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The influence of lipophilicity upon the nasal absorption of a series of hexapeptides

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Abstract

A series of glycine (G) and phenylalanine (F) containing hexapeptides have been synthesised that vary only in the proportions of their constituent amino acids. The peptides FGGGGG (F_1G_5), FFGGGG (F_2G_4) and FFFGGG (F_3G_3) were characterised by their distribution coefficients and it was found that a linear relationship existed between logD and the number of phenylalanine residues. Metabolism by aminopeptidases is a major barrier to the nasal absorption of labile peptides. To overcome this the peptides were synthesised with D-phenylalanine that rendered the peptides totally resistant to metabolism by leucine aminopeptidase. The extent of nasal absorption of the peptides was measured by a nasal perfusion technique. The peptides were generally poorly absorbed with only 20.2% (D-F_1G_5), 19.7% (D-F_2G_4) and 25.9% (D-F_3G_3) disappearing from the perfusate after 105 min. The results also illustrate that the extent of absorption does not correspond closely with differences in lipophilicity of the peptides.

Keywords: Nasal absorption; Peptides; Leucine aminopeptidase; Metabolism; Stability; Lipophilicity

1. Introduction

The understanding and development of nasal drug delivery systems has advanced rapidly during recent years. In addition to peptides and proteins many disparate compounds have been studied as candidates for nasal drug delivery, from highly lipophilic steroids (Hussain et al., 1981; Corbo et al., 1988), to extremely hydrophilic quaternary ammonium compounds (Su et al., 1984). Consequently the nasal route is now routinely used for the systemic delivery of many non-peptide and peptide drugs such as oxytocin, vasopressin, desmopressin, and the LHRH analogue buserelin (Harris, 1986).

The rate and extent of nasal drug absorption may depend upon many physicochemical factors including the aqueous to lipid partition coefficient of the drug, the pK_a and the molecular weight of the drug. Several authors (McMartin et al., 1987; Maitani et al., 1989; Donovan et al., 1990a; Donovan et al., 1990b) have investigated the effects of molecular weight upon the nasal absorption of non-peptide compounds, and others

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(Corbo et al., 1989) have investigated the effects of lipophilicity, also with non-peptide compounds. However, there is a paucity of information available regarding whether lipophilicity affects the nasal absorption of peptides.

The numerous proteolytic enzymes in the gastrointestinal tract provide a formidable barrier to the oral delivery of peptide drugs (Lee, 1988). Degradation can occur luminally, at the brush border or intracellulary. To overcome such stability and absorption problems, other delivery sites are under investigation. It was initially believed that the nasal mucosa was deficient in enzyme activity but it has now been shown that this tissue possesses many enzymes including a variety of proteases (Dodda Kashi and Lee, 1986; Stratford and Lee, 1986). The lack of effective nasal absorption of peptides at this site prompted assessment of its proteolytic activity in both in vivo (Hussain et al., 1985) and in situ (Hirai et al., 1981) systems. The presence of both endo- and exopeptidases has been demonstrated and enzymes include aminopeptidases A, B, N, leucine aminopeptidase and microsomal aminopeptidase (Stratford and Lee, 1986; Sarkar, 1992). Eighty-five per cent of enkephalin hydrolysis in nasal mucosa homogenate was found to be due to aminopeptidases (Dodda Kashi and Lee, 1986). The presence of dipeptidylpeptidase, diaminopeptidase, postprolyl cleaving enzyme, angiotensin-converting enzyme and endopeptidases has also been indicated (Hayakawa et al., 1987; Lee et al., 1987). This high enzyme activity may explain why it has been found that enhancing the lipophilicity of a peptidase labile peptide would probably not have any great effect on its bioavailability from the nasal cavity (Hussain et al., 1991). The increase in lipophilicity may simply increase the partitioning of the peptides into areas of higher enzyme activity where they are metabolised. Consequently any series of peptides that are to be used to solely investigate the effects of lipophilicity upon nasal absorption must be resistant to metabolism, especially by aminopeptidases. It is possible to increase the stability of peptides to aminopeptidase by substituting an unnatural D-amino acid on or near the amino terminal of the peptide (Pert et al., 1976).

This study focuses on a series of three hexapeptides that have only glycine (G) and phenylalanine (F) as the constituent amino acids. The peptides FGGGGG (F_1G_5), FFGGGG (F_2G_4) and FFFGGG (F_3G_3) were used to study the effects of lipophilicity upon the nasal absorption of peptides.

2. Materials and methods

The research adhered to the 'Code of Practice for the Housing and Care of Animals Used in Scientific Procedures.' (HMSO publication 1989; HC107) and UFAW Guidelines.

2.1. Synthesis of peptides

All hexapeptides used were synthesised by using repetitive base cleavage of α-amino protective groups in Solid Phase Peptide Synthesis (SPPS). Amino acids used in this SPPS model were protected with 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group. The synthesis was performed using equipment and materials obtained from Applied Biosystems Ltd. The C14 label was incorporated via the 3-glycine unit in all peptides. Fmoc-D-phenylalanine and Fmoc-[^{14}C]glycine were synthesised in house utilising the solution phase reaction between the amino acids and Fmoc-*N*-hydroxysuccinimide (Sigma, Poole, UK.). The peptides were identified by proton-NMR, mass spectroscopy and elemental analysis.

2.2. HPLC assays for peptides

The HPLC system consisted of an LDC Constametric 3200 pump, a Milton Roy, Promis auto sampler (fitted with 100 μl loop), a reverse phase chromasil ODS2 column (Hichrom Ltd), LDC spectromonitor D and Berthold LB506-c-1 HPLC radiochemical detector.

Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade obtained from Fisons Scientific Equipment (Loughborough, UK). Liquid scintillation fluid for the HPLC was Quickszint 302 scintillation cocktail obtained from Zinsser Analytic Ltd (Maidenhead, UK).

Table 1
Incubation conditions used to investigate the metabolism of peptides by leucine aminopeptidase

Peptide	Concentration (μM/ml)	Leucine aminopeptidase conc. (units/ml)	Incubation time
L-FG_5	0.5, 0.4, 0.3, 0.2, 0.1	1.0	10 min
D-FG_5	0.5	>1.0	24 h
L-F_2G_4	1.5, 1.0, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1	0.1	10 min
D-F_2G_4	0.5	>1.0	24 h
D-F_3G_3	0.5	>1.0	24 h

The mobile phases consisted of acetonitrile/water mixtures containing 0.1% TFA. The percentage of acetonitrile in each mobile phase for the peptides was as follows; 7.5%, 17.5% and 25% for F_1G_5, F_2G_4 and F_3G_3, respectively. The flow rates were 1.5 ml/min (F_1G_5 and F_2G_4) and 1.4 ml/min (F_3G_3). Mobile phases were filtered and degassed before use. Retention times were 4.5 min for F_1G_5, 5.0 min for F_2G_4 and 6.0 min for F_3G_3. Detection was conducted both spectrometrically (λ = 214 nm) and radiochemically (solid scintillator cell or liquid admixture method depending upon sensitivity required, with a scintillant flow rate at 3 ml/min).

2.3. Incubation studies

Leucine aminopeptidase was obtained from Sigma and had an activity of 180 units/mg protein. Carboxypeptidase A and carboxypeptidase B were also obtained from Sigma. All other reagents including Krebs salts used were SLR grade obtained from Fisons Scientific Equipment.

Solutions of peptides in Krebs solution were incubated with solutions of leucine aminopeptidase also in Krebs. Krebs solution contains sufficient amounts of magnesium for aminopeptidase activity (1.18 mmol). Five concentrations of each peptide were incubated at 37°C (n = 4 for each concentration, two standards at each concentration) according to the conditions in Table 1. For peptides containing L-amino acids samples were taken at 1 min intervals. For the other peptides samples were taken at 0 and 24 h.

The total volume of the reaction mixture was 1 ml. The reaction was stopped by inactivating the enzyme with the addition of 100 μl 2.5% TFA and chilling. The concentration of peptide remaining was assayed by HPLC as described previously. The initial rate of reaction was then corrected for protein content (the concentration of enzyme present). Using the results V_{max} and K_m values were determined by plotting [S] against V and fitting the curve $V = V_{max} \times [S]/(K_m + [S])$ using a non-linear regression programme (Minim, R. D. Purves, University of Otago).

The appearance of phenylalanine can also be detected by HPLC, the method used is the same as for F_1G_5 (retention time for phenylalanine, 6.5 min).

Solutions of all the peptides in Krebs were also incubated with carboxypeptidase A and B in Krebs at varying concentrations. Samples were assayed for all peptides at 0 and 24 h.

2.4. ClogP and logD values

The method of calculating logP used was that proposed by Akamatsu and Fujita (1992) who devised an equation for calculating the logP of various peptides based upon measured logP values of 124 di- to pentapeptides composed of amino acids having un-ionisable side chains. The method takes into account parameters relevant to peptides such as steric effects of side chain constituents, chain length and β-turn potential. The authors suggest that with adaptations the equation may be used to calculate logP values for higher peptides. The peptides of interest in this case consist of six amino acids and it was assumed that major adaptations would not be necessary in order to obtain reasonable predictions.

The water/octanol distribution coefficients logD were measured for two peptides in duplicate at four concentrations. n-Octanol of spectrograde was obtained from Fisons Scientific Equipment.

n-Octanol saturated aqueous solutions of each peptide (1 ml for D-F_3G_3 and D-F_2G_4) were shaken with a suitable volume of water saturated *n*-octanol (5 ml for D-F_3G_3, 20 ml for D-F_2G_4) in glass screw-capped tubes or bottles overnight. The mixtures were shaken at 37°C and protected from light. After centrifugation to ensure total separation of the two phases the aqueous phase was assayed for peptide concentration by HPLC.

2.5. In situ perfusion studies

The in situ perfusion technique used was first described by Hirai et al. (1981) and later by Huang (1985). Male Wistar rats weighing between 200 and 300 g were anaesthetised by an i.p. injection of 0.824 ml/kg body weight of Hypnorm™ and 0.832 ml/kg body weight of Hypnovel™. The anaesthetic state was maintained by a further injection of Hypnorm™ 0.1 ml/rat every 30 min. The rat was placed in a supine position, an incision made in the neck and the trachea cannulated with a polyethylene tube (Portex Ltd) to maintain respiration. Another polyethylene tube of smaller diameter was inserted through the oesophagus toward the posterior part of the nasal cavity and ligated. The naso-palatine tract was sealed with an adhesive agent (cyanoacrylate glue, Fisons Scientific Equipment) to prevent drainage of the solution from the nasal cavity to the mouth. The oesophageal cannula was connected to peristaltic tubing (Tygon tubing) which served to deliver the perfusion solution to the nasal cavity. The perfusion medium, which consisted of a solution of the peptide in isotonic phosphate buffer pH 7.4, was recirculated by means of a peristaltic pump at a flow rate of 1.9 ml/min. The rate of nasal absorption is independent of perfusion rate at values greater than 1.5 ml/min (Huang, 1985). The perfusing solution passed from the nostrils through a funnel back into the reservoir. The temperature of the reservoir was maintained at 37°C throughout the course of the experiment. The body temperature of the rat was also maintained by means of a lamp. The initial volume of the perfusate was 10 ml.

Samples (100 μl) were taken at the following time intervals: 5, 10, 15, 20, 30, 40, 50, 60, 75, 90 and 105 min, with the 100-μl sample replaced with 100 μl buffer. The sample was then added to 3 ml scintillation fluid (HiSafe 3, Fisons Scientific Equipment) and assayed for peptide content by scintillation counting. Scintillation counting was used instead of HPLC because it was found that with the small changes in peptide concentration observed the prolonged counting time possible with scintillation counting reduces the results variability.

3. Results

3.1. Stability Studies

Initially peptides containing L-phenylalanine were synthesised and their stability to leucine aminopeptidase was determined from incubation studies. For L-F_1G_5 and L-F_2G_4 the rate of metabolism of the more lipophilic of the two was much greater than that of the peptide containing only one phenylalanine group. However, these peptides were both found to be much too unstable to be suitable candidates for the perfusion studies. As a result of these studies the peptides containing the non-endogenous isomer D-phenylalanine were synthesised. Table 2 illustrates the striking differences between the peptides regarding susceptibility to aminopeptidase metabolism. The hexapeptides containing D-phenylalanine were far more resistant to degradation than the equivalent peptides containing the corresponding L-amino acid. This increase in stability was so substantial that after exposure to high concentrations of the enzyme for 24 h no metabolism (< 2%) of D-F_1G_5, D-F_2G_4 and D-F_3G_3 was detected. As ex-

Table 2
The rate of metabolism of the hexapeptides by leucine aminopeptidase

Peptide	V_{max} (μM min^{-1} mg^{-1})	K_m (μM)
L-F_1G_5	1.107	0.280
D-F_1G_5	NMD	NMD
L-F_2G_4	12.963	0.232
D-F_2G_4	NMD	NMD
D-F_3G_3	NMD	NMD

NMD, no metabolism detected after 24 h.

Table 3
Calculated and observed octanol/water distribution coefficients of the hexapeptides studied

Peptide	LogD(observed)	ClogP(Akamatsu and Fujita (1992))
D-F_1G_5	NPD	−4.06
D-F_2G_4	−2.41	−2.50
D-F_3G_3	−0.88	−0.93

NPD, no partitioning detected.

pected because of the small glycine unit on the carboxy terminal none of the peptides showed any susceptibility to metabolism by carboxypeptidases.

3.2. Distribution coefficients and ClogP values

As a result of the incubation studies it was decided to continue work with only the most stable peptides. The octanol/water distribution coefficients of these remaining peptides were therefore determined. Table 3 illustrates the results of observed and calculated distribution and partition coefficients. No value of observed logD was obtained for D-F_1G_5 because of the high volumes of octanol expected to be required to achieve a measurable concentration difference in the aqueous phase. Calculated logP values for the peptides were similar to the observed logD. This is further evidence for the suitability of the method proposed by Akamatsu and Fujita (1992) for the prediction of the logD values for peptides. The linear relationship between number of phenylalanine groups and logP of the compounds shows that this series of glycine- and phenylalanine-containing peptides were suitable candidates for investigating the effects of lipophilicity on nasal absorption of peptides.

3.3. In situ nasal perfusion

Finally to determine the effects of lipophilicity upon the nasal absorption of the model hexapeptides the stable peptides were radiolabelled and solutions perfused through the nasal cavity. The total perfusion time was 105 min and the gradual decrease in concentration produced by absorption of the peptide is illustrated in Fig. 1. The results were represented as a percentage of the initial peptide concentration remaining in the perfusate. It can be seen that after 105 min, 20.2% of the peptide D-F_1G_5 ($k = 1.66 \times 10^{-3}$ % min $^{-1}$) and 19.7% of D-F_2G_4 ($k = 1.54 \times 10^{-3}$ % min $^{-1}$) were absorbed. D-F_3G_3 was absorbed to a slightly greater extent with a decrease of 25.9% being observed ($k = 2.35 \times 10^{-3}$ % min $^{-1}$). A range of concentrations (0.2–0.02 mM) was used for each peptide studied to determine if the percentage of compound absorbed varies with concentration. A series of blank experiments was conducted to ensure that extensive evaporation of water was not occurring from the perfusate solution, that the peptides were not adsorbing onto the system and that the peptides were stable in solution for the duration of the experiment. None of the peptides showed differences in the extent of absorption when perfused at different concentrations and all the controls demonstrated that there was no change in peptide concentration in the absence of the rat. However, a correction factor was included to allow for the addition of buffer to the sample and the loss of water due to nasal water absorption. Statistical analysis (ANOVA) of the extent

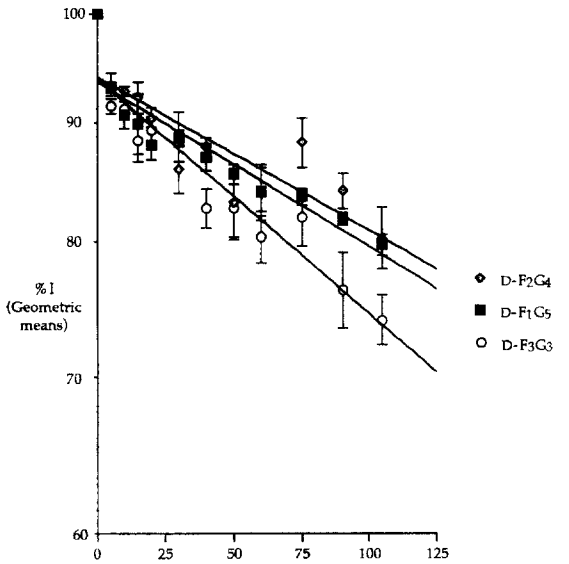

Fig. 1. Graph illustrating the nasal absorption of D-F_1G_5, D-F_2G_4, D-F_3G_3, using the in situ perfusion technique. (Geometric means ± S.E.M., $n = 5$.)

of absorption after 105 min or rate of absorption (k) showed that there were no statistical differences between the absorption of D-F_1G_5 and D-F_2G_4 ($P < 0.05$). However, both were significantly different from D-F_3G_3 ($P < 0.05$). The results therefore show that as expected the most lipophilic peptide is the one most extensively absorbed. However, there was no clearly defined pattern apparent within this small series due to the similarities observed with the two more hydrophilic compounds. Following termination of the perfusion the perfusate was assayed by HPLC with radiochemical detection in order to illustrate that the peptide was not being metabolised during the course of the experiment and that the radiochemical activity being measured was produced solely by the peptide. This was confirmed by the fact that only one radiolabelled peak appeared on each HPLC trace. Furthermore in order to determine that the decrease in peptide concentration was produced by absorption of the peptide into the systemic circulation a series of blank experiments was conducted to ensure absorption of the peptide onto the materials being used was not occurring. Secondly, in order to check that the peptides were not merely sticking to the nasal mucosal tissue this was removed from the rat, homogenised and assayed for any radiochemical content by scintillation counting following termination of the experiment. The results revealed no traces (i.e. $< 1.0\%$ of dose administered) of the peptides or radiolabelled metabolites within the nasal homogenate. This suggests that the peptides were absorbed and not merely adhering to the nasal mucosa.

4. Discussion

The results have demonstrated how a series of peptides, with different lipophilic properties, likely to have a high resistance to metabolism in the nasal cavity have been developed. Generally the peptides were poorly absorbed from this site with the octanol/water partition coefficient exhibiting only a minor influence on peptide absorption. The extent of absorption was unaffected by peptide concentration indicating that absorption was occurring mainly by passive diffusion. The octanol/water partition coefficient may not be the only parameter to predict the transcellular absorption of the peptides. Other parameters that may influence absorption could be the hydrogen bonding potential of the peptides as described by Burton et al. (1992), steric effects or other physicochemical differences such as solubility. Alternatively the peptides may not be absorbed by a transcellular route but by a paracellular route where the lipophilicity is not a determinant factor.

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