were starved for 20 h before experimentation. Anaesthesia was induced with a combination of Hypnorm (20 μl, i.m., Janssen Pharmaceuticals Ltd, Oxford, U.K.) and sodium pentabarbitone (3 mg 100 g^{-1}, i.v., May and Baker Ltd, Dagenham, U.K.) and maintained by additional i.v. sodium pentabarbitone, when required. The left carotid artery was cannulated for blood sampling. The abdomen was opened to allow access to the gastrointestinal (GI) tract. The proximal colonic segment was identified and isolated by ligation. The segment was flushed out with 2.0 × Hanks buffer (NaCl, 16 g l^{-1}; $NaHCO_3$, 0.7 g l^{-1}; KCl, 0.8 g l^{-1}; KH_2PO_4, 0.12 g l^{-1} and Na_2HPO_4, 0.095 g l^{-1}) before the distal end was tied off.

Doses

A 0.1 mg ml^{-1} hCT solution was prepared in 240 mmol l^{-1} mannitol: 150 mmol l^{-1} NaCl. A 10 mg ml^{-1} solution of HRP was prepared in 2.0 × Hanks buffer with [^{14}C]PEG 4000 (10–20 μCi $mmol^{-1}$) at tracer concentrations. The osmolarities of the mannitol saline and the Hanks buffer were matched at approx. 500 mOsm. The equimolar concentrations of monoolein and sodium taurocholate over a concentration range of 5–40 mM were included in the vehicle when required. All dosage forms were clear.

Administration

The hCT or HRP doses were administered at 100 μl 100 g^{-1} animal weight via a needle inserted through a proximal incision in the colon. The needle was tied in before administration and the ligature tightened as the needle was withdrawn. For hCT, an i.v. dose was administered (10 μg kg^{-1}) in 150 mmol l^{-1} NaCl via the tail vein, with no laparotomy.

Blood samples

A 250 μl zero time blood sample was taken. Further 250 μl aliquots were withdrawn at 5, 15, 30, 60 and 120 min after the hCT administration and 5, 10, 15 and 30 min after HRP administration. All samples were kept at 0°C. The aliquots were prevented from clotting by the addition of 3 μl (15 U) of heparin (Evans Medical Ltd, Horsham, U.K.). After each sample the volume withdrawn from the animal was made up with physiological saline with 50 U ml^{-1} heparin. The samples were centrifuged and the plasma stored at −80°C.

Histology and immunohistochemistry

Two tissue samples were taken for examination from each animal, a test region which had been exposed to the formulation and a control region immediately distal to the dosed segment. The tissues from the hCT dosed animals were fixed in Bouins fluid for 24 h, stored in 70%

methanol, processed and paraffin wax embedded. Sections were cut on a rotary microtome and stained with haematoxylin and eosin for histopathological examination. The tissues from the HRP dosed animals were fixed in 10% neutral buffered formalin solution for a minimum of 48 h and dehydrated through an ascending series of ethanol. The tissue was cleared in xylene and embedded in paraffin wax. Sections (4 μm) were attached to glass microscope slides, dewaxed and hydrated before immunohistochemical staining. The peroxidase activity was blocked with methanol-H_2O_2. The sections were washed with 0.2 M Tris-HCl/1% normal donkey serum and incubated at 4°C overnight in a humidity chamber with rabbit antisera against type II HRP. The sections were rewashed before exposure to the donkey anti-rabbit IgG. This second antibody was conjugated to peroxidase. Diaminobenzidine (37°C for 2 h) was used to locate the antibody complexes. All sections were haematoxylin counterstained. Positive and negative controls were completed.

Assays

Plasma hCT and calcium were determined using commercially available kits (see Hastewell et al., 1992). Plasma PEG 4000 was determined by scintillation counting in Lumagel using a Beckman LS 1801, with quench correction. Plasma HRP was determined by an immunospecific assay (Ambler and Peters, 1984) using the colour development system of Gallati and Pracht (1985).

Expression of results

The results are presented as μmol calcium ml^{-1} plasma and pg hCT or HRP ml^{-1} plasma. Results are presented as the mean $\pm$ SEM (n = 6). Statistical comparisons were carried out using Student's t-test, one and two-way analysis of variance.

Results

Colonic administration

The absorption of hCT and HRP across colonic mucosa is presented in Figs 1 and 2, respectively.

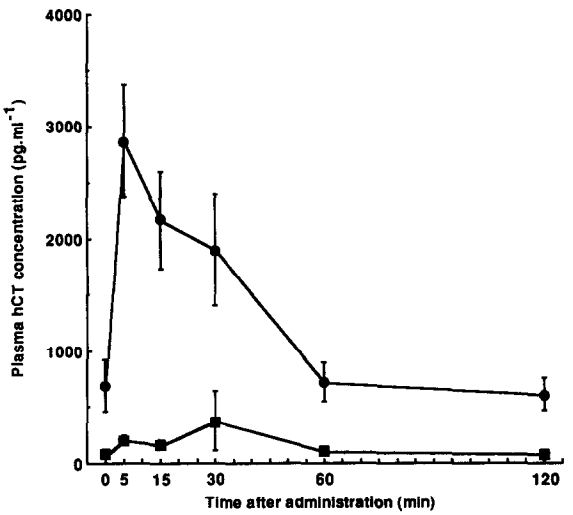

Fig. 1. The plasma appearance of hCT after intracolonic administration. (■) Control with no lipidic enhancers; (●) in the presence of 40 mM monoolein:40 mM sodium taurocholate lipidic enhancers.

The data show that absorption of both hCT and HRP is significantly ($p < 0.001$) increased by the coadministration of the 40 mM monoolein:40 mM sodium taurocholate enhancer formulation. Table 1 shows that although the molecular weights

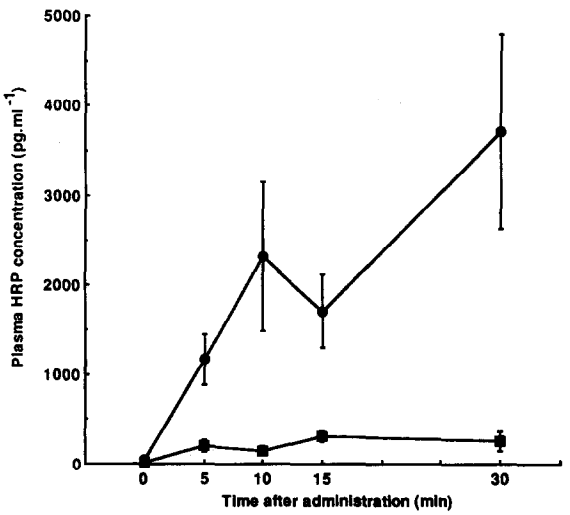

Fig. 2. The plasma appearance of HRP after intracolonic administration. (■) Control with no lipidic enhancers; (●) in the presence of 40 mM monoolein:40 mM sodium taurocholate lipidic enhancers.

TABLE 1

The comparision of colonic absorption in the presence and absence of 40 mM monoolein : 40 mM sodium taurocholate

Transport marker	Molecular weight	AUC(0–30)		Enhancement ratio
		Control	+40 mM enhancers	
hCT	3418	6016 (2133)	87632 (17180)	11.8 (1.3)
PEG 4000	4000	2108 (738)	12990 (3348)	6.4 (1.7)
HRP	40000	6820 (1294)	62414 (10989)	9.2 (1.6)

AUC(0–30), Area under the curve between 0 and 30 min, calculated by the trapezoid rule; values in parentheses represent SE of meaned data ($n = 6$). The enhancement was independent of transport marker ($p > 0.1$).

of hCT and HRP differ by 10-fold the enhancement ratios are similar ($p > 0.1$), and that the absorption of PEG 4000 is also increased by this formulation.

In the case of hCT the enhancer formulation increases the basal colonic bioavailability from 0.2 to 2.0%. This increased availability elevates the C_{max} 8-fold to 2851 ± 500 pg ml^{-1}. We have previously shown (Hastewell et al., 1992) that a plasma level of at least 600 pg ml^{-1} hCT is needed to elicit a maximal calcium lowering response in rats. Therefore, use of the enhancer formulation makes the 0.1 mg kg^{-1} hCT dose pharmacodynamically active (Fig. 3).

The influence of reducing the concentration of the enhancer formulation on hCT absorption is shown in Fig. 4. The data indicate that 40 mM monoolein : 40 mM sodium taurocholate was needed for significant enhancement ($p < 0.001$).

Light microscopy

The histopathology data showed that there was no influence of the lipidic enhancers on the morphology of the colon as judged by luminal debris, cellular morphology and cell loss from the epithelial layer.

The histochemical studies showed that HRP staining occurred at the apical pole of the epithelial cell cytoplasm. The degree of this intracellu-

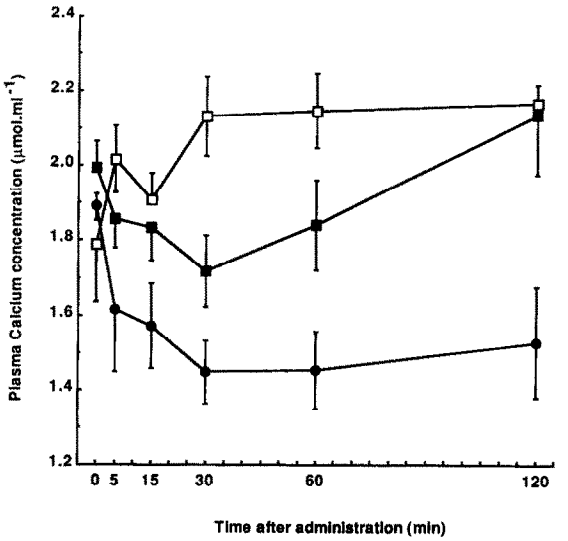

Fig. 3. The plasma calcium concentration after intracolonic administration of hCT. (□) Vehicle (no hCT) with 40 mM monoolein : 40 mM sodium taurocholate lipidic enhancers; (■) hCT alone; (●) hCT with 40 mM monoolein : 40 mM sodium taurocholate lipidic enhancers.

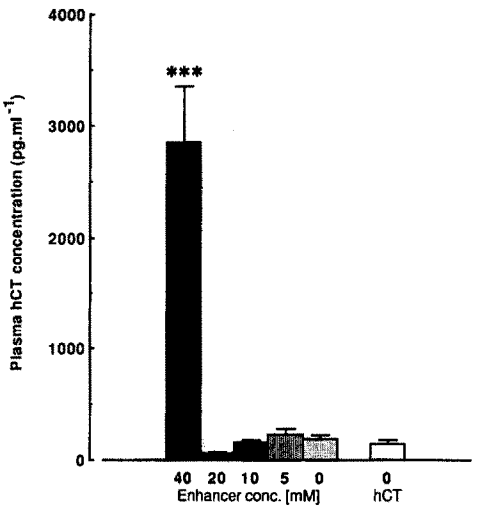

Fig. 4. The plasma appearance of hCT after intracolonic administration. (filled bars) Influence of concentration of equimolar formulations of monoolein : sodium taurocholate lipidic enhancers on hCT absorption. (open bar) Control, 0 hCT in the presence of 40 mM sodium taurocholate : 40 mM monoolein lipidic enhancers. *** $p < 0.001$ when compared with control (no enhancers).

lar staining was increased by the use of 40 mM monoolein : 40 mM sodium taurocholate.

Discussion

Absorption enhancers have been used to increase the bioavailability of drugs following oral administration. Most of this work has focused on increasing the bioavailability of therapeutic peptides and proteins (Van Hoogdalem et al., 1989; Muranishi, 1990). Several types of enhancer have been used. These include non-steroidal anti-inflammatory drugs, for example, salicylates (Peters et al., 1987), water-oil-water emulsions (Shichiri et al., 1978), surfactants (Galloway and Root, 1972), bile salts (Matsumura and Saito, 1989), surfactant-lipid mixed micelles (Yoshikawa et al., 1984) and promotion effects of azone and fusogenic fatty acids (Fukui et al., 1986).

Muranishi (1985) developed the mixed micellar system used in this study. It was shown that the absorption enhancement of macromolecules by mixed micelles was more effective in the colon than in the small intestine. This resulted in higher and faster peak plasma levels. Explanation of the enhancing mechanism of mixed micelles include mucous effects and transcellular mechanisms.

Our results confirm those of Muranishi. The bioavailability of hCT, HRP and PEG 4000 was increased following co-administration of lipidic enhancers. Whilst the mixed micelles described increase absorption, there have been few attempts to determine the predominant mechanism of action or the toxic effects of chronic application. Many enhancers perturb the epithelial membrane. The histochemical data after HRP administration with enhancers supports the hypothesis that the cellular uptake of macromolecules is influenced by the enhancers. This raises the possiblity that molecules other than the drug may also be absorbed. The extent of this co-absorption will be enhancer and concentration dependent.

The data show that there is little discrimination over the molecular weight range of 4000 to 40000. This suggests that there is scope for interaction between the soluble peptide and protein contents of the GI tract and the enhancers. This potential lack of specificity for the lipidic class of absorption enhancer needs to be addressed in terms of their pharmaceutical acceptability. The long term toxicity implications can only be assessed in chronic studies. However, in this study we have shown that there is no acute toxicity as a result of administration of the lipidic enchancers. This is in keeping with data from Richardson et al. (1991) who showed that 0.5% L-α-lysophosphatidylglycerol caused little epithelial damage after 24 h contact with the vaginal epithelium of rats. In addition to the potential local epithelial damage the co-absorption of macromolecules (for instance enzymes or antigenic material) from the GI tract could lead to clinical pathologies. A better understanding of how the lipidic enhancers work should lead to the development of systems which do not cause epithelial damage and are selective for the drug.

We conclude that the absorption enhancer used in this study can increase intestinal absorption of a range of molecules without causing acute tissue damage. Further work is aimed at assessing the safety of this system with a view to testing in man.

References

Ambler, L. and Peters, G.E., An immunospecific enzyme assay for horseradish peroxidase. *Anal. Biochem.*, 137 (1984) 66–68.

Antonin, K.H., Saano V., Bieck, P., Hastewell, J., Fox, R., Lowe, P. and Mackay, M., Colonic absorption of human calcitonin in man. *Clin. Sci.*, 83 (1992) 627–631.

Austin, L.A. and Heath, H., Calcitonin, physiology and pathophysiology. *N. Engl. J. Med.*, 304 (1981) 269–278.

Beglinger, C., Born, W., Muff, R., Drewe, J., Dreyfuss, J.L., Bock, A., Mackay, M. and Fischer, J.A., Intracolonic bioavailability of human calcitonin in man. *Eur. J. Pharmacol.*, 43 (1992) 527–531

Fukui, H., Murakami, M., Yoshikawa, H., Takada, K. and Muranishi, S., Combinative promotion effect of azone and fusogenic fatty acid on the large intestinal absorption in rat. *Int. J. Pharm.*, 31 (1986) 239–246.

Gallati, V.H. and Pracht, I., Horseradish peroxidase: Kinetic studies and optimisation of the peroxidase activity determination with the substrate H_2O_2 and 3,3′,5,5′-tetramethylbenzidine. *J. Clin. Chem. Clin. Biochem.*, 23 (1985) 453–460.

Galloway, J.A. and Root, M.A., New forms of insulin. *Diabetes,* 21 (1972) 637–648.

Gennari, C., Passeri, M., Chierichetti, S.M. and Piolini, M., Side effects of synthetic salmon and human calcitonin. *Lancet,* i (1983) 594–595.

Greenberg, P.B., Doyle, F.H., Fisher, M.T., Hillyard, C.J., Joplin, G.F., Pennock, J. and MacIntyre, I., Treatment of Paget's disease of bone with synthetic human calcitonin. *Am. J. Med.,* 56 (1974) 867–871.

Hastewell, J., Lynch, S., Williamson, I., Fox, R. and Mackay, M., Absorption of human calcitonin across the rat colon. *Clin. Sci.,* 82 (1992) 589–594.

Horwith, M., Suh, S.M., Torun, B., et al., Synthetic human calcitonin in the treatment of hereditary bone dysplasia (Hyperphosphatasaemia). In MacIntyre, I. (Ed.), *Human Calcitonin and Paget's Disease,* Hans Huber, Berne, 1977, pp. 207–215.

Matsumura, M. and Saito, S., Effect of bile salts on the plasma concentration of immunoreactive vasoactive intestinal polypeptide in man. *Endocrin. Jap.,* 36 (1989) 15–21.

McDermott, M.T. and Kidd, G.S., The role of calcitonin in the development and treatment of osteoporosis. *Endocrin. Rev.,* 8 (1987) 377–390.

Muff, R., Dambacher, M.A., Perrenould, A., Simon, C. and Fischer, J.A., Efficacy of intranasal human calcitonin in patients with Paget's disease refractory to salmon calcitonin. *Am. J. Med.,* 89 (1990) 181–184.

Muranishi, S., Modification of intestinal absorption of drugs by lipoidal adjuvants. *Pharm. Res.,* 2 (1985) 108–118.

Muranishi, S., Absorption enhancers. *Crit. Rev. Ther. Drug Carrier Systems,* 7 (1990) 1–34.

Overgaard, K., Hansen, M.A., Jensen, S.B. and Christiansen, C., Effect of salcatonin given intranasally on bone mass and fracture rates in established osteoporosis: a dose response study. *Br. Med. J.,* 305 (1992) 556–561.

Overgaard, K., Riis, B.J., Christiansen, C. and Hansen, M.A., Effect of salcatonin given intranasally on early postmenopausal bone loss. *Br. Med. J.,* 299 (1989) 477–479.

Peters, G.E., Hutchinson, L.E.F., Hyde, R., McMartin, C. and Metcalfe, S.B., Effects of sodium 5-methoxysalicylate on macromolecule absorption and mucosal morphology in a vascularly perfused rat gut preparation in vivo. *J. Pharm. Sci.,* 76 (1987) 857–861.

Reginster, J.Y., Albert, A. and Franchimont, P., Salmon-calcitonin nasal spray in Paget's disease in bone: preliminary results in five patients. *Calcif. Tissue Int.,* 37 (1985) 577–561.

Reginster, J.Y. and Franchimont, P., Side effects of synthetic salmon calcitonin given by intranasal spray compared with intramuscular injection. *Clin. Exp. Rheumatol.,* 3 (1985) 155–157.

Reginster, J.Y., Jeugmans-Huynen, A.M., Wouters, M., Sarlet, N., McIntyre, H.D. and Franchimont, P., The effect of nasal hCT on bone turnover in Paget's disease of bone – implications for the treatment of other metabolic bone diseases. *Br. J. Rheumatol.,* 31 (1992) 35–39.

Richardson, J.L., Thomas, N.W. and Illum, L., Recovery of rat vaginal epithelium from the histological effects of absorption enhancers. *Int. J. Pharm.,* 77 (1991) 75–78.

Shichira, M., Kawamori, R., Goriya, Y.I., Kikuchi, M., Yamasaki, Y., Shigeta, Y. and Abe, H., Increased intestinal absorption of insulin in a micellar solution: water-oil-water insulin micelles. *Acta Diabetol. Lett.,* 15 (1978) 175–183.

Van Hoogdalem, E.J., de Boer, A.G. and Breimer, D.D., Intestinal drug absorption enhancement: an overview. *Pharm. Ther.,* 44 (1989) 407–443.

Vega, E., Gonzalez, D., Ghiringheli, G. and Mautalen C., Acute effect of the intranasal administration of salmon calcitonin in osteoporotic women. *Bone Miner.,* 7 (1989) 267–273.

Yoshikawa, H., Takada, K., Muranishi, S., Satoh, Y. and Naruse, N., A method to potentiate enteral absorption of interferon and selective delivery into lymphatics. *J. Pharmacobiol. Dyn.,* 7 (1984) 59.

Ziegler, R., Holtz, G., Raue, F. and Streibl, W., Nasal application of human calcitonin in Paget's disease in bone. In MacIntyre, I. and Szelke, M. (Eds), *Molecular Endocrinology,* Elsevier, Amsterdam, 1979, pp. 293–299.