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Effect of L-α-lysophosphatidylcholine on the nasal absorption of human growth hormone in three animal species

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Summary

The effect of L-α-lysophosphatidylcholine (LPC) on the nasal absorption of human growth hormone (hGH) in anaesthetised rats, conscious rabbits and sheep, has been examined. LPC was shown to be an effective enhancer of nasal hGH absorption in three different animal models. In the rat, concentrations of LPC in the range 0–1.0% co-administered with hGH gave absorption values, relative to subcutaneous administration (S/C), from 2.3 to 16.3%. In the rabbit, concentrations of LPC of 0 and 0.2% co-administered with hGH gave absorption values, relative to S/C, of 1.4 and 72.8% respectively. In the sheep, concentrations of LPC in the range 0–0.59% co-administered with hGH gave absorption values, relative to S/C, from 0.2 to 16.0%. The amount of hGH absorbed was directly proportional to the concentration of LPC co-administered, and in sheep this relationship was linear, with a correlation coefficient of 0.99. The shapes of the plasma concentration vs time curves were similar in all three species.

Introduction

The nasal route is currently being investigated as a potential alternative for the administration of the many novel bioengineered compounds (Chien et al., 1989; Fisher, 1990) particularly biologically active peptides and proteins (Eppstein and Longenecker, 1988). There are many advantages to using the nasal cavity for the administration of peptides including: the avoidance of degradation in, or while traversing, the gut, the reduction of 'first pass' metabolism, the potential of an extensive absorptive surface in the nose and the possibility of better patient compliance when compared with parenteral therapy. However, due to the physicochemical properties of these peptides they are often poorly absorbed, with typical bioavailabilities in the range of 1–2%. To overcome this problem many peptides have been ad-

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ministered in combination with absorption enhancers (Chien et al., 1989; Fisher, 1990). The range of agents used to enhance absorption has included nonionic surfactants, natural and synthetic bile salts, fatty acids, chelating agents (Lee, 1986) and mucoadhesive delivery systems (Illum et al., 1988). It has been suggested that the enhancing agents could act by various mechanisms, such as increasing membrane fluidity, opening up membrane 'tight junctions', inhibiting proteolytic enzymes, reducing mucus viscosity, or, in the case of the mucoadhesive agents, by increasing the clearance time from the nasal cavity. In some cases a combination of these actions appears to operate.

Previously, the effect of a range of absorption enhancers on the nasal absorption of recombinant human growth hormone (hGH) in the rat has been investigated (Daugherty et al., 1988; O'Hagan et al., 1990). In these laboratories it was shown that L-α-lysophosphatidylcholine (LPC) was an effective enhancer of the nasal absorption of hGH in the rat, increasing the relative bioavailability to about 26% (O'Hagan et al., 1990). Lysophospholipids have some special advantages as enhancing agents. They are surface active amphiphiles which are present, at low concentrations, in most biological membranes. They are active as enhancers at low concentrations and are converted in the cell to normal cell components. A recent review by Stafford and Dennis (1988) has discussed the physical characteristics and biological properties of these compounds. In the preliminary studies (O'Hagan et al., 1990) the effect of only one concentration of LPC (0.2%) on the enhancement of hGH absorption was investigated. Moreover, the experiments were only performed in the anaesthetised rat model where clearance mechanisms are minimised and hence absorption would be expected to be higher than in a conscious animal model. In another series of studies, LPC was also shown to be effective in increasing the bioavailability of nasally applied gentamicin (Illum et al., 1988) and insulin (Illum et al., 1989; Farraj et al., 1990). When the major constituents of LPC, lysophosphatidylcholine palmitoyl and lysophosphatidylcholine stearoyl, were administered separately, and the effect compared with the effect of the native (mixed) LPC on nasal insulin absorption, no differences were seen (Illum et al., 1989).

The aim of the present study was to investigate the effect of LPC on the nasal absorption of hGH in different animal species, both in the anaesthetised and in the conscious states. hGH was chosen as a model peptide compound with a relatively high molecular mass, approximately 22000 Da, which shows very low absorption when administered nasally in simple solution formulations.

Growth hormone was originally extracted from human pituitaries and used for the treatment of growth hormone deficiency in children. Serious side effects have been reported with this pituitary derived material (Brown et al., 1985; Gibbs et al., 1985; Koch et al., 1985). Using recombinant DNA technology synthetic human growth hormone (hGH) has been produced (Martial et al., 1979) and such material is now preferred in growth deficient children. The problem with hGH therapy is that the usual means of administration entails regular, painful injections to children. Therefore, an alternative route of administration, such as the nasal route, would be an advantage for both current and proposed uses (Moore et al., 1986).

The animal models used in the present study were the anaesthetised rat and the conscious rabbit and sheep. These are commonly used species for nasal absorption studies. In order to investigate the mechanism of action of LPC the compound was administered to both rats and sheep in a range of concentrations.

Materials and Methods

Materials

Biosynthetic hGH, guinea pig anti-hGH IgG and guinea pig anti-hGH F_{ab} were obtained from Novo-Nordisk, Gentofte, Denmark. L-α-Lysophosphatidylcholine (LPC), *o*-phenylenediamine dihydrochloride (OPD) and human serum albumin (HSA) (Fraction V) were purchased from Sigma, Poole, U.K. All other materials were of reagent grade.

Preparation of hGH formulations

For the preparation of the hGH dose solutions, the supplied vials of hGH were reconstituted in 1/75 M potassium phosphate pH 7.4 (rats) or distilled water (rabbits and sheep) in the presence of the required amount of LPC. These solutions were prepared freshly on the morning of the study.

Animal experiments

Rat studies The rat model used was a modification of the in vivo technique of Hirai et al. (1981) as described by Fisher et al. (1987). Male Wistar rats (JABU, Sutton Bonington, U.K.) weighing approximately 250 g were used. These were anaesthetised with a suitable volume of 15 mg/ml pentobarbitone (Sagatal, May and Baker, Dagenham, U.K., diluted with sterile isotonic saline) administered intravenously via an in-dwelling needle in a caudal vein. The rats were tracheotomised, the oesophagus sealed and the carotid artery cannulated. Eight groups of rats with at least three animals in each group were used in these studies. Seven groups were dosed directly into the nasal cavity, via one nostril, with 100 μl/kg of hGH solution to give a final dose of 2.93 IU/kg (1 mg/kg). One of these groups received hGH without added LPC, and the other groups were dosed with hGH plus LPC at concentrations of 0.05, 0.1, 0.2, 0.25, 0.5 or 1% (0.05, 0.1, 0.2, 0.25, 0.5 or 1 mg/kg, respectively). The eighth group was dosed subcutaneously, into a flank, with hGH at 1.46 IU/kg (0.5 mg/kg). Blood samples (approximately 0.2 ml) were collected from the carotid cannula into tubes containing 10 μl of 150 IU/ml heparinised saline prior to dosing and at 5, 10, 20, 30, 40, 50, 60, 90, 120, 180 and 240 min after dosing. The volume of blood collected was replaced with an equal volume of isotonic saline administered via the in-dwelling needle in the caudal vein. Blood samples were kept on ice until the plasma was separated by centrifugation, and plasma samples were then stored at $-20\,^{\circ}$C until analysed.

Rabbit studies A rabbit model was used that has been developed by this group. New Zealand white rabbits (JABU, Sutton Bonington, U.K.) weighing approximately 3 kg were divided into three groups of, at least, three animals in each group. Two groups were dosed intranasally with hGH at 0.9 IU/kg (0.3075 mg/kg), one group received hGH alone and the other group received hGH in combination with LPC at 0.2% (0.067 mg/kg). The animals were gently restrained, inverted so that they were ventral side up, and 0.05 ml of the appropriate dose solution was instilled into each nostril using an automatic pipette. The third group received hGH subcutaneously at 0.1 IU/kg (0.0342 mg/kg) into the scruff of the neck. Blood samples (approximately 0.8 ml) were collected by venepuncture of a marginal ear vein, into lithium heparin tubes, prior to dosing and at 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, 180, 240 and 300 min after dosing. Blood samples were kept on ice until the plasma was separated by centrifugation, and plasma samples were then stored at $-20\,^{\circ}$C until analysed.

Sheep studies Seventeen cross-bred (Suffolk and Texel) sheep of known weight (approximately 40 kg) were used in this study. An in-dwelling Viggo secalon cannula was placed into one of the external jugular veins of each animal on the first day of the study and, whenever necessary, was kept patent by flushing it with heparinised normal saline (25 IU/ml). This cannula was removed upon the completion of the study.

The sheep were divided into five groups of three animals each and one group of five animals. The sheep were sedated with an IV dose of ketamine hydrochloride at 2.0 mg/kg. This was intended as a counter-measure against the animal sneezing during administration of the formulation. The anaesthesia lasted for about 3 min. For intranasal administration of the solutions, a blue-line umbilical cannula (size 6FG, Portex Ltd) was inserted into the nostril of the sheep before the delivery of the solution from a 1 ml syringe. Five groups of sheep were dosed with hGH at 0.9 IU/kg (0.3075 mg/kg) in combination with LPC in the dose solution at 0, 0.029, 0.17, 0.29 or 0.59% (0, 0.005, 0.02, 0.05 or 0.1 mg/kg, respectively). The volume administered was approximately 17 μl/kg, divided equally between the two nostrils. The last group of sheep was dosed subcutaneously, into a flank, with hGH at 0.1 IU/kg (0.0342 mg/kg). Blood samples (2.5 ml) were

collected in heparinised tubes (Li Heparin, Sarstedt, Leicester, U.K.), from the cannulated jugular vein of the sheep, prior to the hGH administration and at 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, 180, 240 and 300 min post-administration. The tubes were temporarily stored on crushed ice. Plasma was collected by centrifugation at 4 °C, and then stored at −20 °C awaiting analysis.

Determination of plasma concentrations of hGH by enzyme linked immunosorbent assay

The plasma concentrations of hGH were determined using an enzyme linked immunosorbent assay (ELISA) which was previously described by Dinesen and Anderson (1984) and modified by O'Hagan et al. (1990). Briefly, 125 μl of guinea pig anti-hGH IgG, diluted 1/1000 in sodium carbonate (pH 9.8) was incubated in a microtitre plate (NUNC Immunoplate I, NUNC, Denmark) for 3 days at 4 °C. The plate was triple washed in 0.9% (normal, isotonic) saline solution, 0.1% Tween (SAL/TWE). Then 125 μl of the samples and the standards, in an equivalent dilution of plasma, diluted in sodium phosphate buffer pH 7.4 containing 0.1 M sodium chloride solution, 0.5% human serum albumin and 0.05% Tween 20 (SAL/TWE/HSA) were added to the wells and the plate incubated for 2 h at room temperature (RT). Standard concentrations of hGH were prepared in diluted blank plasma (0.11–7.0 ng/ml) and the experimental samples were diluted to within this range for the assay. The plate was triple washed in SAL/TWE and 125 μl guinea pig anti-hGH IgG F_{ab}-peroxidase, diluted 1/1500 in SAL/TWE/HSA, was added to the wells and incubated for 2 h at RT. The plate was triple washed in SAL/TWE and 125 μl of enzyme substrate (40 mg *o*-phenylenediamine dihydrochloride (OPD) in 25 ml citrate/phosphate buffer pH 5.0 and 20 μl 30% hydrogen peroxide) was added and the plate was incubated in the dark for 1 h. The reaction was stopped by the addition of 150 μl of 2.5 M sulphuric acid to each well and the absorbance was read at 492 nm in a Titertek Multiscan (Flow Laboratories). Plasma concentrations of hGH were calculated using the Titersoft program (Flow) with reference to the standard curve constructed for the standard concentrations of hGH in plasma.

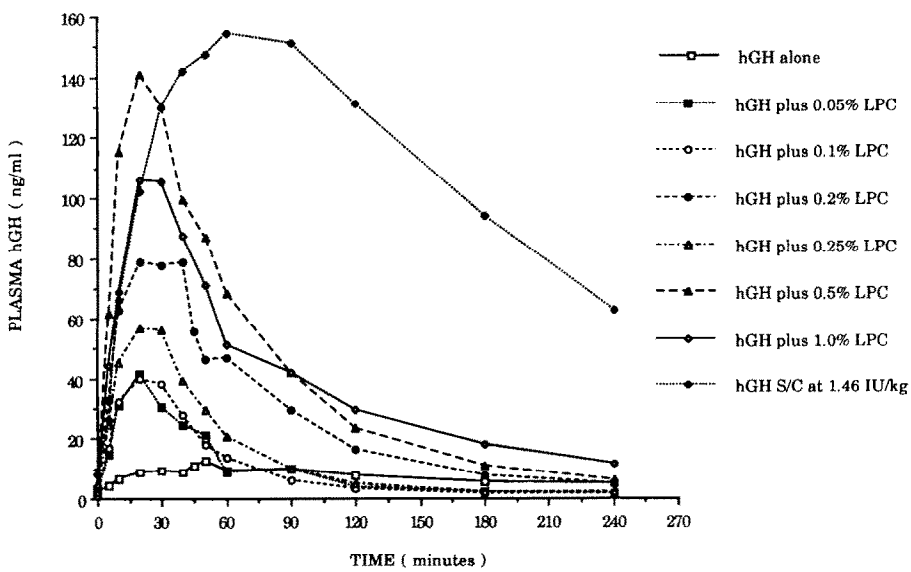

Fig. 1. Mean plasma concentrations of hGH after IN administration to rats of hGH at 2.93 IU/kg (1 mg/kg), plus LPC at different concentrations.

Calculations

Means, standard deviations and standard errors were calculated using standard statistical methods. Areas under the plasma concentration curves (AUCs) were calculated using the trapezoidal method. Relative bioavailabilities were estimated by comparison of AUCs, with adjustment for the different doses administered.

Results and Discussion

Rat

The mean plasma concentrations of hGH following administration to the rat are illustrated in Fig. 1. The time of peak plasma concentration of hGH and the peak concentration, together with the area under the plasma concentration curve (AUC) and absorption relative to subcutaneous administration are shown in Table 1.

It can be seen from Table 1 that there was considerable biological variation between groups. After intranasal (IN) administration of hGH alone there was only a small and general rise in plasma concentrations of hGH to a broad peak between 10 and 120 min after dosing. When LPC was co-administered with hGH there was a well defined peak in plasma concentrations of hGH between 20 and 40 min after dosing.

Following peak concentrations there was a rapid decline to control values. This sharp early peak was similar to that previously reported from these laboratories (O'Hagan et al., 1990) and that shown following the co-administration of hGH with sodium tauro-24,25-dihydrofusidate (STDHF) in rats reported by Baldwin et al. (1990). Where dose levels of LPC can be compared (0.2%), O'Hagan et al. (1990) showed a significantly higher absorption of hGH (26%) than is shown here (11%). When hGH was administered

TABLE 1

Summary of plasma data following administration of hGH in different formulations, via different routes, to rats, rabbits and sheep

Species	Formulation	hGH Dose [IU/kg (mg/kg)]	Peak plasma time [min (±SE)]	Peak plasma concentration [ng/ml (±SE)]	Area under curve [ng/ml per min (±SE)]	Relative bioavailibility [%(±SE)]
Rat	IN hGH alone	2.93 (1)	32.0 (16.5)	12.7 (1.26)	1197 (225)	2.3 (0.43)
	IN hGH plus 0.05% LPC	2.93 (1)	16.7 (3.34)	41.4 (5.87)	1942 (291)	3.7 (0.56)
	IN hGH plus 0.1% LPC	2.93 (1)	20.0 (4.08)	43.7 (4.08)	2084 (295)	4.0 (0.57)
	IN hGH plus 0.2% LPC	2.93 (1)	37.8 (9.23)	89.7 (10.57)	6155 (272)	11.8 (0.52)
	IN hGH plus 0.25% LPC	2.93 (1)	24.0 (1.34)	62.3 (6.75)	2921 (392)	5.6 (0.75)
	IN hGH plus 0.5% LPC	2.93 (1)	31.1 (4.55)	159.4 (35.44)	9128 (2062)	17.5 (3.89)
	IN hGH plus 1.0% LPC	2.93 (1)	26.7 (3.34)	114.5 (9.29)	8470 (1783)	16.3 (3.42)
	SC hGH	1.46 (0.5)	66.7 (12.03)	162.7 (10.47)	26035 (1808)	100
Rabbit	IN hGH alone	0.9 (0.3075)	40.4 (4.08)	6.3 (1.87)	263 (156)	1.4 (0.62)
	IN hGH plus 0.2% LPC	0.9 (0.3075)	43.3 (8.83)	107.7 (4.87)	23427 (2457)	72.8 (7.63)
	SC hGH	0.1 (0.0342)	58.0 (2.24)	36.9 (2.63)	3572 (547)	100
Sheep	IN hGH alone	0.9 (0.3075)	42.0 (6.62)	1.5 (0.34)	60 (27)	0.2 (0.10)
	IN hGH plus 0.0293% LPC	0.9 (0.3075)	75.3 (14.12)	1.0 (0.15)	59 (32)	0.2 (0.10)
	IN hGH plus 0.171% LPC	0.9 (0.3075)	26.7 (6.67)	14.5 (4.39)	952 (456)	2.9 (1.37)
	IN hGH plus 0.293% LPC	0.9 (0.3075)	33.3 (3.34)	24.4 (8.51)	2053 (950)	6.2 (2.86)
	IN hGH plus 0.585% LPC	0.9 (0.3075)	51.7 (13.03)	51.6 (7.61)	5325 (569)	16.0 (1.71)
	SC hGH	0.1 (0.0342)	70.0 (5.01)	23.9 (4.19)	3662 (984)	100

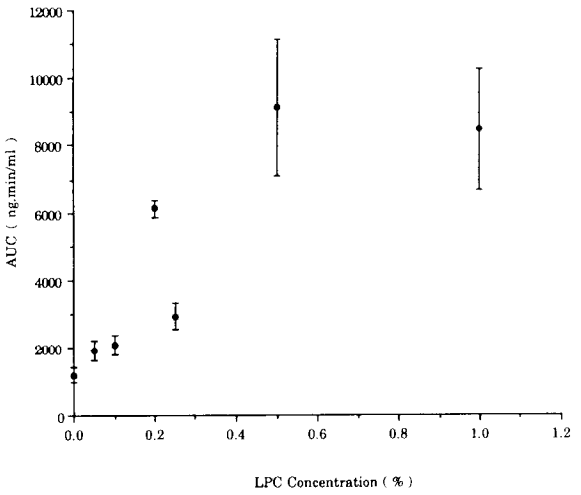

Fig. 2. Relationship between mean AUC (±SE) and concentration of LPC added to a fixed dose (2.93 IU/kg) of hGH administered IN to rats.

sub-cutaneously (S/C) a broad peak in plasma concentrations at about 60 min was obtained. The shape of this curve was different from that shown by Baldwin et al. (1990), who obtained a plateau in concentrations from about 20 min after dosing; however, these authors only collected samples up to 150 min after dosing as compared to 240 min in this study.

The relationship between AUC and LPC concentration in the dose solution is shown in Fig. 2. No satisfactory line could be simply fitted to these points; all curve-fitting attempts gave poor correlation coefficients. However, although there was considerable variation between the groups, as the concentration of LPC in the dose solution increased the AUC increased.

Rabbit

The mean plasma concentrations of hGH following administration to the rabbit are illustrated in Fig. 3. The time of peak plasma concentration of hGH and the concentration at that time, together with the AUCs, and absorption relative to subcutaneous administration are shown in Table 1. When hGH was administered alone there was very little increase in plasma concentrations of hGH. This was comparable to the rat results described above.

In combination with LPC there was an early broad peak between 30 and 60 min after dosing followed by a rapid decline to control values. The peak was broader, and occurred later, than that reported in anaesthetised rabbits by Baldwin et al. (1990) with STDHF. Following S/C adminis-

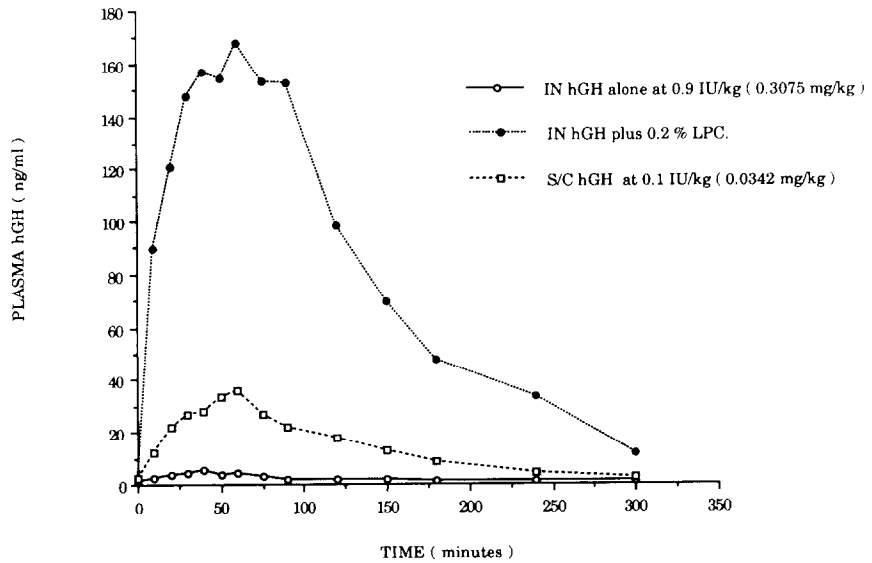

Fig. 3. Mean plasma concentrations of hGH after IN administration, with and without LPC, and S/C administration to rabbits.

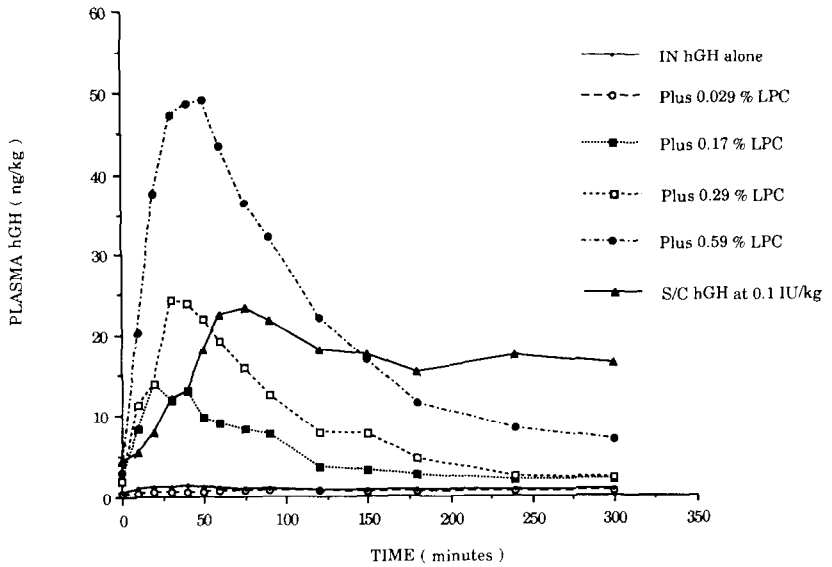

Fig. 4. Mean plasma concentrations of hGH after IN administration to sheep of hGH at 0.9 IU/kg (0.3075 mg/kg), plus LPC at different concentrations.

tration a very broad peak in plasma concentrations was seen.

Sheep

The mean plasma concentrations of hGH following administration to the sheep are illustrated in Fig. 4. The time of peak plasma concentration of hGH and the peak concentration, together with the AUCs, and absorption relative to subcutaneous administration are shown in Table 1.

There was no significant change in the plasma concentrations of hGH following the co-administration of LPC at 0.029% (0.005 mg/kg) when compared to a simple hGH solution. At this low concentration LPC did not enhance the nasal absorption of hGH. With the addition of higher concentrations of LPC, sharp peaks in plasma concentration of hGH occurred between 30 and 60 min with subsequent steady declines in concentrations. These results were again similar to those obtained in sheep by Baldwin et al. (1990).

The linear ($r = 0.994$) relationship between AUC and LPC concentration in the dose solution is shown in Fig. 5.

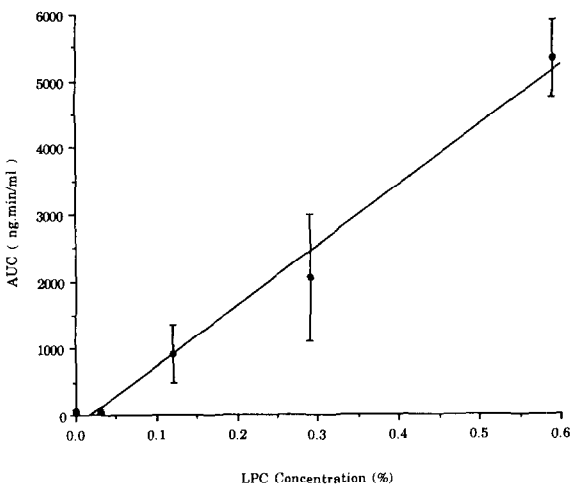

Fig. 5. Relationship between mean AUC (±SE) and concentration of LPC added to a fixed dose (0.9 IU/kg) of hGH administered IN to sheep.

Effect of LPC on hGH absorption

When LPC was co-administered with hGH it consistently increased the nasal absorption of hGH in the three species examined. Fig. 6 illustrates the effect of similar dose concentrations of LPC on the plasma concentrations of hGH in all three animal species. In rats and sheep the relative bioavailibility of hGH was in the range of approximately 3–16%, when LPC was co-administered at concentrations of approximately 0.2–

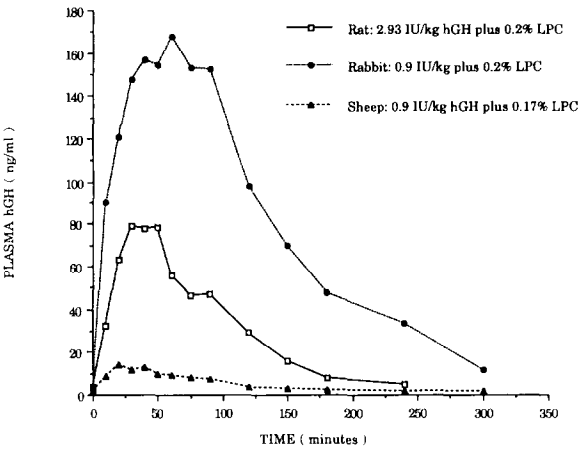

Fig. 6. Comparison of the mean plasma concentrations of hGH in all three animal species after co-administration of hGH with similar concentrations of LPC.

1.0%. Thus there was good agreement between the two different species, and the two different models, which was unexpected. Absorption in the rat was predicted to be higher, as the nasal clearance mechanisms were reduced. There was a considerable increase in hGH absorption in the rabbit model at the one concentration of LPC studied. As the rabbit was a conscious model the hGH absorption would be expected to fall relative to the rat. This difference is attributed to the different models and species. These results disagree with those of Baldwin et al. (1990), who showed that in their four different models at a fixed concentration of STDHF, the absorption profiles in rats dosed via the nasopharynx, and anaesthetised rabbits were similar, whereas the bioavailibility in conscious sheep was increased by a factor of two. These authors also showed that hGH absorption in the rat after nasopharyngeal administration was always higher than after administration via the nares; the latter route was the one used in this present study.

Differences in species and model are important when considering the nasal absorption of peptides. The differences in these two factors shown in the present study with hGH have also been seen with other compounds. Deurloo et al. (1989) investigated the nasal absorption of insulin with and without STDHF in rats and rabbits. These workers kept their dose formulations constant between the two species which led to a twenty fold difference in the doses. Without STDHF, insulin was absorbed more in rabbits (0.9%) than in rats (0.3%). However, with the enhancer, insulin was absorbed more in rats (18%) than in rabbits (5.2%). Igawa et al. (1990) investigated the nasal absorption of human fibroblast interferon when co-administered with sodium glycocholate in mouse, rat, rabbit and dog. In this study the doses per kilogram were similar in all four species. However, the shapes of the plasma concentration of human fibroblast interferon vs time curves showed distinct species differences: the rabbit curve showed a sharp early peak, while in rats and dogs concentrations rose to a broad peak or plateau. When the bioavailabilities of human fibroblast interferon were calculated they were similar, as were the elimination rate constants, and the differences in plasma profiles were ascribed to differences in absorption rate constants. The present study with hGH, together with published examples of other peptide compounds, illustrates the necessity to have a well defined model to study nasal absorption, especially when comparisons are being made.

In all animals the plasma concentrations of hGH showed a definite peak followed by a rapid decline. This was also seen by Baldwin et al. (1990), with STDHF as an enhancer. Such a 'pulsate' or 'transient' plasma profile that mimics the natural pulsate release of hGH could be more efficacious than S/C or intramuscular dosing which show prolonged raised plasma profiles (Finkelstein et al., 1972; Albertsson-Wikland and Rosberg, 1988). This suggestion is supported by the work of Clark et al. (1985), who found that growth hormone was more effective in promoting growth in hypophysectomised rats when given as IV pulsate doses than when given either as an IV infusion or a S/C dose.

The LPC used in these studies was derived from egg yolks and was a mixture of phosphatidylcholine with 72% palmitoyl and 24% stearoyl side chains. The critical micelle concentration (CMC) of natural LPCs is very low. Robinson and Saunders (1958) estimated that for egg LPC the CMC was $1-2 \times 10^{-3}$% (approxi-

mately $2-4 \times 10^{-2}$ mM). With the individual constituents, palmitoyl LPC was estimated to have a CMC of 0.7×10^{-2} mM (approximately 0.4×10^{-3}%) by Tanford (1980), or ranging from 0.4 to 0.83×10^{-2} mM (approximately $0.2-0.4 \times 10^{-3}$%) by Stafford and Dennis (1988), and stearoyl 0.04×10^{-2} mM (approximately 0.02×10^{-3}%) (Stafford and Dennis, 1988). Therefore, all the concentrations of LPC used in this study were well above the CMC. As discussed, LPC increased the absorption of nasally applied hGH in all three of the species examined. In the anaesthetised rat and the conscious sheep models an increase in hGH absorption with increasing added doses of LPC was found. This finding disagrees with the results obtained for the relationship between the AUC and the added concentration of STDHF (Baldwin et al., 1990), where the AUC reached a plateau just above the CMC of STDHF. This difference suggests that LPC and STDHF enhance the nasal absorption of hGH via different mechanisms.

The present study has demonstrated the efficient absorption enhancing effect of LPC on the nasal absorption of hGH in three different animal models. The amount of hGH absorbed was directly proportional to the concentration of LPC co-administered. The action of LPC is still not understood, but it appears to have significant potential for enhancing the nasal administration of peptides.

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