JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2003; **46**: 307–318.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.672

Research Article

Preparation via coligand exchange and characterization of [^{99m}Tc-EDDA-HYNIC-D-Phe^{1},Tyr^{3}]Octreotide (^{99m}Tc–EDDA/HYNIC–TOC)

Elisabeth von Guggenberg[1], Bettina Sarg[2], Herbert Lindner[2], Laura Melendez Alafort[3], Stephen J. Mather[3], Roy Moncayo[1] and Clemens Decristoforo[1,*]

[1] *Univ.-Klinik fuer Nuklearmedizin, Innsbruck, Austria*
[2] *Institut fuer Medizinische Chemie und Biochemie, Innsbruck, Austria*
[3] *Nuclear Medicine Research Laboratory, St. Bartholomews Hospital, London, UK*

Summary

[^{99m}Tc-EDDA–HYNIC-D-Phe^{1},Tyr^{3}]octreotide (^{99m}Tc-EDDA/HYNIC–TOC) is a promising new agent with the potential to replace [^{111}In-DTPA-D-Phe^{1}]-octreotide in somatostatin receptor scintigraphy. This hydrazinonicotinic acid derivatized somatostatin complex contains ethylenediamine N,N′ diacetic acid (EDDA) as a coligand resulting in a high *in vitro* and *in vivo* stability. Since direct ^{99m}Tc-labelling of HYNIC–TOC with EDDA results in low labelling yields, in this study we describe the preparation of ^{99m}Tc-EDDA/HYNIC-TOC via coligand exchange from Tricine for EDDA. Exchange of coligands is achieved at elevated temperature and under optimized conditions of pH, EDDA concentration and stannous ion. High labelling yields (mean 95.9%) were achieved at high specific activities (>37GBq/μmol). Characterization via HPLC, receptor binding and LC–MS of the resulting complex is described. The formulation developed enables rapid and simple labelling of ^{99m}Tc-EDDA/

*Correspondence to: C. Decristoforo, Univ.-Klinik Fuer Nuklearmedizin, Anichstrasse 35, A-6020 Innsbruck, Austria. E-mail: Clemens.Decristoforo@uibk.ac.at

Received 14 June 2002
Revised 13 September 2002
Accepted 13 October 2002

HYNIC–TOC in a manner suitable for a clinical setting. Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: technetium-99m; octreotide; EDDA; tricine; HYNIC

Introduction

In vivo imaging of tumours using radiolabelled somatostatin (SST) analogues has become an accepted clinical tool in oncology.[1] Today the radiopharmaceutical of choice for this diagnostic procedure is [^{111}In-DTPA-D-Phe^{1}]-octreotide (^{111}In-DTPA-octreotide).[2] However ^{99m}Tc can be considered as the radiolabel of choice with daily availability from a generator, a 6 h half life and 140 keV monoenergetic gamma ray emission ideal for SPECT-Nuclear Medicine imaging procedures. We have recently described the development of a ^{99m}Tc-labelled SST analogue[3] based on [Tyr^{3}]-octreotide (TOC) as the SST-binding moiety and hydrazinonicotinic acid (HYNIC) as the ligand for ^{99m}Tc. Labelling with ^{99m}Tc is performed using a coligand required to stabilize ^{99m}Tc bound to the hydrazino residue of the peptide conjugate. In the preclinical development we have chosen ethylendiamine *N*, *N* 'diacetic acid (EDDA) as the coligand of choice [4] since it resulted in the lowest lipophilicity and highest *in vivo* stability of the radiolabelled peptides studied. In the first clinical study [^{99m}Tc-EDDA-HYNIC–D-Phe^{1},Tyr^{3}]-octreotide (^{99m}Tc-EDDA/HYNIC–TOC) showed favourable pharmacokinetics and higher uptake in SST-receptor positive tumour sites than ^{111}In-labelled analogues, therefore demonstrated the potential to replace ^{111}In-DTPA-octreotide for SSTR-scintigraphy[5] and was clearly superior to ^{99m}Tc-Tricine/HYNIC–TOC.[6] Direct ^{99m}Tc-labelling of HYNIC-derivatized peptides with EDDA as coligand results in low labelling yields[7] unsuitable for routine clinical application. However recently Liu *et al.*[8] described that in ^{99m}Tc-tricine/HYNIC-complexes tricine can be exchanged for EDDA at elevated temperature. Additionally we wanted to study the use of LC–MS, a method for analysis of the resulting Technetium–complexes, that has recently been applied for analysis of ^{99m}Tc-labelled peptides.[9] In this paper, we describe the development of a labelling approach based on tricine/EDDA ligand exchange which results in quantitative yields allowing the routine preparation of ^{99m}Tc-EDDA/HYNIC–TOC in a clinical setting and the characterization of

the resulting ^{99m}Tc-complex by HPLC, LC–MS and receptor binding analysis.

Experimental

Materials

Reagents were purchased from Aldrich-Sigma Chemical Co except otherwise stated and used as they were received.

HYNIC-[Tyr^3]-octreotide (HYNIC–TOC) was synthesized as described elsewhere.[6]

Na $^{99m}TcO_4$ was obtained from commercial ^{99}Mo/^{99m}Tc generator (ULTRATECHNEKOW, Mallinckrodt, The Netherlands)

Analytical methods

HPLC—Method 1: A Gynkotek M480 low pressure gradient pump with Shimadzu SP6 V variable UV detector and radiometric detection was used for reverse-phase HPLC analysis. An Ultrasphere ODS column (4,6 × 250 mm^2 ID; 5 μm particle size, Beckman, Fullerton, CA), flow rates of 1 ml/min, and UV detection at 220 nm were employed with the following gradient:

Acetonitrile/0.01 N phosphate buffer pH 6; 0–3 min 0%ACN, 3–5 min 0–25%ACN, 5–18 min 25%ACN, 18–22 min 25–70%ACN, 22–24 min 70–0%ACN

TLC: Instant thin layer chromatography on silica gel (ITLC–SG, Gelman Sciences, Ann Arbour, MI) was performed using different mobile phases. MEK was used to determine the amount of free $^{99m}TcO_4^-$(Rf = 1), 0.1 N citrate buffer pH5 to determine non peptide bound ^{99m}Tc-coligand and $^{99m}TcO_4^-$(Rf = 1), 50% acetonitrile/water for ^{99m}Tc-colloid. (Rf = 0).

LC–MS: Samples of ~50 pM were injected onto a PepMap C_{18} column (150 mm × 1 mm ID; 3 μm particle size; ICT, Vienna, Austria). The column eluate was directly coupled to a Finnigan MAT LCQ iontrap instrument (San Jose, CA) equipped with an electrospray source (RP–HPLC–ESI–MS). Samples were chromatographed by HPLC (127 Solvent Module, Beckman Instruments, Palo Alto, CA), flow rate 35 μl/min with the following gradient: water/acetonitrile/0.1% formic acid; 0–10 min: 0%ACN, 10–20 min: 0–51% ACN, 20–55 min: 51%ACN (HPLC method 2).

Experimental conditions

Wet radiolabelling experiments were performed to optimize the amount and concentration of reagents, labelling pH, temperature and reaction time. EDDA was initially dissolved in 0.1 N NaOH (20 mg/ml), tricine in water for injection (40 mg/ml). $^{99m}TcO_4^-$ was used in sterile 0.9% saline solution (0.5–1.0 ml) and $SnCl_2 \cdot 2H_2O$ was immedately dissolved before use in nitrogen purged 0.1 N HCl (1 mg/ml). pH was adjusted with 0.1 N HCl, 0.1 N NaOH or kept constant with 0.2 M phosphate buffer. Volume adjustments were made with 0.9% saline.

Direct labelling with EDDA: 20 µl of 1 mg/ml HYNIC–TOC in water were incubated with 1.0 ml solution of N,N'ethylenediaminediacetic acid (EDDA) (10 mg/ml, pH 7.0), 1.0 ml $^{99m}TcO_4$- solution (1000 MBq) and 5–10 µg $SnCl_2 \cdot 2H_2O$ for 60 min at room temperature.

EDDA/tricine exchange labelling: HYNIC–TOC was incubated with EDDA, tricine, $SnCl_2 \cdot 2H_2O$ and $^{99m}TcO_4^-$ in a volume of 2 ml. The amount of $SnCl_2 \cdot 2H_2O$ (5–200 µg), EDDA concentration (1–10 mg/ml), pH (2.5–8.0), reaction time (5–60 min) and temperature (RT–100°C) were varied to optimize the labelling reaction. The amount of HYNIC–TOC (20 µg), tricine (10 mg/ml) and Na $^{99m}TcO_4$ (1000 MBq) were kept constant.

Reaction solutions were tested for radiochemical purity by HPLC immediately and up to 24 h after preparation.

Preparation of Tc-EDDA/HYNIC–TOC for LC–MS experiments: Direct labelling and EDDA/tricine exchange labelling for LC–MS experiments was performed as described above except for the use of 50 µg of peptide and Na $^{99m}TcO_4^-$ eluate with low specific activity containing about 100 ng technetium/labelling-experiment (based on theoretical calculation from generator ingrown). The reaction solution was purified by HPLC method 1 to separate ^{99m}Tc-EDDA/HYNIC–TOC from excess HYNIC–TOC and the radioactive peptide peak was isolated. After removal of acetonitrile by nitrogen purging a calculated amount of 20 pmol Tc-EDDA/HYNIC–TOC was injected onto the HPLC–MS system (HPLC method 2).

Receptor binding: To evaluate possible peptide degradation in the labelling process receptor binding studies of ^{99m}Tc-EDDA/HYNIC–TOC were performed as previously described.[7] The specific binding of the ^{99m}Tc-labelled peptide was determined by competition against unmodified TOC. Rat pancreatic (AR4-2J cells) tumour cell membranes were used as a source for SST-receptors, bound was separated from free

radioligand by filtration through glass fibre filters (Whatman GF/C) and IC_{50} values calculated following nonlinear regression with Origin software (Microcal Origin 5.0, Northampton, MA).

Results and Discussion

Wet labelling: Direct ^{99m}Tc-labelling of HYNIC–TOC with tricine as coligand took place rapidly at room temperature, after 10 min >90% labelling was observed. Exchange labelling from ^{99m}Tc-Tricine/HYNIC–TOC to the EDDA complex was achieved at higher reaction temperatures and resulted in a complex with retention time on HPLC identical to that of ^{99m}Tc-EDDA/HYNIC–TOC prepared by direct labelling (see Figure 1(A)). Radio-HPLC analysis of ^{99m}Tc-Tricine/HYNIC–TOC is shown in Figure. 1(B). After 20 min at 50°C (Figure 1(C)) labelling yields were about 50%, while after 30 min at 75°C (Figure 1(D)) or 10 min at 100°C (Figure 1(E)) less than 5% tricine complex was found. Labelling yields >90% were achieved by heating longer than 30 min at 75°C or 10 min at 100°C. Heating in boiling water for 10 min did not result in any shift in HPLC-retention times of the ^{99m}Tc-EDDA/HYNIC–TOC peak or detectable changes in receptor binding. Figure 2 summarizes the influence of EDDA concentration and pH on the labelling process. In formulations with EDDA concentrations below 5 mg/ml residual amounts of ^{99m}Tc-tricine/HYNIC–TOC were found, at 1 mg/ml up to >40% (Figure 2(a)). Neither EDDA concentration nor temperature had a significant influence on the amount of other possible impurities such as free pertechnetate, Tc-coligand complexes (^{99m}Tc–EDDA or ^{99m}Tc–Tricine) or Tc–colloid (data not shown). Ten micrograms of $SnCl_2 \cdot 2H_2O$ were required to achieve reliable reduction of $^{99m}TcO4^-$, >100 μg decreased labelling yields significantly. Control of pH of the labelling mixture was crucial to achieve good labelling (Figure 2(b)). Highest labelling yields were achieved at pH 6.5. At lower pH high amounts of free pertechnetate were detected. Overall the following formulation and labelling conditions with reproducible labelling yields of >90% (mean 95.9% $\pm$ 1.9, $n = 5$) was established: 20 μg HYNIC–TOC, 10 mg EDDA, 20 mg tricine, 10 μg $SnCl_22H_2O$, pH 6.5–7.5, labelled with 1000 MBq $^{99m}TcO_4^-$ in a total volume of 2 ml, reaction time: 10 min in boiling water. Radiochemical purity remained stable up to 24 h p.i. Mean values determined with HPLC method 1

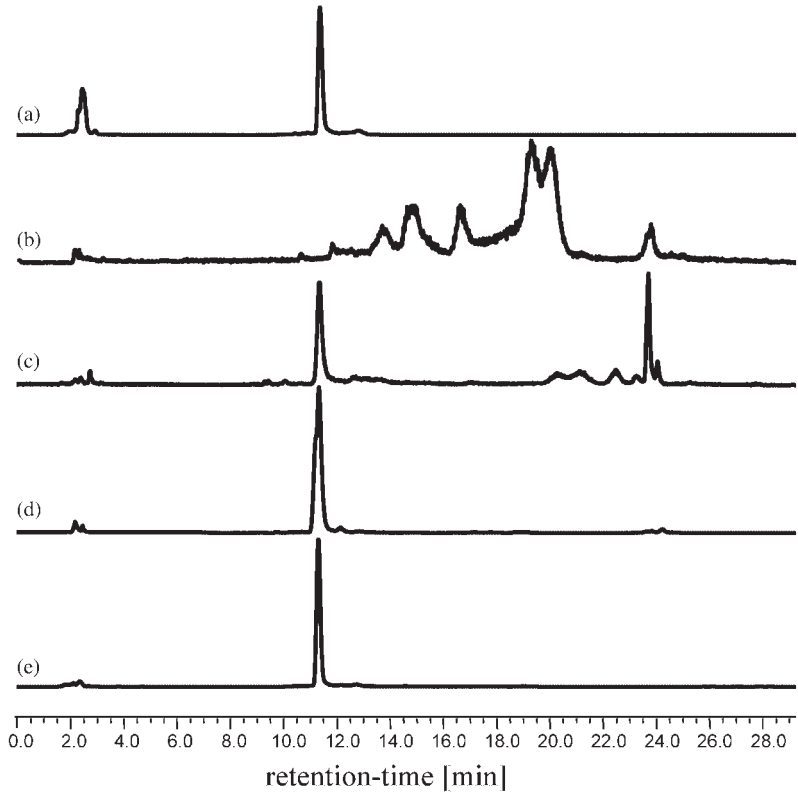


Figure 1. Radiochromatograms of labelling mixtures of different ^{99m}Tc–HYNIC–TOC preparations analysed by HPLC method 1. (A) ^{99m}Tc–EDDA/HYNIC–TOC prepared via direct labelling, (B) ^{99m}Tc–tricine/HYNIC–TOC, (C)–(E) ^{99m}Tc–EDDA/HYNIC–TOC prepared via tricine/EDDA exchange, (C)—50°C 20 min, (D)—75°C 30 min, (E)—100°C 10 min

were 95.89% $\pm$ 2.15 ($n = 5$) after 4 h and 94.83% $\pm$ 2.89 ($n = 5$) after 24 h. Receptor binding assays showed a high receptor affinity of ^{99m}Tc-EDDA/HYNIC–TOC prepared via this method.

LC–MS: Figure 3(a) shows the total ion current trace of carrier added Tc-EDDA/HYNIC–TOC after purification with HPLC method 1, indicating a high purity of the isolated Tc-peptide-complexes. Figure 3(b) shows the electrospray-ionization mass spectrum of the HPLC-peak observed in Figure 3(a). The major peak was found to be 1616.1 Da $(M + H)^+$ (peak 1). In addition variable amounts of a minor peak of 1440.2 Da $(M + H)^+$ were detected (peak 2). The major ion of 1616.1 Da was selected for further analysis by tandem mass spectrometric analysis. The resulting MS/MS spectrum revealed a major

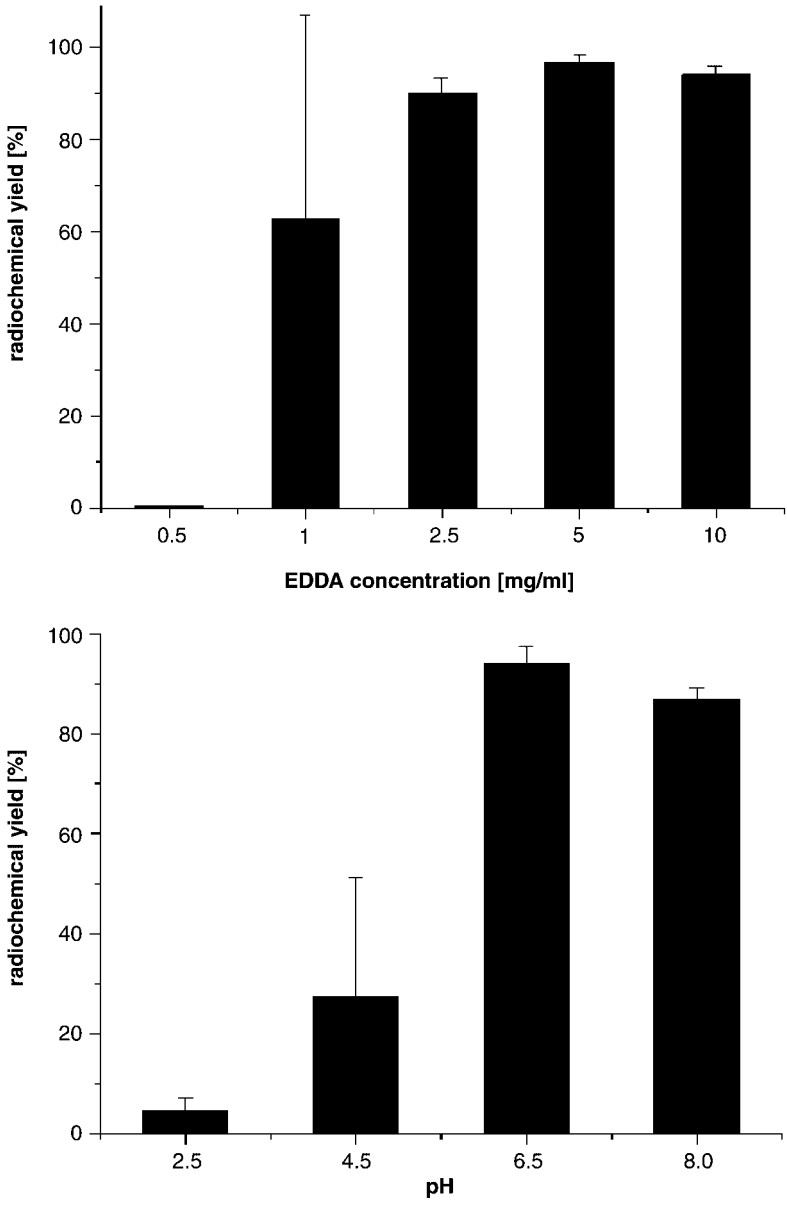
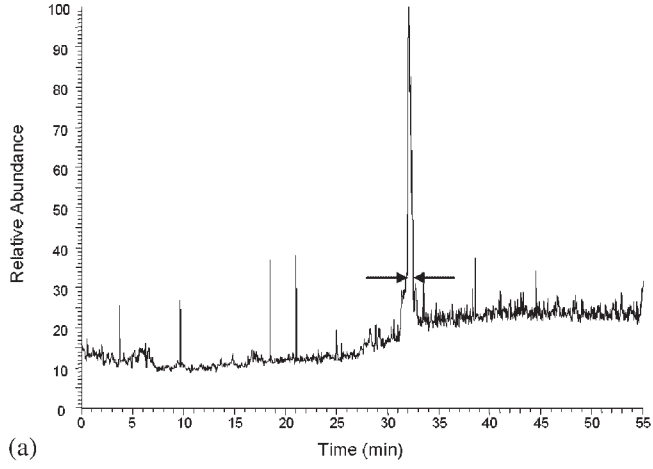
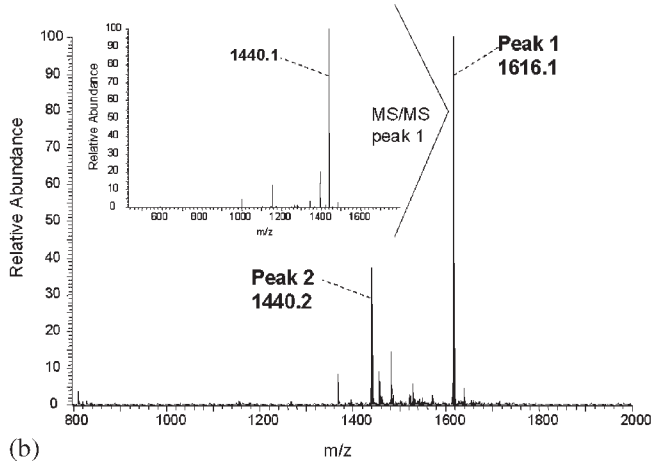


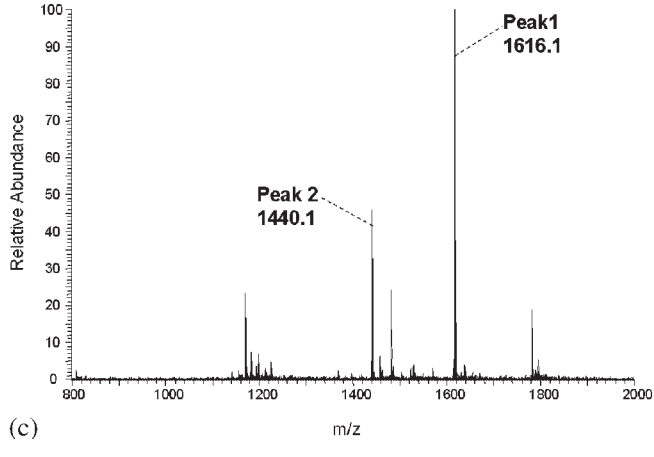
Figure 2. Influence of coligand concentration and pH on radiochemical yield of ^{99m}Tc–EDDA/HYNIC–TOC-preparations via tricine/EDDA ligand exchange. (A) EDDA-concentration: mean ± SD of *n*=3; (C) pH: mean ± SD of *n*=3. Standard conditions for labelling: 20 μg HYNIC–TOC, 1000 MBq ^{99m}Tc-pertechnetate, 10 μg $SnCl_2 \cdot 2H_2O$, total volume 2 ml, 10 min 100°C, analysis by HPLC–method 1, determination of ^{99m}Tc–colloid by TLC



(a)



(b)



(c)

fragment ion with a mass of 1440.1 Da (inset Figure 3(a)). Figure 3(c) shows the mass spectrum of Tc-EDDA/HYNIC–TOC prepared via direct labelling also showing two peaks at 1616.1 and 1440.1 Da.

Table 1 compares the experimental results with the calculated masses of possible complexes.

Discussion: For the development of a clinically useful formulation of a ^{99m}Tc-labelled peptide a number of considerations have to be taken into account. First the formulation should result in a product with high radiochemical purity, ideally $>95\%$, thus avoiding the need for purification prior to use. This aim was not achieved by a direct labelling approach with EDDA as coligand even at elevated temperature.[7] Secondly the amount of ligand required (i.e. the peptide) should be low to avoid possible receptor saturation and undesirable pharmacological effects. Thirdly the amount of activity for radiolabelling should be sufficient to enable reasonable count statistics during the scintigraphic study, taking into account physical decay of ^{99m}Tc during storage and performance of the study. Additionally the final product should have a sufficient stability to enable scintigraphy even if the study has to be delayed.

We therefore chose a fixed amount of peptide and radioactivity in our attempt to develop a suitable clinical formulation for the preparation of ^{99m}Tc-EDDA/HYNIC–TOC. In previous experiments we have shown that specific activities greater than 37 GBq/μmol could be achieved.[7] We considered it necessary in the labelling process to use a ^{99m}Tc-activity of at least 1000 MBq. Patients receive a radioactive dose of 300–400 MBq for a diagnostic scan.[5] One thousand megabecquerels would therefore be sufficient for two patient doses, even if the second patient is injected up to 4 h after preparation. For labelling experiments we chose a fixed amount of 20 μg HYNIC–TOC. This is a comparable amount of peptide present in other formulations of radiolabelled somatostatin analogues such as ^{111}In-DTPA-octreotide (Octreoscan[10]) or ^{99m}Tc-depreotide (NeoSpect[11]), and when labelled with 1000 MBq patients would receive about 10 μg of peptide, a dose, that has been used safely in a pilot study[5] and is also being used in an ongoing

Figure 3. LC–MS analysis of carrier-added Tc–EDDA/HYNIC–TOC. (A) Total ion current trace of RP–HPLC–ESI–MS analysis, (B) mass spectrum of main HPLC peak (Rt 44 min) prepared via Tricine/EDDA ligand exchange, MS/MS spectrum of the ion at *mlz* 1616.1, (C) mass spectrum of Tc-EDDA/HYNIC-TOC prepared via direct EDDA labelling.

Table 1. Detected mass compared to theoretical complex composition and calculated mass of Tc–EDDA/HYNIC–TOC complexes. The calculated masses include the loss of four protons during complex formation

Method of preparation	Peak	Found M + 1	Suggested theoretical composition	Formula of suggested composition	Exact mass of suggested composition
Direct EDDA labelling	Main peak	1616.1	[Tc (EDDA)$_2$ (HYNIC–TOC)]	$C_{67}H_{91}N_{17}O_{20}S_2Tc$	1615.55
	Minor peak	1440.1	[Tc (EDDA) (HYNIC–TOC)]	$C_{61}H_{79}N_{15}O_{16}S_2Tc$	1439.55
Tricine/EDDA exchange labelling	Main peak	1616.1	[Tc (EDDA)$_2$ (HYNIC–TOC)]	$C_{67}H_{91}N_{17}O_{20}S_2Tc$	1615.55
	Minor peak	1440.2	[Tc (EDDA) (HYNIC–TOC)]	$C_{61}H_{79}N_{15}O_{16}S_2Tc$	1439.55

Phase II trial. To achieve high labelling yields we employed an exchange labelling procedure starting from the relatively unstable ^{99m}Tc-tricine/HYNIC–TOC complex, that preclinically and in a patient study proofed to be unsuitable for clinical use.[3,6] Liu et al.[8] have shown the tricine coligand can be exchanged for EDDA to prepare a more stable complex. The advantage of such an approach lies in the fact that ^{99m}Tc-tricine/HYNIC–TOC is rapidly formed with almost quantitative labelling yields under mild reaction conditions. We could show that exchange labelling at optimal pH and EDDA concentration rapidly takes place at elevated temperature and is completed after 10 min in a boiling water bath. In contrast to Liu et al.[8] we found that no excess of EDDA over tricine was necessary for this reaction. Radiochemical purity after 4 h was not significantly lower than immediately after labelling. This time point was chosen as the shelf life of the preparation not only for stability reasons but also to limit the mass of injected peptide. In contrast to previous reports[12,13] we could not observe any changes of the radiolabelled peptide neither in HPLC retention time nor in receptor binding which might be caused by the cleavage of the disulfide bridge at the elevated temperatures employed. LC–MS analysis of carrier-added samples showed that the ^{99m}Tc–HYNIC–TOC complex formed via tricine/EDDA ligand exchange had the same mass as the complex prepared via direct EDDA labelling. This confirms that no tricine/EDDA ternary complexes are formed, such as have been described for tricine/phosphine and tricine/pyridine coligand systems.[14,15] However, although only one peak was present in the HPLC, two species were found in LC–MS with m/z of 1616.1 and 1440.1. The mass of the ion found at m/z 1616.1 corresponds to the complex [Tc $(EDDA)_2$ (HYNIC–TOC)] and that at 1440.1 Da corresponds to [Tc (EDDA) (HYNIC–TOC)].[8] However, applying further tandem mass spectrometry to the 1616.1 peak also results in the formation of a fragment ion with a mass of 1440.1 Da. This suggests that the species formed during labelling contains two EDDA molecules/complex in contrast to previous reports suggesting only one[8], but that the mono-EDDA complex is formed as a result of the MS analysis. Although analysis at no carrier-added level has been performed successfully with a dedicated Radio-LC–MS system[13], using our experimental set-up with Radio-HPLC purification followed by LC–MS-analysis sensitivity was too low to confirm the composition of the complex at no carrier-added level.

Conclusion

In this study we have shown that ^{99m}Tc-EDDA/HYNIC–TOC can be prepared *via* tricine/EDDA ligand exchange under suitable conditions in a clinical setting. This method of preparation has been safely used in more than 100 patients in ongoing clinical trials at the University Hospital Innsbruck. Our results form the basis for a kit formulation that would allow a wider application of this promising radiopharmaceutical.

References

1. Eising EG, Bier D, Knust EJ, Reiners C. Radiologe 1996; **36**: 81–88.
2. Bakker WH, Albert R, Bruns C, *et al*. Life Sciences 1991; **49**: 1583–1591.
3. Decristoforo C, Melendez-Alafort L, Sosabowski JK, Mather SJ. J Nucl Med 2000; **41**: 1114–1119.
4. Decristoforo C, Mather SJ. *Eur J Nucl Med* 1999; **26**: 869–876.
5. Decristoforo C, Mather SJ, Cholewinski W, *et al*. *Eur J Nucl Med* 2000; **27**: 1318–1325.
6. Decristoforo C, Mather SJ, Cholewinski W, Donnemiller E, Riccabona G, Moncayo R. *Eur J Nucl Med* 2000; **27**: 1580.
7. Decristoforo C, Mather SJ. *Bioconjug Chem* 1999; **10**: 431–438.
8. Liu S, Edwards DS, Looby RJ, *et al*. *Bioconjug Chem* 1996; **7**: 63–71.
9. Liu S, Ziegler MC, Edwards DS. *Bioconjug Chem* 2000; **11**:113–117
10. Summary of Product Characteristics Octreoscan, Mallinckrodt Inc., January 1997.
11. Summary of Product Characteristics NeoSPECT, Amersham Health AS, 2001.
12. Kolan H, Li J, Thakur ML. Peptide Res 1996; **9**: 144–150.
13. Uehara T, Arano Y, Ono M, *et al*. *Nucl Med Biol* 1999; **26**: 883–890.
14. Edwards DS, Liu S, Barrett JA, *et al*. *Bioconjug Chem* 1997; **8**: 146–154.
15. Liu S, Edwards DS, Harris AR. *Bioconjug Chem* 1998; **9**: 583–595.

