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How do HYNIC-conjugated peptides bind technetium? Insights from LC-MS and stability studies†,‡

Robert C. King^a, M. Bashir-Uddin Surfraz^b, Stefano C. G. Biagini^c, Philip J. Blower^d, and Stephen J. Mather^a

^aCancer Research UK, Department of Nuclear Medicine, St Bartholomew's Hospital, London, UK EC1A 7BE

^bCancer Research Group, Biosciences Dept., University of Kent, Canterbury, Kent, UK CT2 7NJ

^cFunctional Materials Group, School of Physical Sciences, University of Kent, Canterbury, Kent, UK CT2 7NJ

^dDivision of Imaging Sciences, Kings College London, 5th Floor Thomas Guy House, Guy's Campus, London, UK SE1 9RT. E-mail: Philip.Blower@kcl.ac.uk; Fax: 0207 188 5442; Tel: 0207 188 9513

Abstract

Hydrazinonicotinamide (HYNIC) is an established bifunctional complexing agent for technetium-99m (^{99m}Tc) but the structure of the technetium coordination sphere remains uncertain. To gain further insight into this, we have prepared conjugates of HYNIC and hydrazinobenzoic acid (HYBA) with a model peptide, and radiolabelled them with ^{99m}Tc using three well-established co-ligand systems: EDDA, tricine and tricine–nicotinic acid. The labelled peptides were studied by LC-MS and by subjecting them to serum stability and protein binding assays. For each co-ligand system, HYNIC conjugates formed fewer and more stable labelled species than the corresponding HYBA conjugates. LC-MS analysis showed that all conjugates contained one hydrazine moiety bound to Tc, that binding of Tc to HYNIC–peptide and co-ligand occurs with displacement of 5H⁺ indicating a Tc formal oxidation state of +5, and that the Tc has no oxo- or halide ligands. LC-MS also shows that complexes formed with the HYNIC conjugate contain fewer coordinating co-ligand molecules than the HYBA conjugate indicating that HYNIC is able to more effectively satisfy the coordination requirement of technetium, perhaps by binding in chelating mode.

Introduction

Targeted scintigraphy is a promising tool for the diagnosis and staging of cancer. The success achieved in targeting somatostatin receptor-expressing cancers has led to interest in targeting other high-incidence receptors that are over-expressed in various tumour types.¹ The CCK-2 (formally CCK-B/gastrin) receptor has been found in high incidence in samples of medullary thyroid carcinoma, small cell lung cancer, stromal ovarian cancers, and in gastrointestinal neuroendocrine tumours.² Radiolabelled analogues of the CCK-2 receptor ligands, gastrin and CCK, have shown promise in the detection of CCK-2 receptor-positive tissue in humans.³ For example, [DTPA-D₅-Glu¹]-minigastrin radiolabelled with ¹¹¹In for

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detection and ^{90}Y for therapeutic purposes have been evaluated clinically in MTC.⁴ For diagnostic applications, Tc-99m remains the radionuclide of choice in scintigraphic imaging as it has a number of favourable characteristics including low cost, low radiation dose to the patient, optimum gamma-energy profile, and wide commercial availability *via* generators.⁵ High specific activity Tc-99m labelling can be achieved using bifunctional coupling agents, and one of the most widely employed is 6-hydrazinonicotinic acid (HYNIC).⁶ HYNIC is typically attached to the amino group at the N-terminus of the peptide, but can also be coupled to Fmoc-Lys and subsequently inserted into the peptide sequence *via* solid phase peptide synthesis (SPPS),⁷ which allows greater flexibility and control in the positioning of HYNIC within the peptide sequence. HYNIC-peptide conjugates can be readily labelled with technetium-99m using a number of co-ligand systems. The exact nature of the $^{99\text{m}}\text{Tc}$ -HYNIC complex is unclear, though it is thought that the technetium atom binds the hydrazine group through its terminal nitrogen and the other coordination sites are occupied by atoms from one or more co-ligands. Most publications depict a monodentate HYNIC coordination as shown in Fig. 1(A),⁸⁻¹⁰ rather than chelating as in Fig. 1(B), although there is no direct evidence for this assumption. The coordination mode of the HYNIC, the oxidation state of the technetium, the number and coordination mode of co-ligands, the possible presence of other ligands such as oxo- and halide ligands, and the possibility of coordinate bonds with amino acid side chains, remain indeterminate.

Structural studies of model technetium and rhenium hydrazinopyridine complexes¹¹⁻¹⁴ show that HYNIC is capable of coordinating in either monodentate mode through the terminal hydrazine nitrogen atom or in a chelating mode with the pyridine nitrogen atom also coordinated, and in both modes simultaneously when two HYNIC ligands are coordinated. In order to arrive at an ideal HYNIC-based binding site for technetium to produce more stable, homogeneous radiopharmaceuticals, a more complete understanding of the coordination sphere around the technetium is required. Because of the low concentrations of the metal complexes under no-carrier added conditions, it is not possible to use structural and spectroscopic analytical methods such as NMR or crystallography. In this study we have therefore sought to address this by comparing the stability properties and LC-MS of labelled conjugates of HYNIC and its non-heterocyclic analogue 4-hydrazinobenzoic acid (HYBA). We herein describe the use of radio-chromatographic methods to assess Tc-99m radiolabelling and *in vitro* stability of HYNIC and HYBA conjugated to a truncated form of the peptide gastrin, derived from the C-terminal domain and termed "nanogastrin" with the sequence [Lys(R)-Glu-Ala-Tyr-Gly-Trp-Met-Asp-PheNH₂] where R = HYNIC or HYBA (Fig. 2), using tricine, tricine-nicotinic acid, and EDDA as co-ligands. These peptide conjugates are termed "HYNIC-nanogastrin" and "HYBA-nanogastrin," respectively. In addition, Tc-99 complexation was performed with an analogue of HYNIC-nanogastrin, termed HYNIC-[gly⁸]nanogastrin, in which the glutamate residue adjacent to the HYNIC-lysine residue was replaced with glycine.

Results

HPLC

$^{99\text{m}}\text{Tc}$ radiolabelling of HYNIC-nanogastrin using tricine as co-ligand with a reaction time of 10 min at room temperature ($\sim 20^\circ\text{C}$) resulted in $>99\%$ radiolabelling efficiency (*i.e.* total peptide binding). Several distinct species were resolved by radioHPLC (Fig. 3). Over the following 24 h, a shift in the balance of two groups of peaks occurred, with later-eluting species (21.2 and 21.3 min) dominating at early incubation times, and earlier-eluting peaks (21.1 and 20.2 min) dominating after longer incubation (Fig. 3). Changing the radiolabelling conditions to 95°C accelerated this change, leading to complete disappearance of the more hydrophobic group of species by 30 min (Fig. 4) and giving a product that was more stable on incubation with serum (see below). A more gentle and extended elution gradient allowed

clear resolution of five or more species in the 95 °C sample. These labelling conditions (95 °C, 30 min, pH 5–6) were adopted as standard in subsequent experiments reported below, unless otherwise indicated, giving >98% radioligand purity as determined using HPLC, and <0.1% ^{99m}Tc colloid (by thin layer chromatography).

Addition of a monodentate ligand, nicotinic acid (NA), to the radiolabelling of the ^{99m}Tc –tricine–HYNIC–nanogastrin resulted in a radiochromatogram in which only one radioactive species was clearly resolved, with a labelling yield >99%. The shoulder or slightly asymmetric peak shape suggests that there are other unresolved species present (see ESI).[‡] Using EDDA alone as co-ligand resulted in a lower labelling yield (~80% labelled peptide and ~20% pertechnetate), even at elevated temperature. However, using an exchange labelling approach, with both tricine and EDDA present together, increased the radiolabelling yield to >95%. The radiopeptide envelope of the chromatogram consisted of a major peak (approx. 75%, 20.95 min) preceded by four resolved minor peaks (approx. 25% total, 19.4–20.7 min). The retention times of the radiolabelled peptide peaks from ^{99m}Tc –HYNIC–nanogastrin labelled using either EDDA alone or EDDA–tricine as co-ligand were identical, suggesting that tricine does not feature in the complex when EDDA is present.

Radiolabelling of the HYBA–nanogastrin using tricine as co-ligand required a minimum reaction time of 30 min at 50 °C to achieve a >95% yield. The radiolabelled HYBA–nanogastrin conjugates produced an increased number of labelled species compared to the HYNIC conjugates. These species were best resolved using a very gentle, extended elution gradient (method 2, see Fig. 4). The retention times of the radiolabelled HYBA conjugates were longer than those of the HYNIC conjugates for each of the co-ligand systems studied (see Fig. 4 and ESI[‡]).

Radiolabelling of the nanogastrin peptide sequence lacking either HYNIC or HYBA was also performed as a control to test non-specific binding of Tc-99m to the peptide. This showed no binding of Tc-99m to the peptide sequence by HPLC and TLC.

Mass spectrometry

Increased concentrations of peptide and technetium (up to *ca.* 50 μM), compared to the radiolabelling conditions described above (no-carrier-added, approx. 10 nM) were required to generate good quality mass spectra. This was achieved using Tc-99 pertechnetate as well as Tc-99m generator eluate. To check that this change in concentration did not alter the radiolabelled species formed, radiochromatograms were obtained at both concentration levels. Although in many cases the radiolabelling efficiency was lower than that obtained with ^{99m}Tc (*i.e.* significant amounts of $^{99m}\text{TcO}_4^-$ pertechnetate remained), the pattern and retention times of the labelled peptide HPLC peaks in all cases were identical (see ESI[‡]). A similar check was performed in reverse, by progressively reducing the carrier-added labelling concentration by up to 100 fold (to 500 nM) and observing the LC-MS. Again no qualitative changes occurred. Thus, it is reasonable to conclude that the mass spectra obtained at the higher concentration, described below, reflect the identity of the species obtained at no-carrier-added concentration. The limit for reliable ES-MS detection of identifiable ions was shown in this way to be of the order of 10^{-11} to 10^{-10} moles per sample (injection volume 100 μL).

Unlabelled peptides—LC-MS of the free HYNIC- or HYBA-conjugated peptides was performed first, and the labelled forms were subsequently analysed and the results

[‡]Electronic supplementary information (ESI) available: Full details of LC-MS and radioHPLC analysis of $^{99m}/^{99}\text{Tc}$ labelled peptides including HPLC profiles and all significant detected ions. See DOI: 10.1039/b705111e

interpreted in terms of the changes in molecular mass or m/z resulting from labelling. A brief summary of the molecular ions observed is given in Table 1. A full listing of observed ions and assignments is provided in the ESI.[†] In positive ion mode (method C), HYNIC-gastrin gave a molecular ion M^+ at m/z 1280.5 ($[M + H]^+$) and in negative mode, M^- was observed at 1278.5 ($[M - H]^-$). Analysis of HYBA-gastrin in negative mode gave, in addition to the expected ion at 1277.5 ($[M - H]^-$), an ion at 1275.5 which may be accounted for by an oxidation of the hydrazine group to give a diazene occurring during ionization.

Tricine as co-ligand—ES-MS of the multiple radioHPLC peaks observed upon labelling HYNIC-gastrin with Tc in the presence of tricine all gave the same m/z molecular ion at 1553.2 (positive mode) and 1551.4 (negative mode, see Table 1) corresponding to the initial HYNIC-gastrin peptide becoming conjugated to a single Tc^{5+} and a single tricine molecule, with the concomitant displacement of $5H^+$. Other ions were observed, also assignable as originating from these molecular species (*e.g.* the corresponding $[M + 2H]^{2+}$ and trifluoroacetic acid adducts, see ESI[†]). The analysis was repeated on numerous occasions to give a statistically reliable confirmation of the precise number of protons displaced on Tc binding since, importantly, this gives a clear indication of the oxidation state of the technetium. The ES-MS profiles are shown in ESI[†] at high resolution to illustrate the precision and resolution attainable. There was no evidence for the presence of any Tc-conjugates other than those corresponding to [peptide + Tc + single tricine – $5H$].

In sharp contrast, the eluted HPLC fractions corresponding to labelled peptides after labelling HYBA-gastrin with Tc in the presence of tricine gave ES-MS (methods A and B) consistent with concomitant binding of Tc^{5+} and *two* tricines, again with displacement of $5H^+$ (1552.2 in positive mode, 1550.3 in negative mode, as well as other ions originating from the same stoichiometry listed in ESI[†]). Ions corresponding to a complex containing a single tricine were also detected, but when milder ionisation conditions were used (method C) these ions were diminished and two-tricine species became dominant, suggesting that only two-tricine species are formed on labelling and that one of the tricines dissociated during ionisation under electrospray conditions of methods A and B. By contrast, the HYNIC conjugate did not yield any ions corresponding to two bound tricines, even under the mild conditions of method C.

Tricine and NA as co-ligands—The major HPLC peak obtained by labelling HYNIC-gastrin with Tc in the presence of tricine and nicotinic acid gave ions consistent with one tricine and one NA binding along with Tc, using ES-MS method A. With method B this peak also showed evidence of a less abundant ion containing one tricine and two NA ligands, and under the milder conditions of method C this ion increased markedly in abundance. It is thus possible that the single-nicotinic acid species is derived by loss of a NA during ES ionization, and it cannot be unequivocally concluded that it is present in solution. In the case of the HYBA-conjugated peptide, the dominant molecular ion corresponded to binding of one tricine and two NA molecules under all ES conditions. The same HPLC peak also gave ions with only one NA, although to a much lesser extent than with the HYNIC derivative. While it is clear that a HYBA species with two NA ligands exists in solution, and the relative intensity of its ions increases when the milder method C is applied, the presence of a species with only one NA cannot be excluded.

EDDA as co-ligand—Labelling HYNIC-gastrin in the presence of tricine and EDDA gave a major radioactive peak whose ES-MS indicated two EDDA molecules binding along with Tc, with only the merest trace of single-EDDA ions. Peptide species containing tricine were not present to any significant degree. Among the very minor HPLC fractions eluting earlier a species with only one EDDA bound was detected. Labelling HYBA-gastrin in the presence of tricine and EDDA gave a major HPLC peak whose ES-MS was dominated by a

two-EDDA species but also showed a minor single-EDDA species that was further diminished under the mild method C conditions. Like the HYNIC analogue, ES-MS of a very small earlier eluting peak showed evidence of a single bound EDDA.

HYNIC-[gly⁸]nanogastrin—Studies of a series of model tripeptides incorporating HYNIC, to be published elsewhere, raised the possibility that a glutamate residue close to HYNIC in the amino acid sequence could participate in the coordination sphere of the technetium. To determine whether this might be the case for nanogastrin, which contains a glutamate adjacent to HYNIC-lysine, we repeated the labelling with a peptide analogue in which the glutamate was replaced by glycine. The LC-MS (method C, Table 1) of this species showed ions corresponding to peptide, technetium and both one and two tricine (1481.3 and 1660.9), again with loss of 5H⁺, in contrast to HYNIC-gastrin which gave only single-tricine conjugates.

Intermediate complexes—To study the sequence of reactions leading to the final labelled conjugates, we applied ES-MS to the complexes formed upon reduction of pertechnetate with stannous chloride in the presence of tricine but before the addition of HYNIC-nanogastrin, at room temperature for 20 min. No identifiable molecular ions were observed in positive mode (method D, direct injections without HPLC) but in negative mode (method A) a series of ions was observed corresponding to the formula [(TcO³⁺)-tricine_n - 4H]⁻, with *n* = 1 to 4 (292.1, 471.2, 650.1, 828.8). The number of coordinated tricines is indeterminate because of the formation of non-covalent tricine adducts (these are detected also in the ES-MS of tricine alone, *m/z* = 178.3, 357.1, 536.2, 715.1, 894.2; see ESI[†]). Treatment of this intermediate with HYNIC-gastrin for 20 min at room temperature yields LC-MS results similar to those reported above for the 95 °C labelling, *i.e.* a conjugate consisting of peptide, Tc, and one tricine with loss of 5H⁺. A similar sequence is observed for HYBA-gastrin and HYNIC-[gly⁸]nanogastrin: a bis(tricine) peptide-Tc conjugate was formed rapidly at room temperature, followed by isomerisation on heating, as detected by small shifts in elution time but no change in the ES-MS.

Stability studies

All of the ^{99m}Tc-HYNIC conjugates labelled by incubation at 95 °C for 30 min showed high stability (*i.e.* >97% radiochemical purity over a 4 h incubation) after dilution in PBS either with or without purification (*i.e.* removal of excess stannous chloride and co-ligand) by SEPPAK (see Fig. 5). Among the labelled peptides prepared at 95 °C, the order of stability obtained was ^{99m}Tc-tricine-HYNIC-nanogastrin > ^{99m}Tc-EDDA-HYNIC-nanogastrin > ^{99m}Tc-tricine-NA-HYNIC-nanogastrin. The HYBA conjugates all showed a lower stability than the HYNIC conjugates, especially when tricine was used as co-ligand. Both with and without purification, the stability of the ^{99m}Tc-HYBA conjugates decreased in the order ^{99m}Tc-EDDA-HYBA-nanogastrin > ^{99m}Tc-tricine-NA-HYBA-nanogastrin > ^{99m}Tc-tricine-HYBA-nanogastrin as shown in Fig.5.

The stability of the conjugates in serum was determined by measuring the % of intact radiopeptide using radioHPLC, and by measuring the degree of protein binding of the radiolabel by size exclusion chromatography. The results are shown in Fig. 6. Again the tricine-HYNIC-nanogastrin species was most stable and the HYBA-nanogastrin species were for the most part less stable than their HYNIC analogues. When comparing the HYNIC conjugates labelled with different co-ligands, the extent of protein binding determined by size-exclusion chromatography was tricine > EDDA > tricine-NA. The HYBA conjugates showed significantly a greater degree of protein binding when labelled using tricine or tricine-NA but no significant difference was observed when EDDA was used, see Fig. 6.

Discussion

Since HYNIC was first introduced as a bifunctional complexing agent in combination with secondary coordinating ligands (co-ligands) by Abrams *et al.*,¹³ the exact nature of the Tc-99m–HYNIC–co-ligand complexes formed remains a point of some discussion. Initial studies performed at the macroscopic (Tc-99) level indicated the presence of either one¹² or two^{11,14} hydrazinopyridine groups in the complex. In structurally characterised Tc complexes containing a single hydrazinopyridine, the ligand loses all three hydrazinic hydrogens and acts as a chelating agent (Fig. 7).¹² In complexes with two hydrazinopyridine moieties, invariably one is chelating while the other has a non-coordinated pyridine.^{11,14} Since we have detected no ions in which two peptides are linked by a technetium, only one HYNIC is present in the coordination sphere of the technetium at the low concentrations used here. The same conclusion was also reached by Liu and colleagues on the basis of HPLC studies^{9,15} and by the use of mass spectroscopic analysis of complexes formed at intermediate carrier levels (Tc-99/Tc-99m).¹⁶

Our LC-MS observations also give an indication of the identity and number of co-ligand molecules bound to technetium and how this is affected by the surrounding amino acid side chains and the hydrazine ligand. Whether the co-ligand is tricine, tricine–NA or EDDA, the presence of an oxo-group can be excluded, as can the presence of a coordinated chloride which is often depicted in the literature in the case of EDDA.⁹ In this respect all the labelled conjugates were consistent.

However, the number of co-ligands (tricine, NA and EDDA) present varied between conjugates and co-ligands. Current literature assumes that two tricines are generally present, or a tricine and one monodentate ligand such as NA, but only one EDDA.⁹ In our ES-MS data, however, for HYNIC–nanogastrin with tricine as co-ligand, only one bound tricine was consistently observed in all HPLC fractions. Only if the adjacent glutamate in the sequence was replaced by glycine were two bound tricines found in accordance with the assumptions of Liu and co-workers.⁹ This suggests, in this particular peptide, that a carboxylate group on the adjacent amino acid could participate in technetium coordination, displacing one of the tricines. Similarly, if the HYNIC in HYNIC–nanogastrin is replaced by HYBA, two bound tricines are seen, suggesting the possibility that the pyridine nitrogen of HYNIC could also participate in technetium coordination with displacement of a tricine ligand. Thus it is clear that only in the absence of sufficient internal coordinating groups (*e.g.* the pyridine and glutamate in HYNIC–nanogastrin) are two tricine ligands present in the technetium coordination sphere.

Liu *et al.* proposed that when EDDA is used as co-ligand, only one EDDA is incorporated, although no evidence was presented to support this. However, the results above unequivocally demonstrate that two EDDA molecules are present both in the HYNIC–nanogastrin and the HYBA–nanogastrin conjugates. Liu *et al.* also proposed that when tricine is used together with a second (monodentate) co-ligand (*e.g.* pyridine derivatives such as NA), the technetium binds one tricine and one monodentate ligand, citing the absence of HPLC detectable species with two different bound monodentate ligands when two are present in solution,¹⁴ and also LC-MS evidence.¹⁶ Again our results unequivocally refute this since the ES-MS shows that both the HYNIC and the HYBA conjugates comprise one peptide, one technetium, one tricine and two NA ligands when labelled in the presence of tricine and NA. This conflict may be explained by monodentate ligand dissociation occurring in the ES ionisation experiments of Liu *et al.*, since we have observed that this occurs easily under typical electrospray conditions but can be suppressed by the use of milder conditions (*e.g.* lower cone voltages).

The well-resolved molecular ions shown listed in Table 1 and in more detail in ESI[†] show definitively that the binding of a technetium atom to the HYNIC-peptides and co-ligands is accompanied in all cases by the displacement of five hydrogen atoms. This is effectively equivalent to the binding of a Tc^{5+} ion with displacement of 5H^+ , implicating the presence of technetium formally in oxidation state (v). This is consistent with the observation of the $\text{Tc}^{\text{VO}}(\text{tricine})_n$ species as the intermediate (detected by ES-MS in this work and previously by others¹⁴), which reacts with the HYNIC-peptide without further redox occurring. In this respect the results agree with the conclusion of Liu *et al.* from their LC-MS data.

These observations allow more confidence than could have been exercised previously in assessing the relevance of the model structurally characterised complexes. The technetium oxidation state, the number of HYNIC ligands, and the co-ligands are consistent with the general composition $\text{Tc}(\text{NNpy})\text{X}_2\text{L}_2$ where X is an anionic ligand and L a neutral one. This is modelled by the complexes $[\text{M}(\text{NNPy})\text{Cl}_2(\text{PPh}_3)_2]$ (M = Re, Tc, Fig. 7),¹² which has been characterised by X-ray crystallography and contains a chelating hydrazinopyridine (HYNIC analogue). Here, as in the HYNIC-peptide conjugates, the complex may be viewed formally as derived from replacement of 5H^+ from the ligands (three from the hydrazine and two from HCl molecules) by a Tc^{5+} ion.

Similarly, the ES-MS results obtained for HYBA-nanogastrin conjugates show that formally Tc binds to the hydrazine and co-ligands in the form of Tc^{5+} with displacement of 5H^+ , suggesting that the model arylhydrazine complexes described by Dilworth *et al.*, in which the phenylhydrazine gives rise to a singly-bent, monodentate diazenido ligand (Fig. 7),¹⁷ are relevant to this case.

Although the experimental analogy between these models and the HYNIC-nanogastrin conjugates described here does not definitively demonstrate that HYNIC is a chelator in the peptide conjugates, we may note that there are no structural models of mono-HYNIC analogue complexes in which the hydrazinopyridine group is non-chelating. It is unreasonable, therefore, to assume on the basis only of the number of isomers resolvable by HPLC that the HYNIC does not chelate;^{9,15,16} rather, until definitive evidence to the contrary is available, a chelating mode should be assumed, as indicated by the model complexes (Fig. 7).¹² A number of possible structures may be proposed for the labelled conjugates in accordance with this interpretation, exemplified by those shown in Fig. 8.

The number of species formed when using HYBA conjugates was always greater than the number formed using HYNIC, for all three co-ligand systems. Since the structures proposed in Fig. 8 can generate more than enough coordination isomers to account for the numbers of HPLC peaks, it would be unwise to cite this contrast as evidence for or against chelation by HYNIC. We also observe that the HYNIC conjugates are, for the most part, more stable than their HYBA counterparts. This is consistent with the expectation that the ability of HYNIC to chelate should offer greater kinetic stability compared to HYBA. Once again, however, this argument is to be treated with caution, because for HYNIC to chelate, a 120° Tc–N–N bond angle is enforced which precludes the Tc-bound nitrogen from acting as a three-electron donor by donating its lone pair to form a Tc–N double bond. This might counterbalance the stability advantage of chelation. Nevertheless, from a practical application standpoint, it is helpful to observe that HYNIC is preferable in terms of both greater stability and the smaller number of isomers. We also note that the most stable of all the conjugates was the one in which only one exogenous co-ligand molecule is present and the coordination sphere is most likely to be completed by the HYNIC pyridine and amino acid side chains.

It is important to note the implications of the participation of amino acid side chains in the coordination of technetium. The requirement for an additional tricine ligand, when the glutamate residue adjacent to HYNIC-lysine in the sequence is replaced by glycine, suggests that the glutamate carboxylate group in nanogastrin plays an important role in binding technetium. This is supported by ES-MS studies of a series of model short peptide sequences.¹⁸ We and others have also suggested that an adjacent histidine residue can serve the same purpose^{7,18,19} and is likely to bind even more effectively. The importance of this is two-fold. On one hand, it provides an opportunity to design technetium-binding amino acid sequences within which HYNIC-technetium binding will be optimal in terms of stability and structural homogeneity; as we have seen from the stability experiments, stability is best when the number of additional co-ligands is reduced by the coordination of amino acid side groups.¹⁸ On the other, coordination of amino acid side groups could potentially alter the structure of the peptide, changing its fold or masking amino acid side groups that may be critical to biological activity. These factors should be taken into account in the design of peptide radiopharmaceuticals.

While undertaking this study a number of other interesting observations were made relating to labelling of the HYNIC conjugates, which it is worthwhile to report. The first is that while high labelling yields could be achieved at room temperature using tricine alone as co-ligand (as previously described)⁹ a shift in retention time was observed over the following 24 h. This change could be accelerated by heating the reaction mixture to 95 °C. The solution and serum stability of the complexes formed at 95 °C (and presumably those incubated at room temperature for 24 h) was significantly greater than for those formed during a room-temperature labelling reaction. LC-MS showed that the composition of the conjugates was unaltered by heating, therefore the change is an isomerisation, most likely involving a rearrangement to a more stable coordination isomer. This finding has implications for the clinical application of tricine–HYNIC–peptide complexes since the “mixed press” that such compounds have received (low stability, high protein binding, slow blood clearance)^{9,20–23} has arisen from studies performed using labelling at room-temperature. High temperature labelling results in more hydrophilic, more stable complexes which may well perform better *in vivo*. The second observation is that many of the findings of this study could only be achieved when solutions were carefully analysed by HPLC using gentle, extended mobile-phase gradients which were able to separate out the different complexes formed. Liu *et al.* have previously emphasised the importance of meticulous HPLC analysis⁹ and they employed a variety of mobile-phase systems including acetonitrile–TFA, phosphate and tetrabutylammonium phosphate systems to achieve adequate separation. We were able to achieve good separation using water–acetonitrile–TFA, but only when extended gradients were employed. The final observation is that even when using such discriminating methods of analysis, our results confirm those of von Guggenberg *et al.*²⁴ and Liu *et al.*⁹ which show that ^{99m}Tc–EDDA–HYNIC–peptides can be prepared in high yields using an exchange reaction involving tricine as an intermediate chelator. The HPLC retention times of the complexes prepared using either direct or exchange labelling were identical, indicating that tricine does not contribute to the coordination of the final complex.

Conclusion

These results highlight common features between the Tc-labelled HYNIC conjugates and model Tc and Re hydrazinopyridine complexes,¹² suggesting that the model structures provide a relevant framework within which to interpret the available data on labelled HYNIC–peptide conjugates, and thus increasing confidence with which structures such as those in Fig. 8 may be proposed. The resulting interpretation favours a chelating role for HYNIC in which both the hydrazine and the pyridine groups are coordinated. This in turn

influences the coordination of co-ligands and amino acid side chains and has implications for stability and homogeneity of the radiopharmaceutical. Furthermore we conclude that the HYBA derivative has no advantages over HYNIC as a bifunctional complexing agent and is not attractive for further development as it is inferior on grounds of both homogeneity and stability.

Experimental

Materials

Reagents were purchased from Aldrich-Sigma Chemical Co. unless stated otherwise and used as received. HYNIC-nanogastrin was synthesised using solid phase peptide synthesis at the University of Kent at Canterbury on a Shimadzu PSSM-8 Multiple Peptide Synthesiser using the Fmoc strategy as previously described using lysine-HYNIC as the last amino acid. 7 Standard side chain protecting groups were utilised and the peptide was assembled on TGR amide resin (Novabiochem) using HBTU-mediated coupling. HYBA-nanogastrin was synthesised by the Peptide Synthesis Laboratory, Cancer Research UK using Fmoc strategy. $\text{Na}^{99\text{m}}\text{TcO}_4$ was obtained from a commercial $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ (Drygen, GE Healthcare) generator at St. Bartholomew's Hospital, London. Tc-99 was obtained from Amersham International plc as solid ammonium pertechnetate. It was dissolved in water at a concentration of 2×10^{-4} M and filtered before use.

$^{99\text{m}}\text{Tc}$ -labelling

All Tc-99m work was performed in a radiopharmacy laboratory at the CRUK Nuclear Medicine Dept., St. Bartholomew's Hospital.

HYNIC–HYBA-nanogastrin: tris(hydroxymethyl)methylglycine (tricine) as co-ligand—In a screw top 2.5 mL polypropylene Corning vial, 10 μg of HYNIC- or HYBA-nanogastrin in 15 μL water was incubated with 0.5 mL of tricine solution (100 mg mL^{-1} in water), 0.5 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (>200 MBq), and 10 μL of stannous chloride dihydrate solution (3 mg mL^{-1} in ethanol) for 30 min at 95 °C or 10 min at room temperature.

EDDA as co-ligand—In a screw top 2.5 mL polypropylene Corning vial, 10 μg of HYNIC-/HYBA-nanogastrin in 15 μL water was incubated with 0.25 mL of 0.3 M phosphate buffer, pH 4, 0.25 mL of EDDA solution (10 mg mL^{-1} in 0.1 M NaOH), 0.5 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (>200 MBq), and 10 μL of stannous chloride dihydrate solution (3 mg mL^{-1} in ethanol) for 30 min at 95 °C.

Tricine–ethylenediaminediacetic acid (EDDA) as co-ligand—In a screw top 2.5 mL polypropylene Corning vial, 10 μg of HYNIC-/HYBA-nanogastrin in 15 μL water was incubated with 0.25 mL of tricine solution (20 mg mL^{-1} in 0.3 M sodium dihydrogen phosphate), 0.25 mL of EDDA solution (10 mg mL^{-1} in 0.1 M NaOH), final pH 6.0, 0.5 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (>200 MBq), and 10 μL of stannous chloride dihydrate solution (3 mg mL^{-1} in ethanol) for 30 min at 95 °C.

Tricine–nicotinic acid (NA) as co-ligand—In a screw top 2.5 mL polypropylene Corning vial, 10 μg of HYNIC-/HYBA-nanogastrin in 15 μL water was incubated with 0.4 mL of tricine solution (100 mg mL^{-1} in water), 0.1 mL of NA (90 mg mL^{-1} in water), 0.5 mL of $^{99\text{m}}\text{TcO}_4^-$ solution (>200 MBq), and 10 μL of stannous chloride dihydrate solution (3 mg mL^{-1} in ethanol) for 30 min at 95 °C.

Modifications for MS analysis—To improve detectability in LC-MS analysis, the concentrations of reagents were increased when labelling samples analysed by this method as follows:

Tricine as co-ligand—In a screw top 2.5 mL polypropylene Corning vial, 12.5 µg of HYNIC- or HYBA-nanogastrin in water was incubated with 100 µL tricine (100 mg/350 µL in 30 µL water), 50 µL $^{99}\text{TcO}_4^-$ solution (1×10^{-8} moles), 5 µL $^{99\text{m}}\text{TcO}_4^-$ solution (3 MBq), and 5 µL stannous chloride dihydrate solution (6 mg mL $^{-1}$ in ethanol) for 30 min at 95 °C.

EDDA as co-ligand—In a screw top 2.5 mL polypropylene Corning vial, 12.5 µg of HYNIC- or HYBA-nanogastrin in 30 µL water was incubated with 50 µL tricine (40 mg mL $^{-1}$ in 0.3 M sodium dihydrogen phosphate), 50 µL EDDA (20 mg mL $^{-1}$ in 0.1 M NaOH) (final pH 5.5–6.0), 50 µL $^{99}\text{TcO}_4^-$ solution (1×10^{-8} moles), 5 µL $^{99\text{m}}\text{TcO}_4^-$ solution (3 MBq), and 5 µL stannous chloride dihydrate solution (6 mg mL $^{-1}$ in ethanol) for 30 min at 95 °C.

Tricine–nicotinic acid (NA) as co-ligand—In a screw top 2.5 mL polypropylene Corning vial, 12.5 µg of HYNIC- or HYBA-nanogastrin in 30 µL water was incubated with 85 µL tricine (100 mg/350 µL in water), 15 µL NA (90 mg/150 µL in water), 50 µL $^{99}\text{TcO}_4^-$ solution (1×10^{-8} moles), 5 µL $^{99\text{m}}\text{TcO}_4^-$ solution (3 MBq), and 5 µL stannous chloride dihydrate solution (6 mg mL $^{-1}$ in ethanol) for 30 min at 95 °C.

Purification by solid-phase extraction (SPE)—For purification of the radiolabelled peptide for stability studies, a SPE method was used. The radiolabelling mixture was passed through a conditioned C18-SEPPAK-mini cartridge. The cartridge was washed with 5 mL of water, and the radiolabelled peptide was eluted with 1 mL of 50% ACN.

Analytical methods

Radiolabelled peptides were characterised by HPLC, TLC and LC-MS.

HPLC—A Beckman System Gold running 24 Karat proprietary software was used for RP-radioHPLC analysis with a Phenomenex Jupiter C18 300 A column, 250 × 4.60 mm 5 µm, and a Beckman 168 UV detector in series with a GABI sodium iodide radioactivity monitor (Raytest). A flow rate of 1 mL min $^{-1}$, and UV detection at 220–350 nm were employed with the following gradients: method 1: acetonitrile (ACN) in 0.1% aqueous trifluoroacetic acid (TFA): 0–5 min 0% ACN, 5–25 min 0%–60% ACN, 25–30 min 60% ACN, 30–35 min 60%–100% ACN, 35–37 min 100%–0% CAN; method 2: ACN in 0.1% aqueous TFA: 0 min 15%, 0–70 min 30% ACN, 70–90 min 100% ACN, 90–92 min 15% ACN.

TLC—Instant Thin Layer Chromatography was performed on silica gel (ITLC-SG, Gelman Sciences, Ann Arbor, Mich.) with saline as eluant for detection of $^{99\text{m}}\text{Tc}$ -pertechnetate and soluble non-peptide bound impurities ($R_f = 1$), and 50% acetonitrile-water solution for determination of reduced hydrolysed technetium ($^{99\text{m}}\text{Tc}$ colloid, $R_f = 0$).

LC-MS—Mass spectroscopy on 100 µL of the labelling mixture prepared as described above was performed in both positive and negative ion mode using a Finnigan Mat LCQ ion trap mass spectrometer coupled to a Hewlett-Packard 1100 HPLC system, in the Biomolecular Analysis Laboratory, University of Kent. HPLC parameters were as follows: Phenomenex Polymer PRP-1 column (150 × 2 mm, 5 mm); mobile phase: linear gradient of increasing ACN in 0.05% aqueous TFA: 0–5 min 5% ACN, 5–35 min 5%–100% ACN, 35–40 min 100% ACN, 40–45 min 100%–5% ACN; flow rate: 0.2 mL min $^{-1}$; detection: UV

absorbance at 214 nm and 254 nm. LC-MS was also performed on a series of dilutions (5, 10, 20, 50 and 100 fold) of the ^{99}Tc -HYNIC-tricine preparation in order to determine the sensitivity of the system and to compare the results at a range of concentrations of the complex. RPLCMS analysis of technetium complexes were performed using the following electrospray mass spectrometry analysis parameters: method A: peptide analytical mode, negative mode ionisation with tube lens offset (skimmer) and capillary voltage set at -50 V and -16 V respectively; method B: peptide analytical mode, positive mode ionisation with tube lens offset (skimmer) and capillary voltage set at $+30\text{ V}$ and $+19\text{ V}$ respectively; method C: organic analytical profile mode, positive mode ionisation with tube lens offset (skimmer) and capillary voltage set at 0 V and $+15\text{ V}$ respectively; method D: organic analytical profile method, negative mode, capillary voltage: -11 V and tube lens offset (skimmer) 10 V .

***In vitro* evaluation of radiolabelled peptide stability**

The stability of the radiolabelled peptide in aqueous solution was tested by HPLC after incubation of the reaction mixture, (i) without purification at a peptide concentration of approximately 1500 nmol mL^{-1} in 0.1 M phosphate buffer (pH 7.4) for up to 4 h, (ii) after purification by SPE at a peptide concentration of approximately 7500 nmol mL^{-1} in 0.1 M phosphate buffer for up to 4 h; (iii) in fresh human plasma at $37\text{ }^{\circ}\text{C}$ for up to 5 h. After incubation in plasma, plasma proteins were precipitated with acetonitrile and removed by centrifugation (6500 rev min^{-1} for 5 min). Degradation of the $^{99\text{m}}\text{Tc}$ -peptide complexes to hydrophilic species was assessed by HPLC method 1 (above). Protein binding was determined using size-exclusion chromatography (Microspin G-50 columns; Sephadex G-50), and measurement of retentate (non-bound) and eluate (protein bound) in a gamma counter (Compugamma 1282, LKB WALLAC).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Some possible structures of $^{99\text{m}}\text{Tc}$ -HYNIC-peptide complexes showing monodentate and bidentate binding of HYNIC to technetium.

Fig. 3. RP-HPLC radiochromatograms of $^{99\text{m}}\text{Tc}$ -HYNIC-nanogastrin using tricine as co-ligand, labelled at room temperature for 10 min (top) and 3 h (bottom).

Fig. 4. Sample HPLC profiles of conjugate radiolabelling at 95 °C for 30 min, using tricine as co-ligand, comparing steep (method 1) and gentle (method 2) gradients. From top, ^{99m}Tc -tricine-HYNIC-nanogastrin, (i) method 1 and (ii) method 2; ^{99m}Tc -tricine-HYBA-nanogastrin, (iii) method 1 and (iv) method 2.

Fig. 5. Solution stability of labeled peptides. Top: on dilution in phosphate buffer; bottom: on SEPPAK purification to remove excess co-ligand and stannous chloride.

Fig. 6. Stability of labeled peptides on incubation in human serum. Top, % intact radiopeptide by radioHPLC after precipitation of proteins with MeCN; bottom: % protein binding of radioactivity by size exclusion chromatography.

Fig. 7.
Schematic structures of crystallographically characterised model complexes (ref. 11 and 12).

Fig. 8.
Example schematic structures of possible peptide complexes that are both analogous to crystallographically characterized model complexes and are consistent with ES-MS data; left: Tc-HYNIC-nanogastrin-tricine; right: Tc-HYNIC-nanogastrin-tricine-(nicotinic acid)₂.

Table 1

Selected LC-MS data for peptides and Tc-peptide conjugates. Only monocations and monoanions are listed; many other species derived from the same molecular species in solution (especially $[M + 2H]^{2+}$ ions) were detected often at 100% abundance. These are omitted here for simplicity but are listed in full in the ESI[†]. P = Peptide

Peptide and co-ligands	ES-MS Method	m/z calc.	m/z obs.	Assignment	Inferred solution species
HYNIC-nanogastrin	C	1280.5	1280.5	$[P + H]^+$	$[P]$
HYBA-nanogastrin	C	1277.5	1277.3	$[(P - 2H) + H]^+$	$[P - 2H]$
	C	1279.5	1279.4	$[P + H]^+$	$[P]$
HYNIC-[gly ⁸]nanogastrin	B	1208.2	1208.4	$[P + H]^+$	$[P]$
HYNIC-nanogastrin, tricine	C	1553.5	1553.3	$[(P + Tc + Tri - 5H) + H]^+$	$[P + Tc + Tri - 5H]$
HYNIC-nanogastrin, tricine, nicotinic acid	C	1676.5	1676.1	$[(P + Tc + Tri + Nic - 5H) + H]^+$	$[P + Tc + Tri + Nic - 5H]$
	C	1799.5	1799.9	$[(P + Tc + Tri + 2Nic - 5H) + 2H]^{2+}$	$[P + Tc + Tri + 2Nic - 5H]$
HYNIC-nanogastrin, tricine, EDDA	C	1726.6	1726.1	$[(P + Tc + 2EDDA - 5H) + H]^+$	$[P + Tc + 2EDDA - 5H]$
	C	1550.5	1551.3	$[(P + Tc + EDDA - 5H) + H]^+$	$[P + Tc + EDDA - 5H]$
HYBA-nanogastrin, tricine	A	1550.5	1550.3	$[(P + Tc + Tri - 5H) - H]^-$	$[P + Tc + Tri - 5H]$
HYBA-nanogastrin, tricine	A	1550.5	1550.4	$[(P + Tc + Tri - 5H) - H]^-$	$[P + Tc + Tri - 5H]$
	A	1729.6	1729.2	$[(P + Tc + 2Tri - 5H) - H]^-$	$[P + Tc + 2Tri - 5H]$
HYBA-nanogastrin, tricine, nicotinic acid	A	1796.5	1795.9	$[(P + Tc + Tri + 2Nic - 5H) - 5H]^-$	$[P + Tc + Tri + 2Nic - 5H]$
	A	1673.5	1673.2	$[(P + Tc + Tri + Nic - 5H) - 5H]^-$	$[P + Tc + Tri + Nic - 5H]$
HYBA-nanogastrin, tricine, EDDA	C	1549.5	1549.4	$[(P + Tc + EDDA - 5H) + H]^+$	$[P + Tc + EDDA - 5H]$
	C	1725.6	1725.1	$[(P + Tc + 2EDDA - 5H) + H]^+$	$[P + Tc + 2EDDA - 5H]$
HYNIC-[gly ⁸]nanogastrin, tricine	C	1481.2	1481.3	$[(P + Tc + Tri - 5H) + H]^+$	$[P + Tc + Tri - 5H]$
	C	1660.2	1660.9	$[(P + Tc + 2Tri - 5H) + H]^+$	$[P + Tc + 2Tri - 5H]$