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Kinetics of degradation and oil solubility of ester prodrugs of a model dipeptide (Gly-Phe)

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Abstract

Oil-based depot formulations may constitute a future delivery method for small peptides. Thus, a requirement is attainment of sufficient oil solubility for such active compounds. A model dipeptide (Gly-Phe) has been converted into lipophilic prodrugs by esterification at the C-terminal carboxylic acid group. The decomposition kinetics of octyl ester of Gly-Phe (IV) has been investigated at pH 7.4 (37 °C) and IV was shown to degrade by first-order kinetics via two parallel pathways (1) intramolecular aminolysis resulting in formation of a 2,5-diketopiperazine and (2) hydrolysis of the ester bond producing the dipeptide. The cyclisation reaction was dominating in the decomposition of methyl (II) butyl (III) octyl (IV) decyl (V) and dodecyl (VI) esters of Gly-Phe at pH 7.4. However, this degradation pathway was almost negligible for pH below 6. During degradation of the dipeptide esters in 80% human plasma pH 7.4 (37 °C) a minimal amount of cyclo(-Gly-Phe) was formed. A faster degradation of the esters in 80% human plasma pH 7.4 compared to those in aqueous solution pH 7.4 was suggested to be due to fast cleavage of the peptide bond. Low oil solubilities for Gly-Phe and the hydrochlorides of the dipeptide esters III and VI were observed. Although the solubility of Gly-Phe in oil solutions was enhanced by hydrophobic ion pairing with sodium decyl sulfonate the oil solubility was still less than 1 mg Gly-Phe/ml. By addition of a solubiliser, 10% *N*,*N*-dimethylacetamide (DMA), to Viscoleo the solubility of the HIP complexes increased significantly. The present study indicates that sufficient oil solubility might only be obtained for relatively small peptides by using the prodrug approach in combination with solubility enhancing organic solvents like DMA.

Keywords: Parenteral depots; Dipeptide ester prodrugs; Oil solubility; Hydrophobic ion pair; Intramolecular cyclisation

1. Introduction

Parenteral delivery techniques capable of controlling the release of smaller peptides and peptidomimetics are of considerable interest. Various approaches in the field of parenteral administration of peptide and protein compounds have been investigated (Cleland et al., 2001; Rothen-Weinhold and Gurny, 1997). To this end, the potential applicability of peptide delivery by parenteral oil-based depots has not gain much attention. The feasibility of administering such polar drug substances in the form of oil solutions is governed by the attainment of sufficient oil solubility. Increased lipophilicity of peptide-like compounds has been obtained by the use of the prodrug approach (Bundgaard, 1991). Recently, we have synthesized 4-imidazolidinone prodrugs of the polar local anaesthetic agent prilocaine, which like many peptides contains an α-aminoamide moiety, with the objective to design depot oil injectables (Larsen et al., 2003). In the present study attempts to enhance the oil solubility of a dipeptide by creating lipophilic derivatives were investigated. A model dipeptide (Gly-Phe) was converted into lipophilic prodrugs by esterification at the free carboxylic acid at the C-terminal. A second approach based on formation of hydrophobic ion pairs has previously been applied to increase the solubility of peptides and proteins in organic solvents (Meyer and Manning, 1998). Thus, in order to investigate the effect of "masking" the positive charge of the ester prodrugs on the oil solubility hydrophobic ion pairs have been formed between the positive charged N-terminal of the ester prodrugs and sodium decyl sulfonate. The degradation kinetics including investigation of intramolecular cyclisation of the dipeptide esters has also been studied.

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2. Materials and methods

2.1. Materials

In the synthesis of Gly-Phe esters the following chemicals were used: 4-dimethylaminopyridine 99% and 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride 98% from Aldrich (Germany); *N*,*N*-diisopropylethylamine and Phe-*O*-methyl ester, HCl from Fluka Chemie (Switzer-land); 1-hydroxybenzotriazole, Boc-Phe-OH, Boc-Phe-OH from Novo Nordisk A/S (Denmark); 1-butanol from Rieldel de Häen (Germany); 1-decanol and from Lancaster (England); 1-octanol and *n*-dodecanol from Merck (Germany). Cyclo(-Gly-Phe) was obtained from Bachem (Switzerland) and Gly-Phe and castor oil were from Sigma (USA). Fractionated coconut oil (Viscoleo®) and *N*,*N*-dimethylacetamide 99% (DMA) were purchased from P. Broeste A/S (Denmark) and Acros Organics (USA), respectively. Sodium decyl sulfonate (1-decanesulfonic acid sodium salt) was obtained from Buchem bv (Holland). Other chemicals and solvents were of analytical or chromatographic grade. Demineralized water was used throughout.

2.2. Synthesis of hydrochlorides of Gly-Phe esters

2.2.1. Esterification of Phe

2.2.1.1. Boc-Phe-O-butyl ester. To an ice-cold stirred solution of Boc-Phe-OH (1.91 g, 7.2 mmol), 1-butanol (0.66 ml, 7.2 mmol) and 4-dimethylaminopyridine (0.44 g, 3.6 mmol) in 50 ml of dichloromethane was added 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (1.38 g, 7.2 mmol). The reaction mixture was stirred at 0 °C for 3 h, and overnight at ambient temperature. The reaction mixture was transferred to a separating funnel with 100 ml dichloromethane. The dichloromethane layer was washed with 10% $NaHSO_4$ (50 ml), H_2O (50 ml), saturated aqueous $NaHCO_3$ (50 ml), H_2O (50 ml) and saturated aqueous NaCl (50 ml). The organic layer was dried with anhydrous $MgSO_4$ and concentrated under reduced pressure to an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.91 (m, 3H, CH_3), 1.32 (b, 2H, CH_2), 1.42 (s, 9H, *t*-butyl), 1.56 (m, 2H, CH_2), 3.08 (m, 2H, CH_2), 4.09 (m, 2H, CH_2), 4.57 (m, 1H, CH), 4.97 (b, 1H, NH), 7.13–7.31 (m, 5H); LC–MS: R_t = 7.00 min, m/z = 322.4 (m + 1).

2.2.1.2. Phe-O-butyl ester, HCl. Boc-Phe-*O*-butyl ester (2.1 g, 6.6 mmol) was dissolved in ethyl acetate followed by 3.8 M HCl in ethyl acetate. After 30 min the reaction mixture was rotary evaporated under reduced pressure to obtain a white powder. ^{1}H NMR (400 MHz, DMSO) δ: 0.82 (m, 3H, CH_3), 1.18 (m, 2H, CH_2), 1.42 (m, 2H, CH_2), 3.02–3.24 (m, 2H, CH_2), 4.03 (m, 2H, CH_2), 4.24 (m, 1H, CH), 7.24–7.36 (m, 5H), 8.66 (b, 3H, NH_3); LC–MS: R_t = 3.63 min, m/z = 222.4 (m + 1).

2.2.1.3. Boc-Phe-O-octyl ester. Same procedure as with Boc-Phe-*O*-butyl ester. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.89 (m, 3H, CH_3), 1.27 (b, 10H, $(CH_2)_5$), 1.42 (s, 9H, *t*-butyl), 1.56 (m, 2H, CH_2), 3.08 (m, 2H, CH_2), 4.08 (m, 2H, CH_2), 4.57 (m, 1H, CH), 4.97 (b, 1H, NH), 7.12–7.30 (m, 5H); LC–MS: R_t = 8.52 min, m/z = 378.4 (m + 1).

2.2.1.4. Phe-O-octyl ester, HCl. Same procedure as with Phe-*O*-butyl ester, HCl. ^{1}H NMR (400 MHz, DMSO) δ: 0.86 (m, 3H, CH_3), 1.22 (m, 10H, $(CH_2)_5$), 1.42 (m, 2H, CH_2), 3.02–3.25 (m, 2H, CH_2), 4.02 (m, 2H, CH_2), 4.24 (b, 1H, CH), 7.24–7.35 (m, 5H), 8.69 (b, 3H, NH_3); LC–MS: R_t = 5.23 min, m/z = 278.4 (m + 1).

2.2.1.5. Boc-Phe-O-decyl ester. Same procedure as with Boc-Phe-*O*-butyl ester. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.88 (m, 3H, CH_3), 1.27 (b, 14H, $(CH_2)_7$), 1.42 (s, 9H, *t*-butyl), 1.57 (m, 2H, CH_2), 3.08 (m, 2H, CH_2), 4.08 (m, 2H, CH_2), 4.57 (m, 1H, CH), 4.97 (b, 1H, NH), 7.12–7.30 (m, 5H); LC–MS: R_t = 9.12 min, m/z = 406.4 (m + 1).

2.2.1.6. Phe-O-decyl ester, HCl. Same procedure as with Phe-*O*-butyl ester, HCl. ^{1}H NMR (400 MHz, DMSO) δ: 0.86 (m, 3H, CH_3), 1.24 (m, 14H, $(CH_2)_7$), 1.43 (m, 2H, CH_2), 3.02–3.23 (m, 2H, CH_2), 4.02 (m, 2H, CH_2), 4.24 (b, 1H, CH), 7.23–7.35 (m, 5H), 8.62 (b, 3H, NH_3); LC–MS: R_t = 5.35 min, m/z = 306.4 (m + 1).

2.2.1.7. Boc-Phe-O-dodecyl ester. Same procedure as with Boc-Phe-*O*-butyl ester. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.88 (m, 3H, CH_3), 1.26 (b, 18H, $(CH_2)_9$), 1.42 (s, 9H, *t*-butyl), 1.58 (m, 2H, CH_2), 3.08 (m, 2H, CH_2), 4.08 (m, 2H, CH_2), 4.57 (m, 1H, CH), 4.98 (b, 1H, NH), 7.12–7.31 (m, 5H); LC–MS: R_t =9.65 min, m/z = 434.4 (m + 1).

2.2.1.8. Phe-O-dodecyl ester, HCl. Same procedure as with Phe-*O*-butyl ester, HCl. ^{1}H NMR (400 MHz, DMSO) δ: 0.86 (m, 3H, CH_3), 1.24 (m, 18H, $(CH_2)_9$), 1.42 (m, 2H, CH_2), 3.01–3.23 (m, 2H, CH_2), 4.02 (m, 2H, CH_2), 4.25 (m, 1H, CH), 7.23–7.35 (m, 5H), 8.65 (b, 3H, NH_3); LC–MS: R_t =6.53 min, m/z = 334.2 (m + 1).

2.2.2. Synthesis of Gly-Phe esters (Fig. 1)

2.2.2.1. Boc-Gly-Phe-O-methyl ester. A solution of Boc-Gly-OH (1.49 g, 8.5 mmol), 1-hydroxybenzotriazole (1.30 g, 8.5 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (1.63 g, 8.5 mmol) in 30 ml of dimethylformamide was stirred at ambient temperature for 30 min. To the solution *N*,*N*-diisopropylethylamine (2.20 g, 17 mmol) and Phe-*O*-methyl ester, HCl (1.52 g, 8.5 mmol) were added and the reaction mixture was stirred at ambient temperature overnight. The reaction mixture was transferred to a separating funnel with 100 ml ethyl acetate. The ethyl acetate layer was washed with 10% $NaHSO_4$ (50 ml), H_2O (50 ml), saturated aqueous $NaHCO_3$ (50 ml), H_2O (50 ml)

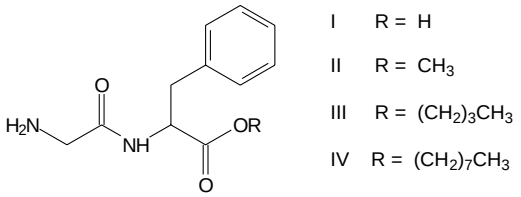


Fig. 1. Chemical structures of Gly-Phe (I) and ester prodrugs thereof (II–VI).

and saturated aqueous NaCl (50 ml). The organic layer was dried with anhydrous $MgSO_4$ and concentrated under reduced pressure to an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.42 (s, 9H, *t*-butyl), 1.42 (s, 3H, CH_3), 1.45 (s, 3H, CH_3), 3.13 (m, 2H, CH_2), 3.70 (m, 3H, CH_3), 4.85 (m, 1H, CH), 4.85 (b, 1H, NH), 6.86 (b, 1H, NH) 7.11–7.30 (m, 5H); LC–MS: $R_t = 4.75$ min, $m/z = 337.4$ ($m + 1$).

2.2.2.2. Gly-Phe-O-methyl ester, HCl (compound II). Boc-Gly-Phe-*O*-methyl ester (2.1 g, 6.3 mmol) was dissolved in ethyl acetate followed by 3.8 M HCl in ethyl acetate. After 30 min a white powder precipitates. The powder was isolated and dried. ^{1}H NMR (400 MHz, DMSO) δ: 3.00 (m, 2H, CH_2), 3.53 (m, 2H, CH_2), 3.62 (s, 3H, CH_3), 4.56 (m, 1H, CH), 7.21–7.32 (m, 5H), 8,13 (s, 3H, NH_3), 8.99 (b, 1H, NH); LC–MS: $R_t = 2.35$ min, $m/z = 237.4$ ($m + 1$); Anal. Calcd. ($C_{12}H_{16}N_2O_3$, HCl): C, 52.85; H, 6.28; N, 10.27; Found: C, 52.50; H, 6.32; N, 10.04; HPLC: $R_t = 16.1$ min (A1), $R_t = 18.2$ min (B1).

2.2.2.3. Boc-Gly-Phe-O-butyl ester. Same procedure as with Boc-Gly-Phe-*O*-methyl ester. LC–MS: $R_t = 5.95$ min, $m/z = 379.4$ ($m + 1$).

2.2.2.4. Gly-Phe-O-butyl ester, HCl (compound III). Same procedure as with Gly-Phe-*O*-methyl ester, HCl. However, after 30 min the reaction mixture was rotary evaporated under reduced pressure to obtain a brown powder. The product was further purified by recrystallization from ethanol. ^{1}H NMR (400 MHz, DMSO) δ: 0.85 (m, 3H, CH_3), 1.24 (m, 2H, CH_2), 1.48 (m, 2H, CH_2), 2.92–3.06 (m, 2H, CH_2), 3.53 (m, 2H, CH_2), 4.01 (m, 2H, CH_2), 4.54 (m, 1H, CH), 7.21–7.32 (m, 5H), 8,09 (b, 3H, NH_3), 8.96 (b, 1H, NH); LC–MS: $R_t = 4.08$ min, $m/z = 279.4$ ($m + 1$); Anal. Calcd. ($C_{15}H_{22}N_2O_3$, HCl): C, 57.23; H, 7.36; N, 8.90; Found: C, 57.61; H, 7.47; N, 8.87; HPLC: $R_t = 27.2$ min (A1), $R_t = 29.1$ min (B1).

2.2.2.5. Boc-Gly-Phe-O-octyl ester. Same procedure as with Boc-Gly-Phe-*O*-methyl ester. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.89 (m, 3H, CH_3), 1.28 (b, 10H, $(CH_2)_5$), 1.44 (s, 8H, *t*-butyl), 1.59 (b, 2.7H, CH_2), 3.12 (m, 2H, CH_2), 3.80 (m, 2H, CH_2), 3.90 (m, 2H, CH_2), 4.88 (m, 1H, CH), 5.03 (b, 1H, NH), 6.46 (b, 1H, NH), 7.09–7.30 (m, 5H); LC–MS: $R_t = 7.72$ min, $m/z = 435.4$ ($m + 1$).

2.2.2.6. Gly-Phe-O-octyl ester, HCl (compound IV). Same procedure as with Gly-Phe-*O*-methyl ester, HCl. However, after 30 min the reaction mixture was rotary evaporated under reduced pressure to obtain a brown powder. The product was further purified by recrystallization from ethanol/*tert*-butylmethylether. ^{1}H NMR (400 MHz, DMSO) δ: 0.86 (m, 3H, CH_3), 1.23 (b, 10H, $(CH_2)_5$), 1.48 (m, 2H, CH_2), 2.99 (m, 2H, CH_2), 3.54 (m, 2H, CH_2), 3.98 (m, 2H, CH_2), 4.53 (m, 1H, CH), 7.21–7.31 (m, 5H), 8.19 (b, 3H, NH_3), 9.02 (b, 1H, NH); LC–MS: $R_t = 5.26$ min, $m/z = 335.4$ ($m + 1$); Anal. Calcd. ($C_{19}H_{30}N_2O_3$, HCl): C, 61.53; H, 8.42; N, 7.55; Found: C, 61.64; H, 8.76; N, 7.56; HPLC: $R_t = 39.7$ min (A1), $R_t = 42.6$ min (B1).

2.2.2.7. Boc-Gly-Phe-O-decyl ester. Same procedure as with Boc-Gly-Phe-*O*-methyl ester. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.88 (m, 3H, CH_3), 1.27 (b, 14H, $(CH_2)_7$), 1.44 (s, 9H, *t*-butyl), 1.59 (b, 2H, CH_2), 3.12 (m, 2H, CH_2), 3.79 (m, 2H, CH_2), 4.09 (m, 2H, CH_2), 4.87 (m, 1H, CH), 5.04 (b, 1H, NH), 6.47 (b, 1H, NH), 7.09–7.30 (m, 5H); LC–MS: $R_t = 8.67$ min, $m/z = 463.6$ ($m + 1$).

2.2.2.8. Gly-Phe-O-decyl ester, HCl (compound V). Same procedure as with Gly-Phe-*O*-methyl ester, HCl. However, after 30 min the reaction mixture was rotary evaporated under reduced pressure to obtain a brown powder. The product was dissolved in water and applied to a C-18 Sep-Pak cartridge (10 g), which had been prewashed with isopropanol and water. Then a gradient of an eluent consisting of water/acetonitril (+0.1% HCl) (10, 20, 30, 40, 50 and 60% acetonitrile in water) was run through the Sep-Pak. The relevant fractions were combined and freeze-dried to obtain a white powder. ^{1}H NMR (400 MHz, DMSO) δ: 0.86 (m, 3H, CH_3), 1.24 (b, 14H, $(CH_2)_7$), 1.48 (m, 2H, CH_2), 2.99 (m, 2H, CH_2), 3.54 (m, 2H, CH_2), 3.99 (m, 2H, CH_2), 4.54 (m, 1H, CH), 7.22–7.31 (m, 5H), 8.08 (b, 3H, NH_3), 8.95 (b, 1H, NH); LC–MS: $R_t = 6.15$ min, $m/z = 363.2$ ($m + 1$); Anal. Calcd. ($C_{21}H_{34}N_2O_3$, HCl): C, 63.22; H, 8.84; N, 7.02; Found: C, 63.41; H, 8.83; N, 6.99; HPLC: $R_t = 44.9$ min (A1), $R_t = 48.6$ min (B1).

2.2.2.9. Boc-Gly-Phe-O-dodecyl ester. Same procedure as with Boc-Gly-Phe-*O*-methyl ester. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.89 (m, 3H, CH_3), 1.26 (b, 18H, $(CH_2)_9$), 1.44 (s, 8H, *t*-butyl), 1.58 (m, 2H, CH_2), 3.12 (m, 2H, CH_2), 3.79 (m, 2H, CH_2), 4.08 (m, 2H, CH_2), 4.86 (m, 1H, CH), 5.05 (b, 1H, NH), 6.48 (b, 1H, NH), 7.09–7.30 (m, 5H); LC–MS: $R_t = 8.92$ min, $m/z = 491.4$ ($m + 1$).

2.2.2.10. Gly-Phe-O-dodecyl ester, HCl (compound VI). Same procedure as with Gly-Phe-*O*-methyl ester, HCl. However, after 30 min the reaction mixture was rotary evaporated

under reduced pressure to obtain a white powder. The product was further purified by recrystallization from ethanol. ^{1}H NMR (400 MHz, DMSO) δ: 0.85 (m, 3H, CH_3), 1.24 (b, 18H, $(CH_2)_9$), 1.48 (m, 2H, CH_2), 2.91–3.05 (m, 2H, CH_2), 3.54 (m, 2H, CH_2), 3.99 (m, 2H, CH_2), 4.54 (m, 1H, CH), 7.22–7.31 (m, 5H), 8.04 (b, 3H, NH_3), 8.92 (b, 1H, NH); LC–MS: $R_t = 7.03$ min, $m/z = 391.2$ ($m + 1$); Anal. Calcd. ($C_{23}H_{38}N_2O_3$, HCl): C, 64.69; H, 9.21; N, 6.56; Found: C, 64.88; H, 9.56; N, 6.54; HPLC: $R_t = 39.79$ min (B6).

2.3. Hydrophobic ion pairing between Gly-Phe esters and sodium decyl sulfonate

Hydrophobic ion pair (HIP) complexes between sodium decyl sulfonate and compounds III and VI were formed by the following procedure: To an aqueous solution of the hydrochloride of the Gly-Phe ester an equimolar amount of sodium decyl sulfonate dissolved in demineralised water was added. The HIP complex precipitated from the solution and after stirring for 5 min the complex was isolated and freeze-dried. The concentration of sodium decyl sulfonate was kept below the critical micelle concentration (CMC: 44 mM (Bromberg and Klibanov, 1994)).

2.4. Kinetic measurements

2.4.1. Degradation in aqueous buffer solutions

The degradation of compound IV was investigated in the pH interval 1.14–9.67 whereas the stability of compounds II, III, V and VI only were investigated at pH 7.38. All rate studies were carried out in aqueous buffer solution at 37 ± 0.5 °C and the buffers used were phosphate (pH 6.02–7.38) and borate (pH 8.22–9,67). At pH below 2.5 hydrochloric acid was used. In the pH range 8.22–9.67, the influence of the borate buffer on the degradation rate was investigated using three different buffer concentrations, 0.02, 0.05 and 0.1 M. At pH 6.02–7.38, the degradation rates were determined at three different phosphate buffer concentrations, 0.005, 0.01 and 0.015 M. A constant ionic strength of 0.5 was maintained for each buffer by adding a calculated amount of potassium chloride. The reactions were in most cases initiated by adding 100 μl of a 1×10^{-2} M stock solution of the compounds in methanol (in the case of compound I and cyclo(-Gly-Phe) an aqueous stock solution was used) to 10.0 ml of preheated buffer solution resulting in a final concentration of approximately 1×10^{-4} M. At appropriate time intervals samples were withdrawn and analysed immediately by HPLC. In case of fast reactions (at pH 10), samples were taken and hydrochloric acid was added to pH 5 prior to analysis in order to stop the degradation process.

Pseudo-first-order degradation rate constants (k_{obs}) were determined from the slopes of linear plots of the logarithm of intact ester prodrug against time. For the slowly proceeding reaction at pH 2.14 and 6.02 the pseudo-first-order rate constant was derived by using the initial rate method (Connors, 1973). These reactions were followed for a time period corresponding to 1–2% degradation of compound IV.

2.4.2. Degradation in human plasma

The stability of the compounds I–VI, cyclo(-Gly-Phe) and Phe-*O*-methyl ester was investigated in human plasma: 0.1 M phosphate pH 7.4 4:1 (v/v). The reactions were initiated by adding 50 μl of a stock solution in methanol (in the case of compound I and cyclo(-Gly-Phe) an aqueous stock solution was used) to 5 ml of preheated plasma to give an initial concentration of 1×10^{-4} to 4×10^{-4} M. The reaction mixture was kept in a water bath at 37 ± 0.5 °C and at appropriate time intervals samples were taken and deproteinised with either acetonitrile or methanol in the ratio of 1:2 (v/v) and 1:3 (v/v), respectively. The mixture was immediately vortexed and after centrifugation for 3 min at 13 000 rpm the supernatant was analysed by HPLC for remaining ester. For the compounds II–VI the amount of cyclo(-Gly-Phe) formed was determined at the end of the study.

2.5. Solubility measurements

The solubility of compound III, VI, Gly-Phe and the decyl sulfonate salts of compound III and VI was measured in Viscoleo, Viscoleo/castor oil 2:1 (v/v) and Viscoleo/DMA 9:1 (v/v). To 1 ml oil solution in a screw capped test tube an amount of the investigated compound was added and the tube was rotated at ambient temperature. Addition of compound was repeated until solid material was remaining after rotating for at least 3 days. The supernatant was transferred to an Eppendorf tube and centrifuged at 13 000 rpm for 20 min. Hundred microliters of the supernatant was adequately diluted with ethanol prior to HPLC analysis. A solution containing less than 5% (v/v) oil in ethanol was applied to the column. The amount of Gly-Phe in the oil solution was quantified by peak area measurements in comparison to those of standards chromatographed under the same conditions.

2.6. Determination of pK_a value of compound III

The pK_a value of compound III was determined by three independent potentiometric titrations running from pH 3 to 10 at 37 ± 0.5 °C and at an ionic strength of $\mu = 0.5$ under a slow argon flow. A Sirius GLpK_a instrument with Sirius pK_a LOGP^{TM} revision 5.2a software from Sirius Analytical Instruments (Forest Row, UK) was used.

2.7. Analyses

2.7.1. ^{1}H NMR, LC/MS and elemental analyses

^{1}H NMR were recorded on a Bruker 400 MHz using $CDCl_3$ or d_6-DMSO as solvent, and chemical shifts (δ) are in ppm relative to tetramethylsilane.

Elemental analyses were obtained from Novo Nordisk Microlab.

The LC–MS method consisting of a HP1100 MSD equipped with binary pump, column compartment, diode array detector and a single quadrupole mass spectrometer detector. Electrospray ionisation was performed in positive ion made mode. The analysis was performed at 40 °C on 3 mm × 50 mm YMC-pack ODS-A column. The mobile phase consisted of mobile phase A: 0.1% TFA in 5% acetonitrile, and mobile phase B: 0.1% TFA in 90% acetonitrile. A linear gradient from 0 to 100% B over 15 min was performed using a flow rate of 1 ml/min.

2.7.2. HPLC methods

Solutions of compounds II–V dissolved in methanol were analysed using two HPLC systems. HPLC (method A1): The HPLC analysis was performed using a Waters 2690 systems fitted with a Waters 996 diode array detector. UV detections were collected at 214, 254, 276, and 301 nm on a 218TP54 4.6 mm × 250 mm × 5 μm C-18 silica column (The Seperations Group, Hesperia), which was eluted at 1 ml/min at 42 °C. The column was equilibrated with 5% acetonitrile in a buffer consisting of 0.1 M ammonium sulphate adjusted to pH 2.5 with 4 M sulphuric acid. After injection the sample was eluted by a gradient of 5–60% acetonitrile in the same aqueous buffer during 50 min. HPLC (method B1): The HPLC-analysis was performed on same system described in method A1 using another gradient system. The column was equilibrated with mobile phase consisting of 0.1% TFA in 5% acetonitrile. After injection the sample was eluted by a gradient of 5–60% acetonitrile (with 0.1% TFA added) during 50 min. The HPLC-analysis of compound VI was performed as described in method B1 however a gradient of 5–90% acetonitrile was used (method B6).

Samples from the stability studies were analysed by a HPLC system consisted of a Merck Hitachi L-6000 pump connected to a Merck Hitachi L-4000 UV detector. Reversed phase chromatography was carried out using an Inertsil ODS-2 (250 mm × 4.6 mm, 5 μm particles) column equipped with an Inertsil ODS-2 precolumn (Chrompack). The mobile phases consisted acetonitrile and 0.1% phosphoric acid in demineralised water which were made 1 mM with respect to triethylamine. The content of acetonitrile in the mobile phase was adjusted to 5–55% (v/v) to give appropriate retention time for the actual compound. The flow rate was 1 ml/min and the column effluent was monitored at 220 nm. Some of the stability studies were performed using a HPLC-system consisting of a Merck Hitachi L-7100 pump connected to a Merck-Hitachi L-7400 UV detector and with an automatically sampling procedure using a Merck Hitachi L-7200 autosampler. These reaction mixtures were kept at constant temperature by using a Merck L-7200 Peltier Sample Cooler.

Samples from the solubility measurements were analysed by a HPLC system consisting of a Shimadzu LC-6A pump and a Shimadzu SPD-6A UV detector operating at 260 nm, otherwise the HPLC analyses were performed at the same settings and conditions as described for the stability studies.

3. Results and discussion

3.1. Synthesis of hydrochlorides of Gly-Phe esters

Structures of the synthesised ester derivatives of Gly-Phe are shown in Fig. 1. The compounds were prepared by the following steps: Esterification of the Boc protected phenylalanine using the appropriate alcohol, removal of the protecting group with hydrochloric acid in ethyl acetate, coupling between the hydrochloride of the phenylalanine ester and the Boc protected glycine, removal of the protecting group with hydrochloric acid in ethyl acetate and if necessary purification by recystallization or by using Sep-Pak cartridge followed by freeze-drying.

The synthesis processes were not optimized thus product yields of the ester derivatives were 40–50%. ^{1}H NMR, LS/MS and elemental analyses confirm the structures of the compounds and the compounds were of high purity (HPLC system A1, B1 and B6 > 99%). The retention times (R_t) of the compounds in the gradient HPLC systems A1 and B1 increased from compound II (16 and 18 min) to compound V (45 and 49 min) and with R_t of compound VI exceeding the analysis time of 50 min. By using another HPLC gradient system (method B6) the lipophilic compound VI could be detected.

3.2. Stability studies

The overall degradation of all ester prodrugs followed apparent first-order kinetics at fixed pH, temperature, ionic strength and buffer concentration. Product analysis (decomposition in 0.1 M phosphate buffer pH 7.38 at 37 ± 0.5 °C) revealed that the disappearance of compound IV was accompanied by parallel formation of Gly-Phe and cyclo(-Gly-Phe) (Fig. 2).

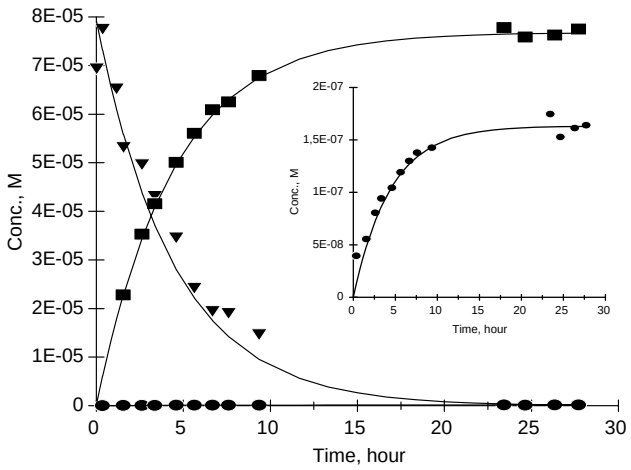
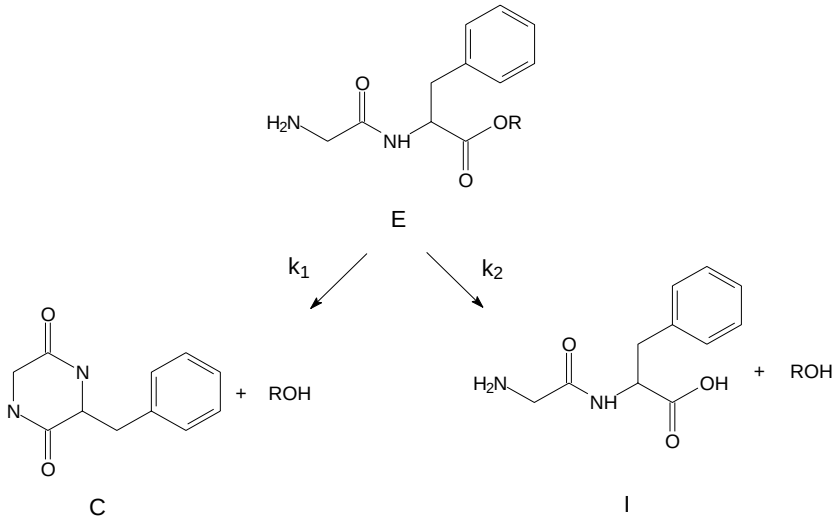


Fig. 2. Time course for compound IV (▼), Gly-Phe (●) and cyclo(-Gly-Phe) (■) during the degradation of compound IV in 0.1 M phosphate buffer at pH 7.38 at 37 ± 0.5 °C. The full lines have been derived using Eqs. (2) and (3).



Scheme 1.

Through the experiment the sum of the concentration of compound IV and the two products was 100 ± 3% and cyclo(-Gly-Phe) was the major degradation product (99.8%) formed. Compound IV is suggested to degrade via two parallel pathways as depicted in Scheme 1; intramolecular aminolysis resulting in formation of a 2,5-diketopiperazine (cyclo(-Gly-Phe)) and direct hydrolysis of the ester bond producing the dipeptide (Gly-Phe). Thus, the overall rate constant k_{obs} can be expressed:

$$k_{obs} = k_1 + k_2 \quad (1)$$

where k_1 and k_2 are the first-order rate constants for the cyclisation process and the hydrolysis of the ester bond, respectively. The rate constants were obtained from the following expressions:

$$\ln([C]_\infty - [C]) = \ln\left(\frac{k_1}{k_{obs}}[E]_0\right) - k_{obs}t \quad (2)$$

$$\ln([I]_\infty - [I]) = \ln\left(\frac{k_2}{k_{obs}}[E]_0\right) - k_{obs}t \quad (3)$$

where $[I]$ and $[C]$ are the concentration of Gly-Phe and cyclo(-Gly-Phe), respectively. Calculation of k_1 and k_2 was performed from the intercepts using the obtained k_{obs} and the initial concentration of compound IV expressed as $[E]_0$. The overall rate constant k_{obs} ($3.6 \times 10^{-3}\,min^{-1}$) calculated by the sum of the obtained k_1 ($3.6 \times 10^{-3}\,min^{-1}$) and k_2 ($6.9 \times 10^{-6}\,min^{-1}$) was in good agreement with the k_{obs} ($3.9 \times 10^{-3}\,min^{-1}$) determined from the slope of the linear plot of the logarithm of intact ester against time demonstrating the adequacy of the suggested degradation mechanism. Alternatively k_1 and k_2 were obtained from linear plots of $[C]/[E]_0$ and $[I]/[E]_0$ against $(1 - e^{-k_{obs}t})$ (Larsen and Bundgaard, 1979) according to:

$$[C] = \frac{k_1}{k_{obs}}[E]_0(1 - e^{-k_{obs}t}) \quad (4)$$

$$[I] = \frac{k_2}{k_{obs}}[E]_0(1 - e^{-k_{obs}t}) \quad (5)$$

with rate constants k_1 ($3.8\times10^{-3}\,min^{-1}$) and k_2 ($7.5 \times 10^{-6}\,min^{-1}$) being in accordance with those obtained by the former method. In conclusion, the results confirm a parallel degradation mechanism of compound IV at the experimental conditions employed. Similar degradation profiles were seen for dipeptide derivatives by Gu and Strickley (1987, 1988) and formation of 2,5-piperazinedione as a result of intramolecular aminolysis has been described for other esters of dipeptides (Jensen and Bundgaard, 1991; Meresaar and Ågren, 1968; Purdie and Benoiton, 1973) and for *p*-nitroaniline amides of a dipeptide (Goolcharran and Borchardt, 1998). In the decomposition of compound IV cyclo(-Gly-Phe) was only formed in significant amounts for pH > 6. Similarly, cyclo(-Gly-Phe) was the major degradation product in the decomposition of compounds II–VI at pH 7.4. These observations suggest that cyclo(-Gly-Phe) formation results from a nucleophilic attack of the free amino group on the carbonyl group of the ester bond.

The degradation of compound IV was subjected to general acid–base catalysis by borate buffers and the estimated rate constants at zero buffer concentration were obtained from the intercepts of the linear plots of k_{obs} versus total buffer concentration (0.02–0.1 M) as described by Larsen and Bundgaard (1977). General acid–base catalysis was also observed by phosphate buffer solutions in the decomposition of all the investigated ester prodrugs at pH 7.4 and of compound IV at pH 6.02 and 6.90. Non-linear relationship between k_{obs} and the total phosphate buffer concentration, which was most pronounced at pH 7.4 are illustrated in Fig. 3. The observed non-linear phosphate buffer-catalytic effect is in accordance with results reported by Jensen and Bundgaard (1991) and the buffer-independent pseudo-first-order rate constants were estimated from these

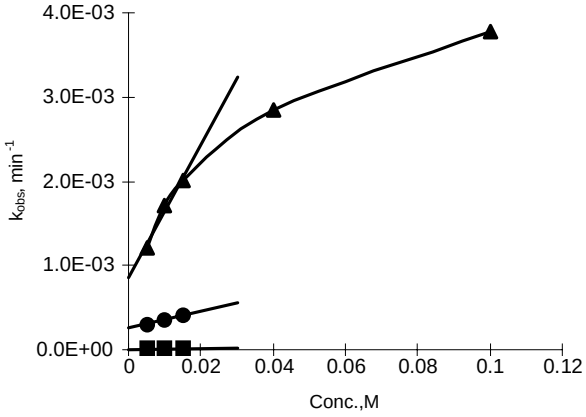


Fig. 3. The effect of phosphate buffer concentration on the pseudo-first-order rate constant k_{obs} for the degradation of compound IV at pH 6.02 (■), 6.90 (●) and 7.38 (▲) ($37 \pm 0.5\,^{\circ}C$ and $\mu = 0.5$).

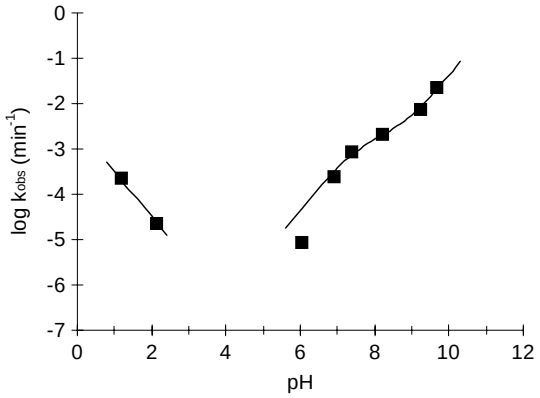


Fig. 5. The pH-rate profile for the degradation of compound IV at $37 \pm 0.5\,^{\circ}C$ and $\mu = 0.5$ (k_{obs}: observed pseudo-first-order rate constant (min^{-1})). The full lines have been calculated from Eq. (6).

plots by using the approximately linear relationship at buffer concentrations of 0.005, 0.01 and 0.015 M.

Although enhanced catalytic effect of phosphate buffer was seen as the pH changed from 6.02 to 7.38 (Fig. 3) a plot of the catalytic rate constants k_{cat} as a function of the fraction of HPO_4^{2-} did not result in a linear relationship. Likewise, non-linear relationship between k_{cat} and the fraction of HPO_4^{2-} was observed for degradation of Phe-Pro-*p*-nitroaniline (Goolcharran and Borchardt, 1998) and dipeptide model prodrugs (Lepist et al., 2000). Moreover, observations in the present study have shown that an increase in the catalytic effect of phosphate buffer was followed by an increase in the amount of cyclo(-Gly-Phe) formed. Consequently, the phosphate buffer is suggested to catalyse primarily the cyclo(-Gly-Phe) formation. Compared to the other ester prodrugs the methyl ester prodrug (compound II) was most sensitive to phosphate buffer catalysis (Fig. 4).

The influence of pH on the rates of degradation of compound IV is shown in Fig. 5 where the logarithms of the buffer-independent pseudo-first-order rate constants (k_{obs}) are plotted against pH.

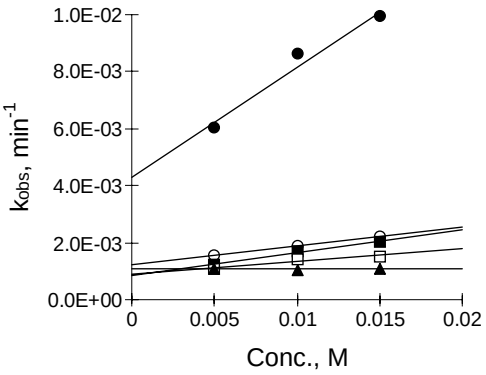


Fig. 4. The effect of phosphate buffer concentration on the pseudo-first-order rate constant k_{obs} at pH 7.4 for the degradation of compound II (●), III (○), IV (■), V (□) and VI (▲) ($37 \pm 0.5\,^{\circ}C$ and $\mu = 0.5$).

The shape of the pH-rate profile of compound IV can be accounted for in terms of a specific acid (k_{H^+}) and specific base (k_{OH^-}) catalysed reaction of the protonated form and specific base (k'_{OH^-}) catalysed reaction of the free base. A spontaneous (or water-catalysed) hydrolysis of the free base kinetically equivalent to the specific base-catalysed reaction of the protonated form may take place. The presence of a spontaneous or water-catalysed hydrolysis of the protonated form has not been investigated. A similar pH-rate profile has been reported for the degradation of glycylglycine benzyl ester (Jensen and Bundgaard, 1991). The full lines in Fig. 5 were obtained from the expression:

$$k_{obs} = k_{H^+} a_{H^+} \frac{a_{H^+}}{a_{H^+} + K_a} + k_{OH^-} a_{OH^-} \frac{a_{H^+}}{a_{H^+} + K_a} + k'_{OH^-} a_{OH^-} \frac{K_a}{a_{H^+} + K_a} \quad (6)$$

where a_{H^+} and a_{OH^-} refer to the hydrogen and hydroxide ion activity, respectively. The best fit was obtained by using $k_{H^+} = 3.2 \times 10^{-3}\,M^{-1}\,min^{-1}$, $k_{OH^-} = 1950\,M^{-1}\,min^{-1}$, $k'_{OH^-} = 170\,M^{-1}\,min^{-1}$ and $K_a = 10^{-7.6}$. The ionisation constant, K_a, for compound IV could not determined by potentiometric titration due to low alkaline solubility. However, the pK_a value for compound III was determined to 7.60 ± 0.002 at $37\,^{\circ}C$ and the magnitude is in accordance with the reported pK_a values of 8.00 ($25\,^{\circ}C$), 7.94 ($25\,^{\circ}C$), 7.8 ($60\,^{\circ}C$) for methyl (Purdie and Benoiton, 1973), ethyl (Meresaar and Ågren, 1968) and benzyl (Jensen and Bundgaard, 1991) esters of Gly-Gly, respectively.

The minor deviation between the observed and calculated k_{obs} values at pH 6 may arise from the complexity of the phosphate buffer catalysis and the uncertainty related to the determination of the buffer-independent pseudo-first-order rate constant by the initial rate method. Complexity in the degradation of dipeptide derivatives using phosphate buffer solutions have also been observed by Goolcharran and Borchardt (1998) and Lepist et al. (2000). In compar-

Table 1
Half-lives of the compounds at pH 7.4 and 37±0.5 °C in aqueous solution at zero buffer concentration and 80% human plasma ($n = 2$)

Compound	$t_{1/2}$ (min)	
	pH 7.4	80% Human plasma
I	Stable[a]	70–73
II	162	8–12
III	555	6–17
IV	819	42–43
V	717	61–62
VI	648	108–116
Cyclo(-Gly-Phe)	Stable[a]	No degradation after 7 h

[a] No degradation after 10 days in 0.02 M phosphate buffer pH 7.4.

Table 2
Solubilities (mean ± standard deviation ($n = 4$)) in Viscoleo (S_V), Viscoleo/castor oil 2:1 (v/v) ($S_{V/C}$) and Viscoleo/DMA 9:1(v/v) ($S_{V/DMA}$) at ambient temperature

Compound	S_V	$S_{V/C}$	$S_{V/DMA}$
Gly-Phe	<0.004	<0.004	<0.004
III	<0.02	0.32 ± 0.01	0.93 ± 0.01
HIP complex of III	0.15 ± 0.02	0.80 ± 0.02	12.52 ± 0.81
VI	<0.01	0.07 ± 0.01	0.18 ± 0.03
HIP complex of VI	0.19 ± 0.03	0.65 ± 0.02	3.79 ± 0.14

The hydrophobic ion pair (HIP) complexes is obtained from sodium decyl sulfonate. The solubility of the compounds is expressed as mg Gly-Phe/ml solution.

ison to the other Gly-Phe esters, the methyl ester derivative (compound II) was degraded much faster at pH 7.4 (Table 1).

The in vitro stability data, per se, may indicate that the esters of Gly-Phe are not to be considered as bioreversible derivatives due to significant intramolecular cyclisation with formation of cyclo(-Gly-Phe) in aqueous buffer solutions at pH > 6. However, in vivo the ester derivatives might function as prodrugs in case cleavage of the ester bond by esterases proceeds much faster than formation of the 2,5-piperazinedione. Compared to aqueous solution pH 7.4 at zero buffer concentration decomposition studies of the compounds in 80% human plasma pH 7.4 revealed much faster disappearance of the ester derivatives (Table 1). Except from compound II less than 1% cyclo(-Gly-Phe) was formed during degradation of the dipeptide esters in 80% human plasma reflecting fast esterase cleavage of the ester bond or fast cleavage of the peptide bond by aminopeptidases since significant amount Gly-Phe was not formed. In order to elucidate the degradation mechanism of the dipeptide esters in human plasma the formation of the degradation products was followed after incubation of compound II in 80% human plasma. In comparison to the other dipeptide esters the degradation of compound II resulted in formation of 15% cyclo(-Gly-Phe). The major degradation product was Phe-*O*-methyl ester (approximately 78%) with less than 1% Gly-Phe formed. Since the degradation rate of Phe-*O*-methyl ester in 80% human plasma was relative slow ($t_{1/2} = 82$ min) compared to the overall degradation rate of compound II ($t_{1/2} = 10$ min) it is suggested that compound II in 80% human plasma is degraded by cleavage of the peptide bond and to minor extent by the cyclisation process. Thus, most likely the half-lives for the degradation of the esters in 80% human plasma shown in Table 1 do not reflect the rate of esterase cleavage. A comparison between the rate of esterase cleavage and the cyclisation process can, however be performed using aminopeptidase stabilised dipeptides.

3.3. Oil solubility

Esterification of the free carboxylic acid at the C-terminal of small active peptides may constitute a means to obtain lipophilic prodrugs with sufficient oil solubility. Optimal solubility of these ester prodrugs in oil is expected when the amino group at the N-terminal is uncharged. However, for the investigated dipeptide esters it is not possible to obtain the free base form due to intramolecular cyclisation. Thus, in order to enhance the oil solubility ester derivatisation in combination with hydrophobic ion pairing (HIP) may be used. Previous studies have shown promising results in the area of hydrophobic ion pairing for enhancement of solubility of proteins in apolar media (Meyer et al., 1995; Meyer et al., 1996; Matsuura et al., 1993). The solubility of insulin in octanol was for example increased from below 0.03 to about 3 mg/ml by ion pairing with sodium dodecyl sulfonate (Matsuura et al., 1993).

Measurements of oil solubilities of compounds III and VI, as representatives for the dipeptide esters, were performed for both the hydrochlorides and the HIP complexes with sodium decyl sulfonate. The solubility studies were done in duplicate. From each experiments two samples were withdrawn and analysed and the results are presented in Table 2, where the solubilities are expressed as mg Gly-Phe per ml oil solution. Three oily solutions were used (i) Viscoleo, (ii) Viscoleo/castor oil 2:1 (v/v) and (iii) Viscoleo/DMA 9:1 (v/v).

As to be expected the solubility of Gly-Phe in the oil solutions was very low (below the limit of detection) and likewise low oil solubilities of the hydrochlorides of Gly-Phe butyl and dodecyl ester were observed. The solubility of Gly-Phe in oil solutions was enhanced by at least a factor 3 by replacement of the chloride anion with decyl sulfonate. The solubilising enhancing effect of hydrophobic ion pairing seems to be more pronounced for the long chain ester derivative (dodecyl) compared to the more short chain ester (butyl) of Gly-Phe.

Previously, a significant solubilising effect of castor oil relative to that of other triglycerides including Viscoleo has been observed (Jumaa and Müller, 2001; Larsen et al., 2002; Riffkin et al., 1964). The presence of hydroxy groups in ricinoleic acid, the dominant fatty acid component in castor oil, might account for the solubilising effect due to their hydrogen bond donor capabilities (Larsen et al., 2002). Castor oil, per se, might be less applicable due to the high viscos-

ity (681 mPa s (Larsen et al., 2001)) resulting in a less convenient syringeability. It has previously been reported that addition of castor oil to Viscoleo results in enhanced oil solubility (Jumaa and Müller, 2001; Larsen et al., 2002) and this solubilising effect of castor oil was also observed in the present study. However, less than 1 mg Gly-Phe was dissolved per ml oil composed of Viscoleo/castor oil 2:1 (v/v). Thus, in order to obtain feasible oil solubility addition of solubilisers might be necessary. By adding 10% DMA, an oil miscible cosolvent used in parenteral product (Spiegel and Noseworthy, 1963; Strickley, 1999), to Viscoleo the solubility of compounds III, VI and their HIP complexes increased by a factor of 18–84. Interestingly, the solubilising effect of DMA was most pronounced for compound III. In the present study the highest oil solubility of 12.5 mg Gly-Phe per ml was obtained for the butyl ester of Gly-Phe ion paired with sodium decyl sulfonate dissolved in Viscoleo containing 10% DMA (Table 2).

Although enhanced oil solubility in this study was observed for the HIP complexes between ester prodrugs of Gly-Phe and decyl sulfonate compared to the hydrochlorides, very low oil solubilities were obtained indicating that for peptides the use of hydrophobic ion pairing may constitute a less promising approach to obtain sufficient oil solubilities. To evaluate the relative effect of a homologous series of ester derivatives with increasing chain length on the oil solubility other model peptides which are not susceptible to intramolecular cyclisation such as tripeptides must be used. For active dipeptides other types of bioreversible derivatisation, e.g. 4-imidazolidinones (Rasmussen and Bundgaard, 1991; Klixbüll and Bundgaard, 1984) and cyclisation by linking the C- and N-terminal functionalities (Borchardt, 1999) might be used in order to increase the lipophilicity and enhance the oil solubility. It appears likely from the preliminary data of this study that in case of relatively small peptides sufficient oil solubility requires involvement of prodrug derivatisation in combination with the use of solubilisers like DMA.

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