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Oral absorption studies of lipidic conjugates of thyrotropin releasing hormone (TRH)[1] and luteinizing hormone-releasing hormone (LHRH)[1]

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Abstract

The lipoamino acids and their oligomers provide an excellent means of enhancing peptide lipophilicity and also increase the biological stability of the peptide by protecting it from enzymatic degradation. The enzymatically labile peptides TRH and LHRH were conjugated to lipoamino acids and lipopeptides. The conjugates were labelled on the N-terminal with a [^{3}H]acetyl group, administered orally to rats and the uptake examined. A high level of radiolabel uptake was observed in the blood, liver, spleen, kidneys, small intestine and large intestine after oral administration. In general the uptake of tripeptide TRH analogues was higher than the decapeptide LHRH analogues. Within the same series, conjugates with two lipidic moieties showed higher uptake than the conjugates with one lipidic unit. The novel conjugates developed have been absorbed and detected after oral administration and appear to be stable for a considerable time in vivo.

Keywords: Lipoamino acids; Lipopeptides; Drug delivery; LHRH; TRH; Oral absorption

1. Introduction

Peptides and proteins must negotiate a multitude of barriers if they are to be administered by the oral route, which is the desired route for most drugs. The gut epithelium presents a number of physical barriers to oral absorption, including hydrophobic membranes and transport processes (Baker et al., 1991), cell junctions (Nellans, 1991), mucus (Lehr et al., 1992) gastric acidity and peristalsis. Yet probably the most formidable barrier to oral uptake is the proteolytic activity (from epithelial, pancreatic and bacterial sources) in the gastrointestinal (GI) tract (Saidel and Edelstein, 1974; Weström et al., 1985 and Lee, 1988). The lipoamino acids and their homo-oligomers, the lipopeptides represent a class of compounds which combine the structural features of lipids with those of amino acids and peptides (Gibbons et al., 1990). They are highly lipophilic due to their long alkyl side chains, yet show polar and

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[1] PyGlu was replaced by Glu.

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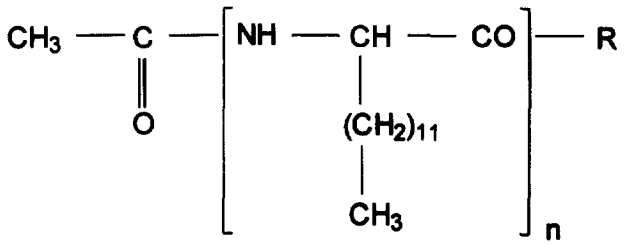

1	n	R
a	0	Glu-His-Pro-NH_2
b	1	Glu-His-Pro-NH_2
c	2	Glu-His-Pro-NH_2
d	0	Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
e	1	Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
f	2	Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

Scheme 1. TRH and LHRH analogues *1a–f*.

conformational behaviour characteristics of amino acids and peptides (Toth et al., 1991). Chemical conjugation of the lipoamino acids to drugs which exhibit poor oral absorption has already been shown to enhance their oral uptake (Toth et al., 1994a), while increased biological stability of peptides against enzymatic degradation has also been observed, when they are modified with lipoamino acids (Toth et al., 1994b).

The tripeptide thyrotropin releasing hormone (TRH), is relatively resistant to proteolytic degradation in the GI tract, and its poor oral activity is probably due to poor absorption and rapid clearance in the bloodstream (Yokohama et al., 1984). TRH is rapidly degraded following first-order kinetics, with a half life in humans of approximately 5 min following i.v. administration (Leppaluoto et al., 1977).

Luteinizing hormone-releasing hormone (LHRH) is a decapeptide that regulates the secretion of both luteinizing hormone and follicle-stimulating hormone from the anterior pituitary. The peptide is too large and too hydrophilic (partition coefficient 0.0451; Banks and Cession, 1985) to cross the GI tract mucosa. It is also highly susceptible to degradation by enzymes and has a very short half-life in the circulation of approximately 3–6 min (Handelsman and Swerdloff, 1986). Attempts have been made to stabilise LHRH against proteolytic cleavage and include the use of D-amino acids at the sixth position and modification of the C-terminus of the peptide chain, which have resulted in superanalogues such as buserelin and leuprolide. Approaches to enhance the oral delivery of LHRH and its analogues include association with colloidal carriers, entrapment in polymeric matrices and liposomes and the use of absorption/penetration enhancers.

Chemical conjugation of the lipoamino acids to the TRH and LHRH peptides could perform two important functions, the first being to increase the lipophilic character of the peptide to facilitate passage across biological membranes

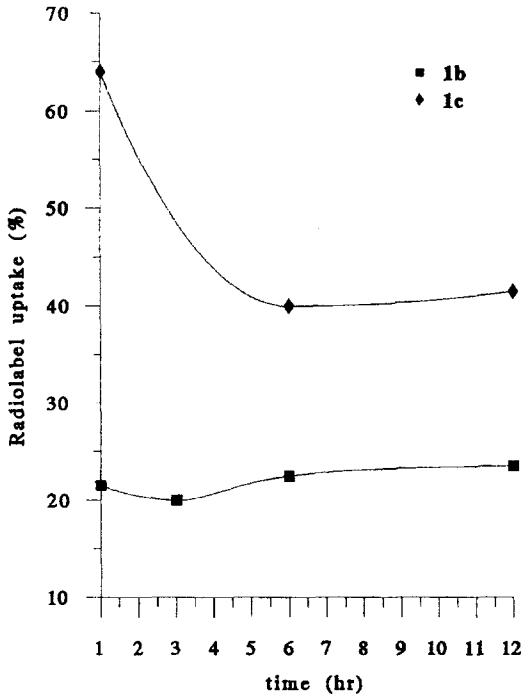

Fig. 1. Total uptake of compounds *1b* and *1c*.

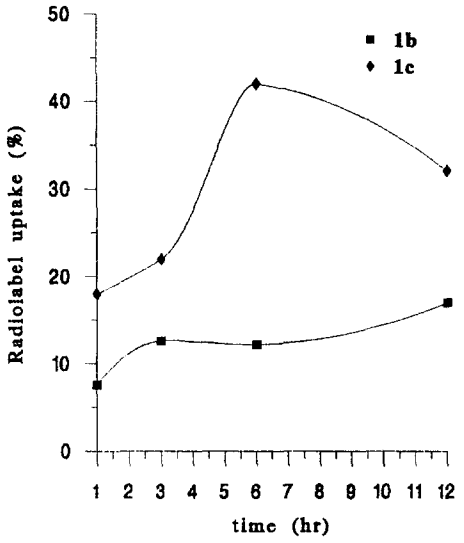

Fig. 2. Blood uptake of compounds *1b* and *1c*.

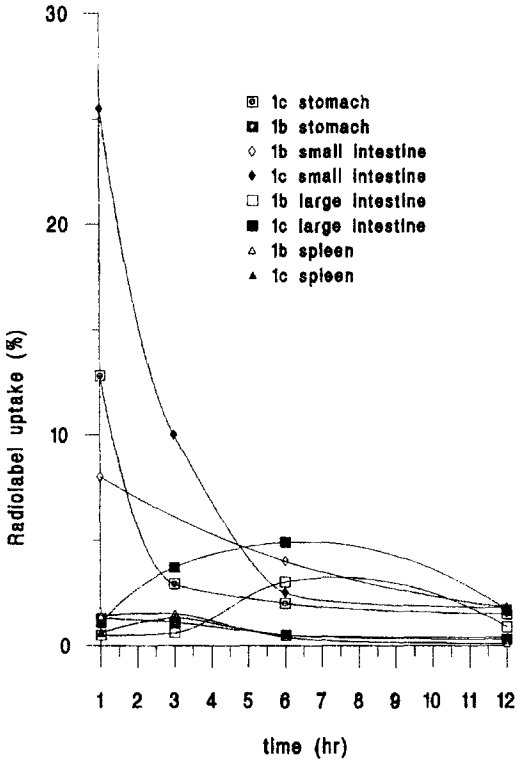

Fig. 3. Stomach, small intestine and spleen uptake of compounds *1b* and *1c*.

and secondly to stabilise and protect the peptide from proteolytic degradation by both epithelial and serum peptidases. Thus, radiolabelled lipidic-TRH and -LHRH conjugates were administered orally to rats and their uptake was examined by liquid scintillation, HPLC and mass spectrometry.

2. Materials and methods

Compounds *1a–1f* (Scheme 1) were synthesised, purified and characterised using the method described by Toth et al. (1994a). Furthermore, for compound separation, a Waters Quanta 4000 Capillary Electrophoresis System was employed, with a 50 mM phosphate running buffer containing 10% methanol (pH 2.5), and for structure elucidation, a Fisons matrix assisted time of flight laser desorption mass spectrometer was used.

2.1. Oral dosing

The respective peptide conjugates were dissolved in 50 mM phosphate buffer (pH 3, containing 2% DMF) to a concentration of 1 mg/ml. A 1-ml aliquot of the solution was then administered to male Wistar rats (approx. 220 g) by oral gavage with a blunt-tipped feeding needle. The animals were then sacrificed at 1, 3, 6 and 12 h and the liver, stomach, kidneys, spleen and small and large intestines were removed along with a sample of blood (4 ml). All digestive matter was removed from the organs and they were thoroughly washed in phosphate buffered saline (PBS). The organs were then homogenised to a fine slurry and 0.5 ml of the homogenate was added to 2 ml of Scintran tissue solubiliser; this was also done for blood. The samples were left for 5 days and allowed to dissolve with the aid of gentle shaking, after which the samples were decolourised using H_2O_2 (30%) and the radiolabel detected by liquid scintillation.

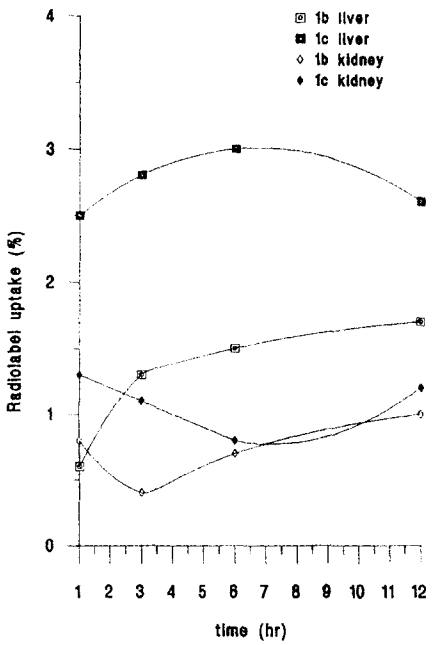

Fig. 4. Liver and kidney uptake of compounds *1b* and *1c*.

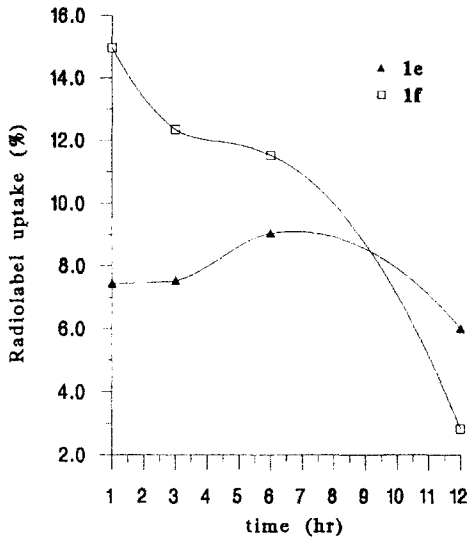

Fig. 5. Total uptake of compounds *1e* and *1f*.

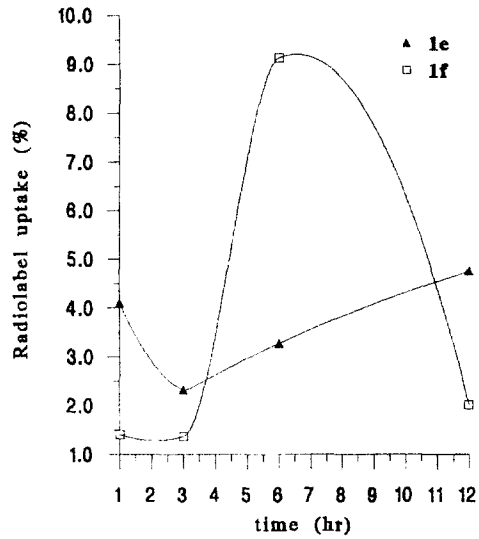

Fig. 6. Blood uptake of compounds *1e* and *1f*.

2.2. Extraction and characterisation of conjugates from blood

Rats were dosed with the conjugates as described above and sacrificed after 3 h using a recommended humane method. Blood samples were taken and the protein denatured using TFA (final concentration, 10%). The denatured blood

was then centrifuged (13 000 rev./min) and the pellet obtained was resuspended in DMF by vortexing. After further centrifugation, the supernatant was removed and the DMF evaporated under reduced pressure in the presence of xylene. The resultant solid was dissolved in 50 mM phosphate buffer (pH 3, containing 20% methanol) and run on HPLC using a C_4 Vydac column with acetonitrile/water as solvents. The target peak was then identified, collected and its identity confirmed by mass spectrometry.

3. Results and discussion

The tripeptide TRH and the decapeptide LHRH were synthesised (Glu was used instead of PyGlu) and acetylated on the N-terminus resulting in compounds *1a* and *1d*. Further samples of peptide were extended on the N-terminus with one or two 2-amino-tetradecanoic acid [synthesised from 1-bromodecane (Gibbons et al., 1990)] moieties, before being acetylated with ^3H acetic anhydride resulting in compounds *1b*, *1e* and *1c*, *1f* respectively. The 'monomer' and 'dimer' conjugates of TRH and LHRH resulted in diastereomeric mixtures of two and four compounds, which were used without separation.

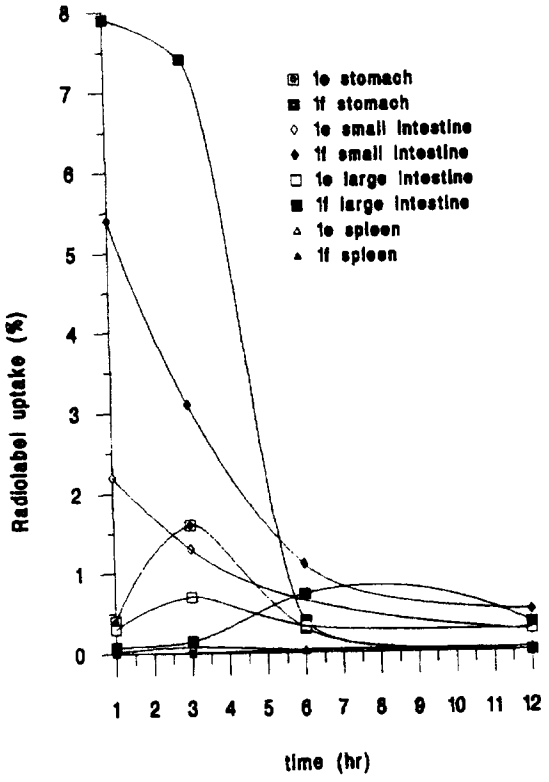

Fig. 7. Stomach, small intestine, large intestine and spleen uptake of compounds *1e* and *1f*.

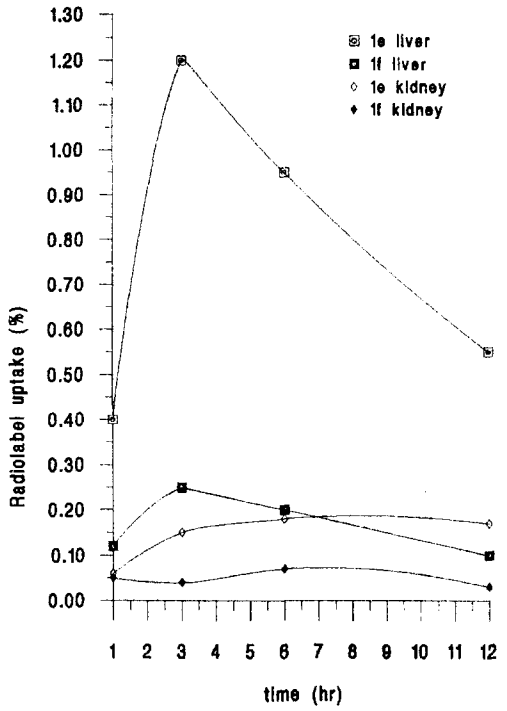

Fig. 8. Liver and kidney uptake of compounds *1e* and *1f*.

Radiolabelled TRH and LHRH analogues *1a–1f* were administered orally to rats and the uptake examined. A high level of radiolabel uptake was observed in the blood, liver, spleen, kidneys, small intestine and large intestine after administration,

Table 1
Comparison of blood uptake of compounds *1e* and *1f*

Compound	Blood uptake (%)	
	Crude homogenate	Extracted, purified compound
1e	2.3	1.7
1f	1.2	1.0

but the non lipidic *1a* and *1d* rapidly degraded in the stomach and gut and only the uptake of radioactive metabolites was observed since the intact *1a* and *1d* could not be detected by HPLC-MS.

The overall uptake of tripeptide TRH analogues *1b* and *1c* was higher than the overall uptake of decapeptide LHRH analogues *1e* and *1f* which is to be expected considering the size difference between the peptides. It was also seen that within the same series, compounds with higher lipophilicity were taken up to a greater extent (uptake of *1c* > uptake of *1b*, and uptake of *1f* > uptake of *1e*), clearly showing the importance of lipophilicity (Figs. 1 and 2).

The blood (Figs. 3 and 4), stomach, small intestine, large intestine and spleen (Figs. 5 and 6) uptake of TRH and LHRH analogues *1b*, *1c* and *1e*, *1f* were in good correlation. The more lipophilic *1c* and *1f* showed higher uptake than the less lipophilic *1b* and *1e* in the blood, stomach, small intestine and large intestine and lower uptake in the spleen. The liver and kidney uptake (Figs. 7 and 8) showed a different trend. The more lipophilic TRH conjugate *1c* was in higher concentration in the liver and kidney than the less lipophilic TRH conjugate *1b*, while the more lipophilic LHRH analogue *1f* was in lower concentration than the less lipophilic analogue *1e* in these organs, showing a slower secretion of compound *1f*. Interestingly, all compounds were present and detectable even 10 h post administration.

In order to confirm the identity of the detected radiolabel, compounds *1b*, *1c*, *1e* and *1f* were extracted from the blood 3 h post administration. The extracted compounds were then purified by HPLC, characterised by MS and requantified by liquid scintillation. The separation of tripeptide TRH conjugates *1b* and *1c* required multiple HPLC and capillary electrophoresis purification (the crude extract contained a large amount of lipoprotein fragments). We were able to detect the presence of intact *1b* and *1c* and obtain mass spectra of the products, but the method was not suitable for quantification.

The purification of decapeptide analogues *1e* and *1f* required less chromatography, so the amount of radiolabel detected in the purified conjugates *1e* and *1f* was in agreement (Table 1) with the detected radiolabel from the initial scintillation counting (Fig. 4) of the crude homogenates.

In summary, the novel conjugates developed have been absorbed and detected after oral administration and appear to be stable for a considerable time in vivo.

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