The Effect of Cyclodextrins on the Stability of Peptides in Nasal Enzymic Systems

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Leucine enkephalin (YGGFL) undergoes rapid degradation in sheep nasal mucosa to yield GGFL which is further degraded to FL. The activity of the nasal mucosal homogenate against YGGFL and GGFL ($t_{1/2}$ 12 and 7 min) was significantly greater than that observed with a nasal wash fluid ($t_{1/2}$ 40 and 13 min). The effect of cyclodextrins on the rate of degradation of FGG and YGGFL by leucine aminopeptidase (LAP) and of GGF by carboxypeptidase A (CPA) was monitored. Little effect was observed with FGG (with LAP) but the half-life of YGGFL (with LAP) was extended from ~44 min to ~75 min in the presence of a 25-fold excess of β-cyclodextrin. The stability of GGF (with CPA) was also enhanced; an effect was observable with a 5-fold excess of cyclodextrin and the half-life could be extended by 40–75%. An equation is presented which allows the estimation of the concentration of free peptide in the peptide-cyclodextrin solutions.

KEY WORDS: cyclodextrin; equilibrium model; nasal delivery; peptides; peptidase; stability.

INTRODUCTION

The bioavailability of therapeutic peptides delivered nasally is often limited by presystemic elimination, due to enzymatic degradation or mucociliary clearance, and by the poor mucosal membrane permeability of large, polar substrates.[1] The nose is now well-recognised as a site of metabolism;[2] significant peptidase activity has been demonstrated both *in vivo*[3] and *in vitro*[4] and both endopeptidases and exopeptidases are present.[5,6] The distribution of subcellular activity has been assessed.[6,7] Various strategies, including prodrug design, the use of bioadhesive particles, coformulation with penetration enhancers and enzyme inhibitors[8] have been used in attempts to increase nasal bioavailability.

The use of cyclodextrins as complexing agents has been extensively documented.[9,10] These cyclic oligosaccharides form inclusion complexes with various lipophilic drugs which may increase aqueous solubility, dissolution rate, bioavailability and stability of the drug.[17,18] Significant increases in peak plasma levels have been observed[8,13–15] and the enhancement of nasal bioavailability has been credited to the solubilising effect of the cyclodextrin on a poorly water-soluble drug and to a direct effect of the cyclodextrin on the epithelial membrane.[16,17] For example, a haemolytic effect on erythrocytes has been demonstrated[17] and mobilisation of membrane-bound enzymes (*e.g.* 5′-nucleotidase) has been shown to occur with dimethyl-β-cyclodextrin (DMβCD) but less so with hydroxypropyl-β-cyclodextrin at the 5% level.[18] Interestingly, although many additives which enhance the mucosal transport of drugs have been shown to exert mucociliotoxicity, DMβCD appears to be an effective enhancer in rats with only a small *in vitro* effect on ciliary movement.[19,20] There may also be other effects operating; these include the possibility of a reduction in the self-association of peptides such as insulin. Although improvement of dissolution rate will enhance bioavailability, complexation *per se* may not necessarily achieve this objective; indeed, it may reduce the rate of delivery as only free drug contributes to the concentration gradient which drives diffusion. Such an effect is seen with antimicrobial agents where the potency of complexes is diminished compared to cyclodextrin-free systems.[21] One other factor which may contribute to the enhancement of nasal delivery caused by cyclodextrins is that of increased stability. This study investigates the ability of cyclodextrins to protect peptides from peptidase activity to determine if this action may contribute to bioavailability enhancement. As substrates, the hydrolysis of the pentapeptide leucine enkephalin (YGGFL) and the tetrapeptide *des*-tyrosine leucine enkephalin (GGFL) were monitored in a sheep nasal wash, and nasal mucosal homogenate and smaller peptides (FGG, GGF), in the presence of aminopeptidase and carboxypeptidase[22] were studied in the presence of β- and γ-cyclodextrin.

MATERIALS AND METHODS

High-Performance Liquid Chromatography

Chromatography of nasal wash and homogenate samples was performed using a Waters Chromatography 815 workstation, comprising a Waters 600E system controller, a WISP 712 autoinjector and a Waters 484 ultraviolet variable wavelength detector operated at 205 nm, using a 5 μm Lichrospher 100 RP-18 end-capped column (250 × 4 mm). Isolated enzymic systems were analysed on an HPLC constructed from an Altex model 110A dual-piston reciprocating pump, a Rheodyne 7125 injection port fitted with a 100 μl loop and a Pye Unicam LC3 variable wavelength ultraviolet detector, operated at 255 nm with a sensitivity of 0.01 AUFS, using a 5 μm ODS-Hypersil (250 × 4.6 mm) reversed-phase column. Chromatograms were integrated with a Hewlett Packard 3390A recording integrator. Guard columns (10 mm), packed with stationary phase, were used. The mobile phase for leucine enkephalin was 30% acetonitrile and 70% dipotassium hydrogen phosphate (0.05M) in water with the pH adjusted to 3.0 with orthophosphoric acid and delivered at 0.8 ml min^{-1}. Retention times were; leucine enkephalin, 6.8 min; *des*-tyrosine leucine enkephalin, 5.3 min. For the tripeptides, the mobile phase comprised aqueous methanol (30%) containing trifluoroacetic acid (0.2%) delivered at 1.0

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ml min^{-1}. Retention times were GGF, 13.5 min; FGG, 9.5 min; F, 6.5 min.

Enzymic Preparations

Nasal Wash

Each half of the nasal cavity of three recently slaughtered sheep was washed with five, 1 ml portions of 0.1 M phosphate buffer (pH 7.4), and the washings were collected and mixed. The washings were stored on ice whilst the combined homogenate of nasal mucosal tissue was prepared. The protein concentration was measured[23] and was typically 0.63 mg ml^{-1}. The wash preparation was equilibrated for 30 min at 37°C immediately prior to use.

Preparation of Nasal Homogenate

The nasal homogenate was prepared as described previously.[22] Briefly, the nasal mucosa was carefully excised from the nasal septum of recently slaughtered sheep and the turbinates were separated. The tissue was rinsed, suspended in 0.1 M phosphate buffer (pH 7.4) and homogenised. Centrifugation at 3020 *g* at 4°C for 10 min removed cellular and nuclear debris and the supernatant, which contained cytosolic, plasma and intracellular fractions, was diluted to a final protein concentration equal to that of the nasal wash; the preparation was equilibrated at 37°C for 30 min then used immediately.

Purified Enzymes

Bovine pancreatic carboxypeptidase A (CPA; EC 3.4.17.1; Sigma): 16.2 mg ml^{-1} of protein with an activity of 1.14 mmol min^{-1} ml^{-1} (1 mg of protein equivalent to 70.5 units; 1 unit hydrolyses 1.0 μmol of hippuryl-L-phenylalanine per min at pH 7.5 and 25°C). Cytosolic porcine kidney leucine aminopeptidase (LAP-C; EC 3.4.11.1; Sigma): 1.9 mg ml^{-1} of protein with an activity of 0.399 mmol min^{-1} ml^{-1} (1 mg of protein equivalent to 210 units; 1 unit hydrolyses 1.0 μmol of L-leucinamide per min at pH 8.5 and 25°C). The enzymes were each diluted in 0.1 M phosphate buffer (pH 7.4) to give a final protein concentration of 10 μg ml^{-1} in the incubation mixture and were equilibrated at 37°C for 30 min prior to initiation by addition of the substrate.

Kinetic Studies

The substrates tested comprised the pentapeptide leucine enkephalin (YGGFL), the tetrapeptide *des*-tyrosine leucine enkephalin (GGFL) and the tripeptides phenylalanylglycylglycine (FGG) and glycylglycylphenylalanine (GGF) with and without the presence of β-cyclodextrin and γ-cyclodextrin.

Nasal Preparations

Aliquots (0.5 ml) of the nasal wash, nasal mucosal homogenate and phosphate buffer were equilibrated at 37°C in a water bath for 30 min. The reaction was initiated by the addition of 100 μl of YGGFL or GGFL solutions (120 μg ml^{-1} in 0.1 M phosphate buffer, pH 7.4) to the enzymic preparations or the buffer, to give a final enkephalin concentration of 20 μg ml^{-1}. At set time points (0–90 min), 100 μl aliquots were removed from the incubation mixture and the reaction quenched, on ice, with 50 μl 0.2 M citrate buffer (pH 2.3). Samples from each quenched solution (20 μl) were injected onto the HPLC column without pretreatment. Standards were prepared in 0.1 M phosphate buffer (pH 7.4) and were treated in the same way as the analytical samples.

Cyclodextrin-Enzyme Preparations

The required amount of β- or γ-cyclodextrin was dissolved in phosphate buffer (3.4 ml, pH 7.4) at 37°C. Concentrations were chosen to give molar ratios of cyclodextrin to peptide of: β-cyclodextrin [0; 1:1 (3.4 mg); 1:5 (17 mg); 1:10 (34 mg); 1:~25 (86 mg), this solution was equivalent to ~80% of the saturated solubility of the cyclodextrin] and γ-cyclodextrin [1:5 (19.5 mg); 1:10 (39 mg); 1:~70 (276 mg), this solution was equivalent to 20% of the saturated solubility of the cyclodextrin]. To the appropriate solution was added the solution of the peptide (GGF, FGG, YGGFL, 5 mM, 0.6 ml) giving a final concentration in this solution of 0.9 mM. The mixtures were stirred overnight. The appropriate amount of the enzyme (*e.g.* 10 and 20 units of carboxypeptidase A for GGF; 5 units and 4 units for FGG by leucine aminopeptidase, so chosen to provide convenient rates of degradation) was incorporated into phosphate buffer (2 ml, pH 7.4) and the solutions were equilibrated for 30 min at 37°C. The degradation was initiated by the addition of the peptide-cyclodextrin solution (4 ml) to provide a final peptide concentration of 0.5 mM). At appropriate time intervals, samples (0.4 ml) were removed and the reaction was quenched on ice and by the addition of 0.1 ml of trifluoroacetic acid. Samples were centrifuged for 10 min at 3000 rpm to precipitate the protein and aliquots (100 μl) were injected onto the HPLC column without any sample pretreatment. Standards were prepared in 0.1 M phosphate buffer (pH 7.4) and were treated with TFA in the same way as the analytical samples. Maximum concentrations of 0.25 μg ml^{-1} were used.

THEORETICAL

Cyclodextrins form complexes by the penetration of a hydrophobic residue of the guest molecule into the cavity of the cyclodextrin torus. This reduces the concentration of free guest in solution and may have a significant effect upon, for example, aqueous solubility and chemical stability. To quantify the effect of system variables on the concentration of free guest, the complexation process, assuming that only one aromatic residue interacts with the cyclodextrin, may be described by the equilibrium:

$$P + CD \rightleftharpoons P - CD \qquad [1]$$

where CD represents the cyclodextrin, P the peptide guest and P-CD the peptide-cyclodextrin complex. The equilibrium position may be quantified using the association constant (K_s) where:

$$K_s = \frac{[P - CD]}{[P] \cdot [CD]} \qquad [2]$$

The total concentration of peptide $[P_0]$ is:

$$[P_0] = [P] + [P - CD] \quad [3]$$

and the total concentration of cyclodextrin ($[CD_0]$) is:

$$[CD_0] = [CD] + [P - CD] \quad [4]$$

which gives:

$$[P - CD] = [P_0] - [P] = [CD_0] - [CD] \quad [5]$$

and thus:

$$[CD] = [CD_0] - [P_0] + [P] \quad [6]$$

Substitution leads to:

$$K_s = \frac{[P_0] - [P]}{[P] \cdot ([CD_0] - [P_0] + [P])} \quad [7]$$

which, on rearrangement, gives:

$$K_s[P]^2 + [P] \cdot (K_s([CD_0] - [P_0]) + 1) - [P_0] = 0 \quad [8]$$

This quadratic may be solved using the standard formula $\{ax^2 + bx + c = 0; x_{1,2} = [-b \pm (b^2 - 4ac)^{1/2}]/2a\}$ to provide the concentration of free peptide [P] as:

$$[P] = \frac{\sqrt{(K_s([CD_0] - [P_0]) + 1)^2 + 4K_s[P_0]} - (K_s([CD_0] - [P_0]) + 1)}{2K_s} \quad [9]$$

For the special case when the initial concentrations are equal ($[CD_0] = [P_0]$) – as, for example, when a 1:1 complex is dissolved, this yields:

$$[P] = \frac{\sqrt{1 + 4K_s \cdot [P_0]} - 1}{2K_s} \quad [10]$$

RESULTS AND DISCUSSION

The substrates YGGFL and GGFL were chemically stable in the absence of enzymes but were readily hydrolysed in the sheep nasal wash and homogenate preparations. In both enzymic systems, degradation of YGGFL occurred predominantly by removal of the tyrosine residue at the N-terminal end to yield GGFL (Figure 1). This tetrapeptide (GGFL) was then further hydrolysed to phenylalanylleucine (FL). Other possible products which could also be determined using the HPLC protocol described included tyrosylgycylglycine (YGG), glycylphenylalanylleucine (GFL) and glycylglycylphenylalanine (GGF); these products, however, were not observed. The principal degradation pathway is, thus, YGGFL → Y + GGFL → GG + FL. The degradation profiles of YGGFL exhibited exponential decline (Figure 2) which followed first order kinetics ($k_{obs} = V_{max}/K_m$ when $K_m \gg S_o$) and the first-order rate constants and half-lives are presented in Table I. In the wash preparation, YGGFL had a half-life of 40 min whilst GGFL was degraded significantly faster ($t_{1/2}$ 13 min). Hydrolysis by the homogenate preparation was more rapid with the same stability trend for the two substrates being observed; GGFL being degraded more rapidly than the parent enkephalin each time. The greater activity of the homogenate compared with the wash may reflect a higher concentration of proteases and access to membrane-bound or intracellular enzymes.

These data contrast with those found in other mammalian systems. In rats, the degradation occurred exclusively at

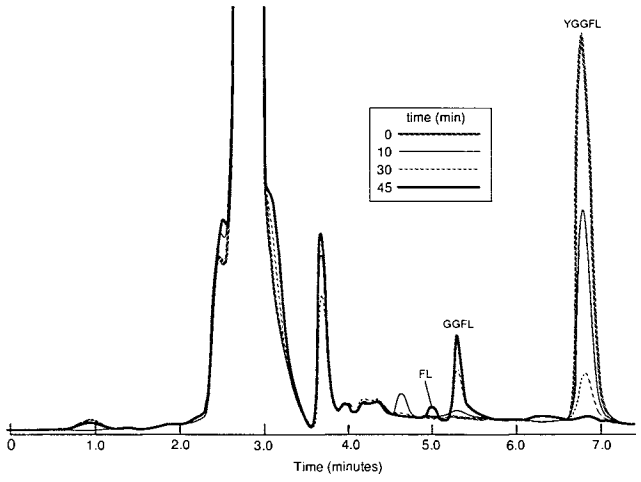

Figure 1. HPLC chromatograms of leucine enkephalin degradation (YGGFL) by the homogenate preparation at times 0, 10, 30 and 45 min. Experiments were performed in 0.1 M phosphate buffer (pH 7.4) at 37°C.

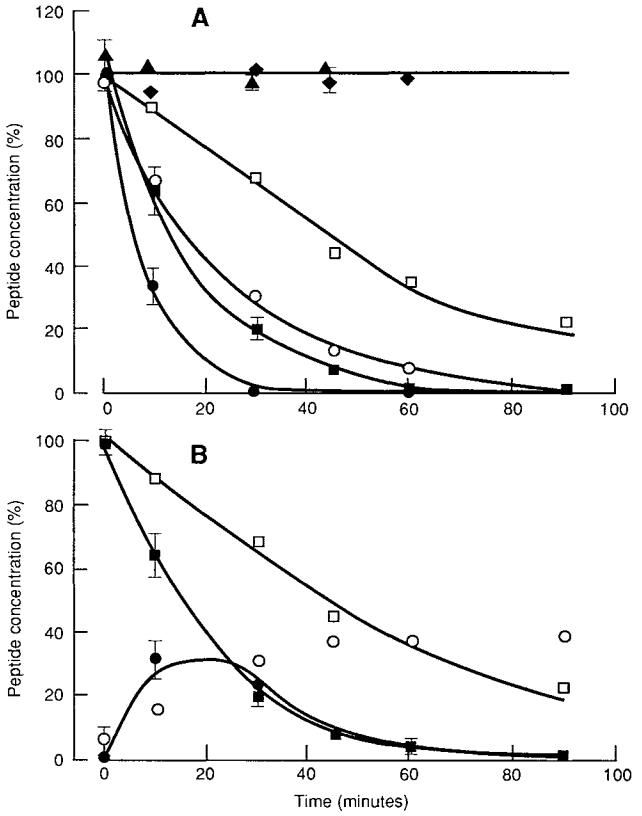

Figure 2. The degradation of leucine enkephalin (YGGFL) and *des*-tyrosine leucine enkephalin (GGFL) using nasal wash and homogenate preparations in 0.1 M phosphate buffer at 37°C. (A, disappearance of YGGFL and GGFL from wash and homogenate preparations; B, reaction profiles for degradation of YGGFL in wash and homogenate preparations. ■, YGGFL homogenate; ●, GGFL homogenate; □, YGGFL wash; ○, GGFL wash; ♦, YGGFL control; ▲, GGFL control. The symbols represent the experimental determinations (mean ± standard error) and lines are the theoretical profiles calculate from the determined first-order rate constants using equations 11 and 12.

Table I. First-Order Kinetic Parameters for the Degradation of Leucine Enkephalin (YGGFL) and *des*-Tyrosine Leucine Enkephalin (GGFL) in Nasal Wash and Homogenate Preparations. Values in Parentheses are Standard Deviations (σ)

Substrate	Preparation	k (min^{-1})	$t_{1/2}$ (min)
YGGFL	Wash	0.0172 (0.0011)	40
YGGFL	Homogenate	0.0581 (0.0016)	12
GGFL	Wash	0.0518 (0.0012)	13
GGFL	Homogenate	0.106 (0.0009)	7

the N-terminal end[3,24] to produce GGFL with the initial rate of hydrolysis of YGGFL being twice as fast as that of GGFL. In rabbits, initial hydrolysis at three sites resulted in the formation of GGFL and tyrosine (Y) as major products together with tyrosylglycine (YG) and tyrosylglycylglycine (YGG). In brain tissue, in addition to loss of the tyrosine residue, cleavage is also observed at the glycine-phenylalanine (G-F) bond. The former action has been attributed to a membrane-bound aminopeptidase[25] and the latter to endopeptidase EC 3.4.24.11.[26] In contrast to these results, GGFL formation was not the major product from YGGFL in human nasal washings[27] and both YGGFL and GGFL were degraded at similar rates. This order is the reverse of that observed in sheep and such differences, in both rates and mechanisms, emphasise the care needed in choosing an animal model for investigating peptide delivery.

The sequential degradation of leucine enkephalin may be modelled by a first-order sequential process in which the initial component A (YGGFL) degrades to B (GGFL) which further reacts to yield C (degradation products). This can be represented by the following reaction scheme:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

The equations which model this system are:

$$A_t = A_0 \exp(-k_1 t) \quad [11]$$

$$B_t = \frac{k_1 A_0}{k_2 - k_1} \cdot [\exp(-k_1 t) - \exp(-k_2 t)] \quad [12]$$

$$C_t = A_0 \cdot \left[1 - \frac{k_2 \exp(-k_1 t) - k_1 \exp(-k_2 t)}{k_2 - k_1}\right] \quad [13]$$

where A_t, B_t, and C_t are the concentrations of components A_t (YGGFL), B_t (GGFL) and C_t (further degradation products) respectively at time t and A_0 is the initial concentration of component A ($YGGFL_0$). The terms k_1 and k_2 are the first-order rate constants for the consecutive reactions. The rate constants, determined by iterative parameter-fitting of the time-concentration data to equations 11 and 12, using non-linear least squares regression analysis,[28] agree closely with those shown in Table I. The fit to this model of the homogenate data also confirms that degradation involves N-terminal hydrolysis.[3,24]

This degradation pathway (YGGFL→GGFL) is consistent with earlier results from sheep mucosal samples[19] where specificity for the bond between an aromatic residue and glycine was observed; loss of the amino terminal residue suggests the involvement of aminopeptidases in the degradation of YGGFL. The activity observed in the wash preparation demonstrates the presence of either loosely bound or luminal enzymes. These include leucine aminopeptidase which was also found to degrade leucine enkephalin, to yield GGFL, *via* first-order kinetics.

When a peptide, which contains aromatic residues, interacts with a cyclodextrin, the situation portrayed by Eq. 1 will pertain (assuming a 1:1 complex) with both free and complexed drug existing together in equilibrium. Phenylalanine forms a complex with β-cyclodextrin but with little agreement on the magnitude of K_s with values of 1000 l mol^{-1},[29] determined by ^{13}C nmr contrasting with a value of ~20 l mol^{-1} obtained from freezing-point depression measurements.[30] In view of the hydrophilic nature of phenylalanine, an estimate at the lower end of this range might be appropriate. Modelling (Eq. 9) of the complexation of phenylalanine-containing peptides (K_s, 50–1000 l mol^{-1}, P_0, 0.5 mM) in the presence of increasing concentrations of cyclodextrin shows that the proportion of free peptide may be substantially reduced by the addition of cyclodextrin. As it is unlikely that complexed peptide can interact effectively with the active site of peptidases, the degradation rate should, thus, be dependent upon the concentration of free peptide and should be reduced compared to a cyclodextrin-free system. This analysis assumes that the enzyme has little effect on the peptide-cyclodextrin equilibrium; additionally, any potential effect may be only observed within certain concentration limits. The rate of an enzyme-catalysed reaction is modelled by the Michaelis-Menten equation (Eq. 14).

$$v = \frac{V_{max}\,[S]}{K_m + [S]} \quad [14]$$

where v is the rate of the reaction at a given time, [S] is the substrate concentration, V_{max} is the maximum reaction rate and K_m is the Michaelis constant, equal to the substrate concentration at which the reaction rate is half its maximum value. If $[S] \gg K_m$, this model approximates to a zero order system ($v = V_{max}$) and the rate is independent of substrate concentration. Thus, if free peptide concentrations greatly exceed the Michaelis constant ($[S] \geq 10K_m$), no effect on the degradation rate would be expected.

Our earlier work on the stability of peptides in sheep nasal enzyme systems[22] has provided estimates of K_m values; these include GGF (12.4×10^{-4} M) and FGG (4.45×10^{-4} M). These estimates were used to determine appropriate concentrations of peptide; 0.5 mM solutions were used in each case and the effect of cyclodextrin on stability was determined. The degradation of FGG at this concentration, by cytosolic leucine aminopeptidase, yielded F and GG; reaction profiles approximated to the first-order case and this model was used to obtain first-order degradation rate constants ($k = V_{max}/K_m$). With no added β-cyclodextrin, a degradation rate constant of 0.0252 min^{-1} was found. Little effect was seen when β-cyclodextrin was added to the system and, across the range of concentrations used (FGG:β-CD; 1:1, 1:5: 1:10, 1:25), a mean rate constant was found to be 0.0278 (σ, 0.00292) min^{-1}. Although the 1:25 combination had a somewhat lower rate (0.0236 min^{-1}), the effect is marginal. In contrast, β-cyclodextrin was found to exert a pro-

Table II. Degradation Rates for the Hydrolysis of Glycylglycylphenylalanine (GGF) by Carboxypeptidase-A (CPA) in the Presence of β-Cyclodextrin (β-CD). Values in Parentheses are Standard Deviations (σ)

	CPA (20 units)		CPA (10 units)	
Ratio β-CD:GGF	k (min^{-1})	$t_{1/2}$ (min)	k (min^{-1})	$t_{1/2}$ (min)
0	0.0232 (0.0022)	29.9	0.0148 (0.0018)	47.0
1:1	0.0238 (0.0014)	29.1	0.0150 (0.0026)	46.1
1:5	0.0223 (0.0007)	31.0	0.0130 (0.0013)	53.5
1:10	0.0163 (0.0009)	42.5	0.0117 (0.0010)	59.5
1:25	0.0164 (0.0010)	42.2	0.0083 (0.0005)	83.1

tective effect against the degradation of GGF. This peptide is a substrate for carboxypeptidase A with fission occurring at the aromatic residue to yield GG and F. Table II records the effect of added β-cyclodextrin on the stability of GGF. With 20 units of carboxypeptidase A, little effect is seen when equal concentrations of peptide and β-cyclodextrin are present but, as further increases in β-cyclodextrin concentration are made, a measurable decrease in degradation rate is seen. At the highest β-cyclodextrin concentration used, which corresponds to ~80% saturation, the half-life was extended by ~40%. When a lower enzyme burden is present (10 units), the reaction rate falls and its dependence on β-cyclodextrin concentration becomes greater. In this instance, an increase in half-life of ~77% was obtained.

A similar series of β-cyclodextrin solutions was used to study the stabilisation of leucine enkephalin against attack by leucine aminopeptidase. The first-order degradation rate constant for YGGFL was found to be 0.0159 min^{-1} ($t_{1/2}$ 43.7 min). Little effect was again seen when β-cyclodextrin was added in 1:1, 1:5 and 1:10 ratios with the mean rate constant for this set being found to be 0.0158 min^{-1} (σ, 0.00025; $t_{1/2}$ 43.9 min). However, when the highest concentration of β-cyclodextrin was used (1:25), a substantial fall in the rate constant, to 0.00929 min^{-1} ($t_{1/2}$ 74.6 min), was observed. This is a significant increase in stability and it is unlikely that it is due to, for example, inactivation of the enzyme by the cyclodextrin as similar conditions had little effect upon the degradation of FGG. In no case did γ-cyclodextrin show a discernable effect upon degradation rate, perhaps because the larger cavity diameter (β-cyclodextrin, 6.0–6.5 nm; γ-cyclodextrin 7.5–8.3 nm) probably results in smaller association constants and less complexed peptide.

This work has shown that a small, but measurable protection against the enzymic degradation of peptides containing an aromatic residue is afforded in the presence of cyclodextrin. Although the magnitude of the effect seen here is insufficient to account for major increases in bioavailability, pointers appropriate to enhancing the protective potential of cyclodextrins may be deduced. Equation 9 show that the concentration of free peptide falls rapidly with increasing cyclodextrin concentration. It is unlikely that isolated complexes (1:1) would be beneficial as there may be very little complexed peptide available on dissolution, particularly if K_s is small; excess cyclodextrin is therefore essential. The use of more soluble cyclodextrins such as hydroxypropyl-β-cyclodextrin and dimethyl-β-cyclodextrin at higher concentrations could furnish greater amounts of complexed peptide. This is compatible, for example, with the use of excess, soluble cyclodextrins[9,10] to enhance the nasal delivery of insulin and an ACTH hexapeptide. Additionally, larger K_s values enhance complexation so that maximisation of this parameter should enhance protection. In the present study, the low association constants leave a large proportion of free drug in solution, even at the most concentrated cyclodextrins systems used. However, although large increases in interaction may not be readily achieved with peptides, compounds which are substrates for alternative enzymic systems (*e.g.* esterases) may offer a greater potential to test this hypothesis. The final parameter of importance is the drug concentration within the nasal cavity. This should probably be as high as possible to maximise trans-membrane concentration gradients. Here, the potential protection afforded by complexation is likely to be poor as $[S] \gg K_m$; however, under these circumstances, unless both K_m and K_s are large, saturation of the enzymatic capacity may be expected.

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